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Design of Fluorescent and Targeted Nanoplatforms for Potential Anticancer Applications: From Chalcone Derivatives to Natural and Repurposed Agents

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
ADC	Antibody-drug conjugate
API	Active pharmaceutical ingredient
AR	Androgen receptor
BCL	Bicalutamide
BCS	Biopharmaceutics Classification System
BL1	Basal-like 1 – <i>breast cancer subtype</i>
BL2	Basal-like 2 – <i>breast cancer subtype</i>
CAC	Critical aggregation concentration
CAM	Chorioallantoic membrane
C-CDP	Cross-linked cyclodextrin polymer
CD	Cyclodextrin
CDP	Cyclodextrin polymer
CD-PR	Cyclodextrin-based polyrotaxane
CGTase	Cyclodextrin glycosyltransferase
CHS	Chalcone synthase
CNS	Central nervous system
COM	Compritol® 888 ATO
CS	Chitosan
DC	Drug content
DDS	Drug delivery system
DGT	First derivative of the TGA curve
DSC	Differential scanning calorimetry

D_H	Hydrodynamic diameter
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DOX	Doxorubicin
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTX	Docetaxel
EAP	Endogenous Alkaline Phosphatase
EE	Encapsulation efficiency
EGFR	Epidermal growth factor receptor
ELS	Electrophoretic light scattering
EPR	Enhanced permeability and retention (effect)
ER	Estrogen receptor
ESF	European Science Foundation
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FITC-CS	Fluorescein isothiocyanate-labeled chitosan
G2-β-CD	6-O-maltosyl- β -cyclodextrin
G-CDP	Grafted cyclodextrin polymer
GRAS	Generally recognized as safe
HA	Hyaluronic acid
HER2	Human epidermal growth factor receptor 2
HP-β-CD	Hydroxypropyl- β -cyclodextrin
ICI	Immune checkpoint inhibitor

IKB	Inhibitor of κ B
IKK	Inhibitor of κ B kinase
IM	Immunomodulatory – <i>breast cancer subtype</i>
iNOS	Inducible nitric oxide synthase
ISO	International Organization for Standardization
LAR	Luminal androgen receptor – <i>breast cancer subtype</i>
LPG	Labrafac™ MC60
M	Mesenchymal – <i>breast cancer subtype</i>
MM-GBSA	Molecular Mechanics Generalized Born Surface Area (method)
MMP	Matrix metalloproteinases
MPS	Mononuclear phagocyte system
MSL	Mesenchymal stem-like – <i>breast cancer subtype</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	Molecular weight cut-off
NLC	Nanostructured lipid carrier
NMR	Nuclear magnetic resonance
NP	Nanoparticle
OD	Optical density
PBS	Phosphate-buffered saline
pCR	Pathological complete response
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
PEGDGE	Poly(ethylene glycol) diglycidyl ether
PIT	Phase inversion temperature

PR	Progesterone receptor
PRCG	Polak-Ribiere Conjugate Gradient (algorithm)
PRR	Pattern recognition receptor
PTX	Paclitaxel
ROS	Reactive oxygen species
RTN	Rutin
SAR	Structure-activity relationship
SBE-β-CD	Sulfobutylether- β -cyclodextrin
SD	Standard deviation
SEEDDS	Self-emulsifying drug delivery system
SG	Sacituzumab govitecan
SLN	Solid lipid nanoparticle
SRB	Sulforhodamine B
Star-CDP	Star-shaped cyclodextrin polymer
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
TKI	Tyrosine kinase inhibitor
TLC	Thin-layer chromatography
TNBC	Triple-negative breast cancer
Trx	Thioredoxin
TrxR	Thioredoxin reductase
USP	United States Pharmacopeia
UV	Ultraviolet
UV-Vis	Ultraviolet-visible

VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
XP	Extra Precision (Glide docking protocol)
ζ	Zeta potential

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GENERAL SCOPE AND INTRODUCTION

Despite decades of advanced research, cancer persists as one of the most pressing health challenges of our era. While progress in prevention, diagnosis, and innovative therapies has significantly improved clinical outcomes, the rising incidence of this disease and the limitations of current treatments underscore the urgent need for more effective and better tolerated strategies. This is particularly true for conventional chemotherapy, which is often plagued by a lack of specificity, leading to severe systemic toxicity. Further limitations include the development of resistance mechanisms, such as the overexpression of ATP-binding cassette transporters, and the inherently poor aqueous solubility of many potent drugs, which severely restricts their bioavailability and therapeutic potential.

To address these complex issues, this doctoral research project focused on the rational design and development of innovative drug delivery systems (DDS) for anticancer therapy. The central aim was to engineer advanced nanoplatforms capable of enhancing solubility, bioavailability, and tumor-targeting specificity of both natural and newly synthesized anticancer agents. Particular emphasis was placed on developing fluorescent DDSs, thereby integrating therapeutic delivery with diagnostic capabilities (theranostics) for real-time tracking and monitoring.

The initial phase of this multidisciplinary work involved the synthesis of a novel series of halogenated chalcones, a class of compounds recognized for their broad pharmacological profile, including anticancer and antiangiogenic activities. The derivatives were designed with different substitution patterns on aromatic ring A (Figure I.1.). The study investigated how the position of electron-withdrawing halogen substituents (*ortho*, *meta*, or *para*) could influence cytotoxicity against five cancer cell lines—follicular thyroid cancer (FTC-133), thyroid carcinoma (8305C),

glioblastoma (U87), hepatocellular carcinoma (HEPG2), and osteosarcoma (U2OS)—and how these effects might correlate with their antitumor properties. The most active derivative (**1e**) was selected as a candidate for nanoformulation within lipid-based nanoparticles (NLCs) coated with fluorescent chitosan (FITC-CS), conceived as carriers able to improve delivery to tumor cells while also enabling real-time visualization through fluorescence imaging.

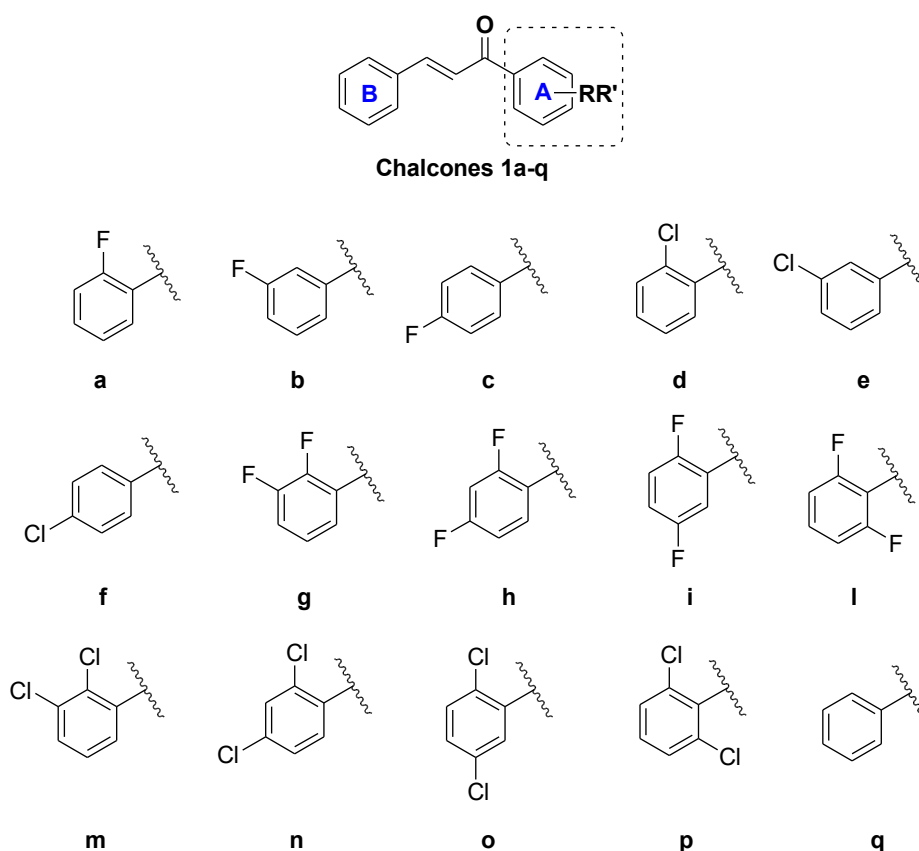


Figure I.1. Design of the novel chalcones **1a-q**.

The second part of the project addressed the critical solubility and bioavailability issues of rutin (RTN), a naturally occurring fluorescent flavonoid with strong antiangiogenic and anticancer potential. To enhance its pharmaceutical applicability, a supramolecular inclusion complex was developed using a novel, biocompatible PEGylated β -cyclodextrin polymer (β -CPCD) provided by the

Centre for Defence Engineering and Physical Sciences at Cranfield University (Shrivenham, UK). This strategy was designed to improve RTN's solubility and stability while maintaining its pharmacological activity, and to provide a suitable nanopatform for combined therapeutic and diagnostic applications. Special attention was given to the assessment of its antiangiogenic activity in the zebrafish model, a well-established and reliable *in vivo* platform for studying angiogenesis and validating antiangiogenic compounds.

The final part of this doctoral project focused on the development of chitosan-based nanoparticles (CS NPs) as carrier systems for breast cancer therapy, specifically intended for the encapsulation of a bicalutamide (BCL)/hydroxypropyl- β -cyclodextrin (HP- β -CD) inclusion complex. While BCL is an established antiandrogen in prostate cancer treatment, emerging preclinical and clinical evidence supports its potential repurposing for other androgen receptor-overexpressing tumors, such as AR-positive triple-negative breast cancer (AR+ TNBC). This formulation strategy was designed to achieve three primary objectives: ensure sustained and controlled release of BCL, enable selective tumor targeting through receptor-mediated mechanisms, and protect the drug from premature degradation. The inclusion of BCL in the form of a host-guest complex with HP- β -CD, rather than as a free drug, was required due to its extremely low aqueous solubility ($<4 \mu\text{g/mL}$), which prevents its direct incorporation into this type of carrier. CS NPs are generally more suitable for the encapsulation of hydrophilic compounds; therefore, the use of a cyclodextrin (CD), specifically HP- β -CD with its improved water solubility compared to the native form, allowed us to overcome this limitation. Moreover, once released from the NPs, BCL would be presented in a form more readily available for dissolution in biological fluids,

thereby improving its subsequent bioavailability in physiological fluids. CS NPs were engineered via ionotropic gelation using HA as a cross-linking agent to actively target CD44 receptors, which are frequently overexpressed in aggressive tumors such as TNBC and are associated with poorer prognosis.

Following the established methodology of this project, all nanosystems underwent comprehensive physicochemical and technological characterization to confirm their suitability as innovative delivery platforms.

A distinctive feature of this work is the selection of three representative models: a newly synthesized compound (halogenated chalcone), a natural product (RTN), and a clinically used drug (BCL). The selection of these three models was not arbitrary but guided by a broader scientific rationale. Investing in the design and synthesis of novel molecules, such as chalcone-based derivatives inspired by natural scaffolds, remains essential for expanding the arsenal of anticancer agents. Natural substances, when properly formulated, can become valuable allies in oncology, potentially complementing drugs already in use in combined therapies. At the same time, drug repurposing represents a powerful strategy to accelerate the identification of new treatment options by exploiting existing pharmacological knowledge.

These three case studies highlight a complementary strategy: designing new molecules inspired by natural scaffolds, drawing on nature, and repurposing existing drugs. When supported by innovative delivery technologies, such an integrated approach holds strong promise for advancing anticancer therapy toward more effective and better tolerated treatments.

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CHAPTER 1. CANCER AND NANOMEDICINE: MOLECULAR MECHANISMS AND ADVANCED THERAPEUTIC STRATEGIES

1.1. Molecular basis and physiopathology of cancer

Cancer is a pathological condition arising from the progressive accumulation of genetic and epigenetic alterations in somatic cells [1]. These alterations lead to uncontrolled proliferation, impaired differentiation, evasion of programmed cell death, and ultimately, tumor formation [2,3]. Central to this process are dysfunctions in two key classes of genes regulating cell cycle progression and genomic stability: proto-oncogenes and tumor suppressor genes [4–6].

Proto-oncogenes encode proteins that normally promote regulated cell growth and division. Upon mutation, they become oncogenes, driving constitutive cellular proliferation. Typically, a single gain-of-function mutation in one allele is sufficient for oncogenic activation [6]. A prominent example is the RAS oncogene, which encodes a GTP-binding protein integral to signal transduction pathways. Oncogenic RAS mutations impair GTP hydrolysis, resulting in constitutive protein activation. This leads to persistent signaling through downstream effectors that promote cell cycle entry, even in the absence of mitogenic stimuli [7].

Conversely, tumor suppressor genes act as negative regulators of the cell cycle, enabling proliferation arrest in response to DNA damage or cellular stress. Tumorigenesis generally requires the inactivation of both alleles (loss-of-function). The RB1 gene (chromosome 13), encoding the retinoblastoma protein (pRb), exemplifies this mechanism. Hypophosphorylated pRb binds and inhibits the E2F transcription factor, blocking the transcription of genes essential for DNA

replication and preventing the G1 to S phase transition. Phosphorylation by the cyclin D-Cdk4/6 complex releases E2F, permitting cell cycle progression. Mutations disrupting pRb function result in unrestrained E2F activity and uncontrolled proliferation. Another critical tumor suppressor is p53, often termed "the guardian of the genome". It monitors genomic integrity and orchestrates responses to damage, including cell cycle arrest, DNA repair facilitation, or induction of apoptosis [6].

Cancer originates from a single somatic cell that accumulates irreversible genetic alterations, leading to a clonal expansion of transformed cells. As the disease progresses, the continued accumulation of mutations gives rise to intratumoral heterogeneity, characterized by distinct subpopulations of cancer cells that differ in morphology, genotype, and behavior [8,9]. This evolutionary process is sustained by genomic instability, a defining feature of malignant transformation that promotes ongoing genetic diversification. As a result, critical processes such as cell cycle regulation, DNA repair, and immune surveillance are disrupted, while tumor cells acquire pro-angiogenic and invasive capabilities that facilitate progression and metastasis [9,10]. Virtually any somatic cell type holds the potential for malignant transformation upon acquiring a critical number and combination of driver mutations. As a result, cancers exhibit immense diversity in histology, clinical behavior, and anatomical site of origin [11–13].

The etiology of cancer is multifactorial, with most cases lacking a single identifiable cause [14,15]. While a limited proportion of tumors stem from endogenous factors, such as inherited mutations or hormonal imbalances (e.g., dysregulation of estrogen or progesterone) [16,17], the majority originate from exogenous carcinogens [14]. These include chemical carcinogens (e.g., aromatic

amines, aflatoxins, arsenic, asbestos, cadmium, chromium, nickel, and polycyclic aromatic hydrocarbons); physical agents like ultraviolet and ionizing radiation; biological agents, notably oncogenic viruses capable of host-genome integration; lifestyle factors such as tobacco use, chronic alcohol consumption, and high-fat diets; and underlying pathologies including liver cirrhosis, chronic peptic ulcers, and ulcerative colitis [14,18]. All of these elements, collectively referred to as carcinogens, trigger the initial phase of carcinogenesis, which typically progresses through three sequential stages: initiation, promotion, and progression [19] (Figure 1.1. [20]).

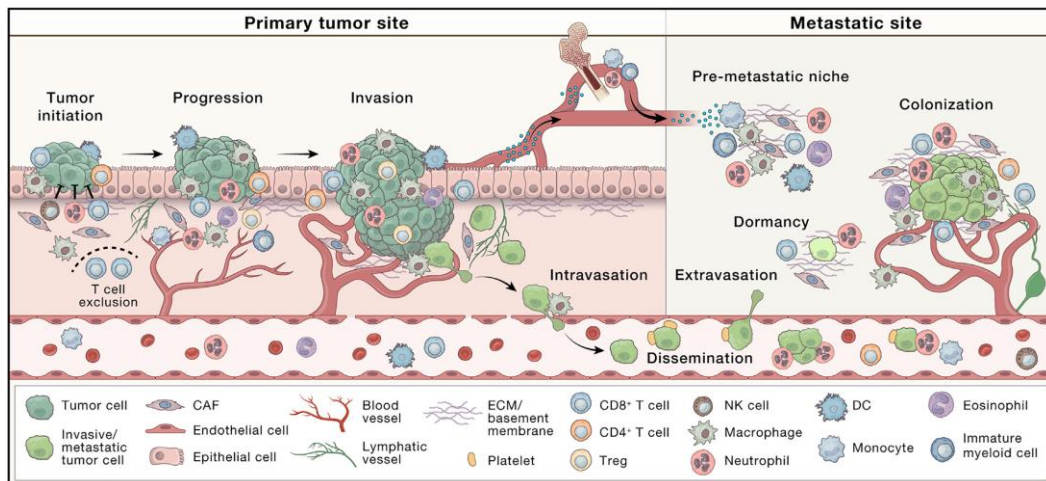


Figure 1.1. The figure provides a comprehensive representation of the multistep process of carcinogenesis, beginning with initiation, followed by promotion and progression at the primary tumor site. It further illustrates how these initial events can lead to metastatic dissemination, detailing key processes such as local invasion, intravasation into the circulation, transport through the bloodstream, extravasation, and eventual colonization of distant organs.

Initiation involves an initial genetic or epigenetic alteration that transforms a normal cell into a latent neoplastic state, not yet capable of autonomous replication

[19]. These alterations may directly modify DNA sequences or indirectly regulate gene expression through chromatin remodeling mechanisms such as histone modification and DNA methylation. When affecting regulatory regions like promoters, such changes can silence tumor suppressors or activate oncogenes, conferring an altered phenotype with newly acquired biological properties [19,21,22].

Promotion entails the clonal expansion of initiated cells driven by accumulated mutations or persistent proliferative stimuli. This stage culminates in clinically detectable tumors [19]. The mutations acquired during promotion fall into two functional categories: driver mutations, which are essential for maintaining malignancy and represent key therapeutic targets in precision oncology, and passenger mutations, which are not essential for tumor survival but may arise due to the genomic instability of cancer cells. These may also be downregulated or altered if recognized by the immune system, as part of immune evasion strategies [23].

Progression marks the transition to aggressive malignancy [19], characterized by phenotypic changes including loss of adhesion molecules. This enables tumor cells to detach from the primary site, invade adjacent tissues, and disseminate to distant organs, establishing the metastatic potential that defines advanced cancer [24].

Tumors are broadly classified as benign or malignant based on morphological features, proliferation dynamics, and interactions with surrounding tissues. Both types arise from cells that have gained autonomous replication through mutational events, yet their biological behavior diverges fundamentally [25].

Benign tumors typically exhibit slow, localized growth while largely retaining the functional identity of their tissue of origin. Though non-invasive, they may still

cause significant clinical complications. These include mass effects (e.g., compression of vital structures) or hormonal hyperactivity, exemplified by pituitary adenomas inducing acromegaly or thyroid adenomas leading to hyperthyroidism [26–29].

In stark contrast, malignant tumors consist of morphologically aberrant cells with aggressive biological properties [30,31]. They demonstrate infiltrative growth, penetrating adjacent tissues rather than displacing them, which complicates complete surgical excision and frequently leads to local recurrence from residual cells [31,32]. Beyond local invasion, their hallmark is metastatic dissemination through multiple pathways: hematogenous spread (via bloodstream), lymphatic permeation, transcoelomic migration (across body cavities), diffusion along anatomical canals, or direct extension to contiguous organs [33,34]. Systemically, malignancies often trigger cancer cachexia, a debilitating metabolic syndrome characterized by profound weight loss, muscle atrophy, and fatigue [35].

1.2. Tumor classification: histogenetic criteria and staging

The etiological classification of tumors remains impractical due to the multifactorial nature of cancer development and the variable tumorigenic potential of oncogenic factors, where a single agent can induce multiple tumor types, and conversely, a single tumor type may arise from diverse causes [13,36]. Therefore, histogenetic classification predominates, assigning each tumor a name that reflects its tissue of origin, regardless of whether it is benign or malignant [36]:

- **Benign tumors of the surface epithelium** (skin and mucosa): Typically present macroscopically as protruding lesions, either sessile or

pedunculated, arising from the tissue of origin. They are generally classified as polyps, papillomas, or warts [36].

- **Malignant tumors of the surface epithelium:** Often exhibit variable macroscopic appearances with poorly defined margins, firmness, and possible hemorrhage or necrosis that may lead to ulceration. These are referred to as carcinomas or epitheliomas [36].
- **Benign tumors of glandular epithelium:** Termed adenomas, these tumors usually preserve much of the gland's architecture. An example is the fibroadenoma of the breast, a common benign mixed tumor characterized by proliferation of both glandular epithelium and connective tissue [36].
- **Malignant tumors of glandular epithelium:** Known as adenocarcinomas when they show some gland-like differentiation despite cytological atypia; poorly differentiated tumors are classified more broadly as carcinomas [36].
- **Benign tumors of connective tissue:** Also called mesenchymal tumors, as mesenchyme is the embryonic tissue that gives rise to connective tissues, blood, and vessels. These tumors are named by combining a tissue-specific prefix with the suffix *-oma* (e.g., lipoma for adipose tissue, fibroma for fibrous connective tissue). Their cytological features typically resemble those of the original tissue [36].
- **Malignant tumors of connective tissue:** The malignant counterparts are collectively termed sarcomas (from Greek *sarkos*, meaning flesh, due to their appearance). When tissue origin is identifiable histologically, they are labeled with a blastic suffix; when undifferentiated, they are called anaplastic [36].

- **Tumors of the hematopoietic and lymphoid systems:** This classification is complex and continuously evolving due to advances in phenotypic characterization and genetic profiling. These neoplasms are broadly divided into leukemias (originating from hematopoietic stem cells) and lymphomas (arising from mature lymphocytes, either B or T lineage) and may involve other lineages such as monocytic or granulocytic. Both categories may present in acute or chronic forms: acute indicates a maturation arrest at an early developmental stage, while chronic refers to a block occurring at more advanced stages of differentiation [36,37].
- **Tumors of the melanophore system:** Derived from melanocyte-lineage cells responsible for melanin synthesis. Benign tumors include nevi, while malignant ones are melanomas, highly aggressive tumors that may arise on the skin, mucosa, or eye. Early diagnosis and identification of driver mutations (e.g., BRAF) are crucial for treatment planning [36,38].
- **Tumors of the central nervous system (CNS):** The classification of brain tumors diverges from the conventional benign/malignant dichotomy typically applied to neoplasms of other organs due to their unique anatomical constraints and biological behavior. Even histologically benign tumors can cause severe neurological damage or death by compressing critical structures or obstructing cerebrospinal fluid flow. Therefore, labeling CNS tumors as “benign” can be misleading [39].

CNS tumors are broadly categorized into primary and secondary types. Primary tumors originate within the brain or spinal cord and encompass a wide range of histological and molecular subtypes. In contrast, secondary tumors (or brain metastases) derive from extracranial malignancies and represent the most

common intracranial neoplasms in adults, frequently arising from primary tumors of the lung, breast, kidney, skin, or colon [40].

Recognizing the complexity of these neoplasms, the World Health Organization (WHO) adopts an integrated approach based on histopathology, molecular features, and clinical behavior. CNS tumors are classified by presumed cell of origin (e.g., astrocytic, oligodendroglial, ependymal, neuronal) and graded on a scale from I to IV, reflecting increasing degrees of aggressiveness, cellular atypia, mitotic activity, and prognosis. The use of molecular markers such as IDH mutations (isocitrate dehydrogenase mutations), 1p/19q codeletion (combined loss of the short arm of chromosome 1 and the long arm of chromosome 19), H3 K27M mutation (histone H3 lysine 27 to methionine mutation), and MGMT promoter methylation (O6-methylguanine-DNA methyltransferase promoter methylation) has become indispensable for accurate diagnosis, prognostic stratification, and treatment decision-making. The spectrum of CNS tumors includes low-grade gliomas (e.g., pilocytic astrocytoma, grade I), aggressive gliomas like glioblastoma (grade IV), and other entities such as meningiomas (arising from arachnoid cap cells), ependymomas, medulloblastomas (more common in children), and tumors of neuronal or mixed glioneuronal lineage [41].

Complementing histogenetic and histopathological classification, anatomical-clinical staging constitutes a fundamental pillar in the evaluation and management of malignant tumors. While histogenetic classification defines the tumor's origin and biological nature, staging quantifies its anatomical spread at the time of diagnosis, offering crucial insight into local, regional, and distant disease extension [42]. The universally adopted system for this purpose is the TNM classification

(Tumor, Node, Metastasis), developed and continuously updated by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC), which aims to standardize the assessment of tumor progression across different organ systems [43]. This system assigns each tumor an alphanumeric code that summarizes its key characteristics: the T category (Primary Tumor) describes the size and/or depth of local invasion, ranging from Tis (carcinoma *in situ*) to T1-T4, depending on increasing dimensions and infiltration into surrounding tissues. The N category (Regional Lymph Nodes) denotes the extent of metastatic involvement in regional lymph nodes, graded from N0 (no nodal metastasis) to N1, N2, or N3, reflecting increasing degrees of nodal burden and features such as the number of affected nodes or extracapsular spread. The M category (Distant Metastasis) identifies the presence (M1) or absence (M0) of metastases in distant organs or non-regional lymph nodes. The MX designation, once used when metastases were not assessable, has become largely obsolete with modern advances in imaging and diagnostic accuracy [44]. By integrating the T, N, and M components, an overall Clinical Stage Group is assigned, expressed in Roman numerals (I-IV), which reflects the tumor's anatomical extent and aggressiveness. For example, a T1N0M0 tumor indicates a small, localized lesion without nodal or distant involvement, typically corresponding to Stage I and associated with a favorable prognosis and curative intent. In contrast, a T4N3M1 tumor denotes an advanced neoplasm with extensive local invasion, substantial nodal involvement, and distant metastases, corresponding to Stage IV, a condition generally requiring systemic and predominantly palliative therapeutic approaches [44]. An example of how this staging framework is applied in clinical practice is illustrated in Figure 1.2. [45], which depicts the TNM-based classification in breast cancer.

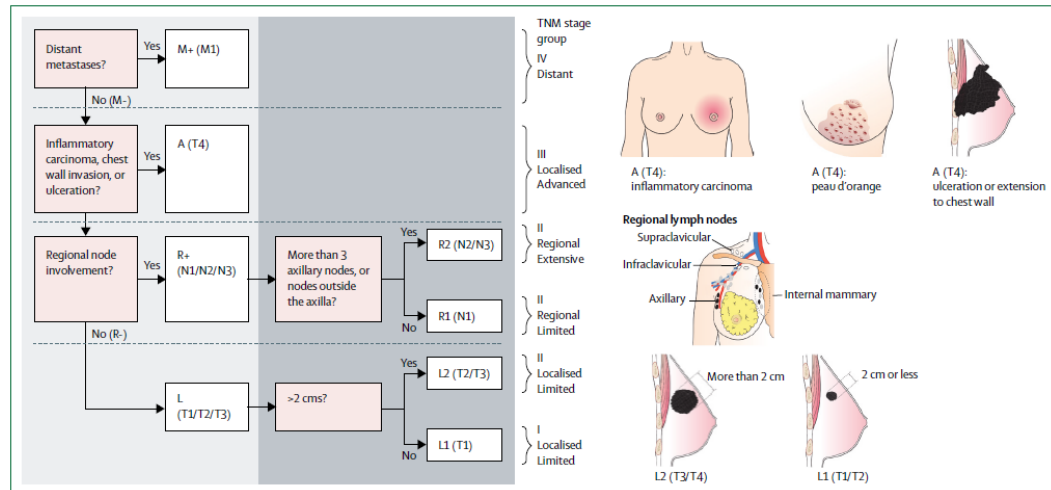


Figure 1.2. Example of clinical staging using the TNM classification system in breast cancer. The diagram illustrates how the combination of tumor size (T), regional lymph node involvement (N), and the presence of distant metastases (M) determines the overall stage group (I-IV).

The TNM staging system plays a central role in oncology, as it offers a solid framework for estimating prognosis, guiding treatment choices (whether curative or palliative), and plan long term follow-up. Furthermore, it provides a shared vocabulary that enhances coordination among multidisciplinary teams, including pathologists, surgeons, medical and radiation oncologists, and radiologists. For all these reasons, the TNM classification represents, alongside histological and molecular characterization, a cornerstone of modern oncology [42].

1.3. Cancer treatments: from conventional therapies to nanomedicine

Malignant neoplasms currently represent one of the greatest challenges in global public health. According to the latest estimates from the WHO, malignant tumors stand out as the second leading cause of death worldwide, surpassed only by cardiovascular diseases, with 10 million deaths per year [46]. The global incidence

of neoplasms continues to rise, driven by a complex interplay of demographic factors, primarily the aging population, environmental exposures including pollution and carcinogenic substances, lifestyle behaviors, and genetic predispositions [47,48]. In this evolving landscape, the therapeutic approach to oncology patients has progressively shifted towards increasingly multimodal and personalized strategies guided by biological and molecular criteria [49].

Surgical resection remains the cornerstone of curative treatment for localized solid tumors, provided that distant metastases are absent and vital structures are not involved [50]. Radiotherapy, particularly advanced techniques like intensity modulated radiation therapy (IMRT), image-guided radiotherapy (IGRT), and stereotactic body radiation therapy (SBRT), plays a crucial role both in curative settings and in palliative care, where symptom relief and quality of life improvement are prioritized [51]. Alongside these traditional therapies, a breakthrough has been achieved with the advent of biological therapies and immunotherapy, which have significantly improved the prognosis of many advanced malignancies [52]. Monoclonal antibodies targeting tumor-specific antigens and immune checkpoint inhibitors (ICIs), such as anti-PD-1/PD-L1/CTLA-4 antibodies, modulate the immune system to recognize and attack cancer cells selectively, leading to prolonged disease control and, in some cases, durable remission [53]. Additionally, adoptive cell therapies like chimeric antigen receptor T-cell (CAR-T) therapy offer promising personalized immunotherapeutic options for hematologic malignancies [54].

Nonetheless, chemotherapy remains the backbone of cancer management. Systemic chemotherapy is employed at various stages of the treatment pathway (neoadjuvant, adjuvant, concurrent, or palliative) and is based on the use of

cytotoxic agents that disrupt cancer cell proliferation through a variety of mechanisms [55]. Key chemotherapeutic agents include antimetabolites like 5-fluorouracil (5-FU), which inhibit nucleotide synthesis and are widely used in colorectal cancer [56]. Taxanes, such as paclitaxel (PTX) and docetaxel (DXT), interfere with microtubule function to block mitosis, while anthracyclines like doxorubicin (DOX) intercalate into DNA and inhibit topoisomerase II [57].

Despite their established clinical efficacy, conventional chemotherapeutic agents are associated with several critical limitations, most notably their lack of specificity for malignant cells [58]. Since these drugs cannot discriminate between neoplastic and healthy rapidly dividing cells, their use is often accompanied by severe systemic toxicity, including myelosuppression, mucositis, alopecia, and cumulative dose-limiting side effects such as the cardiotoxicity commonly observed with anthracyclines [58,59]. Furthermore, many promising anticancer agents suffer from poor physicochemical properties: low aqueous solubility, slow dissolution rates, and chemical instability. These properties negatively affect their pharmacokinetic profile, limit their bioavailability, and reduce overall therapeutic efficacy. Drugs with poor water solubility, for example, often demonstrate erratic absorption and inadequate tumor penetration. Another important issue is the short residence time of the active pharmaceutical ingredient (API) within the therapeutic window, necessitating frequent dosing at regular intervals to maintain effective plasma concentrations [60,61].

In this complex therapeutic landscape, one of the most promising strategies to overcome these limitations involves the development of advanced DDSs [62]. This approach falls within the scope of nanomedicine and leverages nanoscale carriers capable of delivering therapeutic agents directly to the tumor site, thereby

minimizing off-target effects and reducing systemic toxicity. These nanocarriers improve the solubility, stability, and bioavailability of encapsulated drugs, and can be engineered for controlled release and tumor-specific delivery [60,63,64].

A key advantage of nanocarriers in oncology is their ability to exploit the Enhanced Permeability and Retention (EPR) effect. This phenomenon allows nanosystems to preferentially accumulate in tumor tissue due to the leaky vasculature and poor lymphatic drainage typically present in solid tumors. This passive targeting mechanism further increases the therapeutic index of nanomedicine formulations by enhancing drug concentration at the tumor site while minimizing exposure to healthy tissues [63,65].

Among the most widely studied nanomedicine platforms are inclusion complexes with CDs [66], as well as nanostructured carriers such as liposomal vesicles, lipid-based nanoparticles (NPs) [67] and polymeric NPs [68]. These delivery systems offer significant advantages in terms of protecting labile compounds, prolonging systemic circulation time, enabling co-delivery of multiple agents, and facilitating combinatorial therapeutic strategies [69]. Notably, some of these nanocarrier-based formulations have already been approved for clinical use and are available on the market. A well-known example is pegylated liposomal DOX, marketed as Doxil® (Caelyx® in Europe), which exhibits reduced cardiotoxicity and improved pharmacokinetic properties compared to conventional DOX, owing to prolonged circulation and enhanced tumor accumulation via the EPR effect [70].

Other clinically approved nanoformulations include Abraxane®, an albumin-bound NP formulation of PTX, that addresses the solubility issues of traditional PTX. This 130-nm particle formulation utilizes albumin's natural transport properties to enhance tumor penetration and accumulation. Nab-PTX demonstrates

improved pharmacokinetics, increased antitumor efficacy, and a better safety profile compared to conventional PTX. Clinical trials have shown higher response rates, longer time to tumor progression, and improved survival in some patient groups. Nab-PTX has been approved for treating metastatic breast cancer, non-small cell lung cancer, and pancreatic adenocarcinoma [71,72].

Another example is Onivyde®, a nanoliposomal formulation of irinotecan approved for the treatment of metastatic pancreatic cancer, which provides greater drug stability and controlled release [73]. Marqibo®, a liposomal formulation of vincristine sulfate, is approved for acute lymphoblastic leukemia and enhances delivery of vincristine while minimizing neurotoxicity [74]. Additionally Vyxeos®, a liposomal combination of daunorubicin and cytarabine in a fixed molar ratio, has been approved for the treatment of high-risk acute myeloid leukemia, demonstrating improved survival outcomes compared to the free drug combination [75].

These clinically translated examples underscore the potential of nanotechnology-based delivery systems to address major limitations of conventional chemotherapeutics, particularly by improving pharmacokinetics, reducing systemic toxicity, and enhancing tumor-specific drug accumulation.

1.4. Nanomedicine and nanoparticles

Nanomedicine is a branch of nanotechnology that, through a multidisciplinary approach, enables the development and application of nanoscale devices, systems, and materials for medical purposes [76]. In recent years, the European Science Foundation (ESF) has conducted an in-depth analysis of the state of the art in nanomedicine, providing through its Permanent Ethics Committee a widely

accepted definition: “*Nanomedicine is the science and technology of diagnosing, treating, and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body* [77]”.

Within the biomedical field, nanomedicine finds applications in many areas, including the development of advanced nanomaterials and the use of nanotechnology-based biosensors for diagnostics [78]. However, its most transformative impact has emerged in the pharmacological field where nanomedicine plays an increasingly central role, first through the design of sophisticated DDSs [79] and, more recently, through gene therapy, enabling the targeted delivery of nucleic acids (DNA, mRNA, and siRNA) directly into damaged cells [80].

Among the most widely studied platforms in this context are NPs, which are solid or colloidal structures typically ranging from 1 to 100 nm in diameter, as defined by experts from the International Organization for Standardization (ISO) [81]. These systems can be formulated to carry either hydrophilic or lipophilic drugs depending on their composition [82]. Their nanoscale dimensions allow them to reach virtually any biological target, enabling both local and systemic therapeutic action. Thanks to their unique physicochemical properties, NPs not only enhance the aqueous solubility of poorly soluble drugs, thereby improving their bioavailability and therapeutic efficacy, but also offer protection against degradation, prolonging the drug's circulation time [83]. Moreover, they can modulate tissue distribution, facilitate intracellular uptake, and allow for site-specific delivery of the active compound. As a result, the required therapeutic dose can be reduced, minimizing off-target toxicity and limiting adverse effects on

healthy tissues [84]. Importantly, these systems also play a key role in controlling drug release kinetics, allowing for sustained and regulated drug delivery profiles tailored to specific therapeutic needs [82].

The biological behavior and therapeutic efficacy of these NPs are critically influenced by several key parameters, including:

- **Composition, Morphology, and Dimensionality:** NPs exhibit vast diversity in composition, morphology, and dimensionality. They can be spherical, tubular, filamentous, or irregular in shape; crystalline or amorphous; dispersed, agglomerated, or aggregated; with smooth or highly irregular surfaces. These properties profoundly affect their biological interactions [85,86]. Based on their geometry, NPs are classified as zero-dimensional (e.g., quantum dots), one-dimensional (e.g., graphene sheets), two-dimensional (e.g., carbon nanotubes), or three-dimensional (e.g., gold NPs) [87,88].
- **Size:** The size of NPs plays a crucial role in their ability to cross biological barriers. For instance, Sonavane *et al.* demonstrated that gold NPs with a diameter of 15 nm achieved significantly greater tissue penetration than larger particles of 50, 100, or 200 nm [89]. Additional studies confirm that smaller NPs are more effective at traversing mucosal barriers after mucosal administration [90,91].
- **Morphology:** The shape of NPs strongly affects their delivery efficiency and pharmacokinetics [92]. Research highlights that spherical NPs with surface angles greater than 45° exhibit reduced macrophage uptake due to their highly curved geometry, which decreases recognition by the immune

system. Specifically, Champion *et al.* found that nearly perfect spheres demonstrate superior transport properties *in vivo* [93,94].

- **Surface Charge (Zeta Potential, ζ):** High absolute ζ values (± 30 mV) enhance colloidal stability by preventing particle aggregation [95]. Negatively charged NPs tend to evade macrophage uptake, whereas positively charged particles promote stronger interactions with cell membranes but are cleared more rapidly by the mononuclear phagocyte system (MPS) [96].
- **Surface Functionalization:** Modifying NP surfaces with targeting ligands such as monoclonal antibodies [97], folic acid (which targets folate receptor-overexpressing tumors) [98], or hyaluronic acid (HA, which targets the CD44 receptor overexpressed in many cancers) [99] allows active targeting. This approach facilitates selective recognition and binding to target cells, followed by receptor-mediated endocytosis, resulting in enhanced intracellular drug accumulation [100]. An additional fundamental advantage is the ability to prolong systemic circulation time through “stealth” coatings. Functionalization with hydrophilic polymers like polyethylene glycol (PEG) reduces opsonization and subsequent clearance by the MPS. PEGylation forms a steric barrier that significantly improves NP stability and systemic half-life. Studies demonstrate that PEG-coated NPs accumulate in tumors at more than twice the efficiency of uncoated counterparts [101]. Other hydrophilic polymers such as dextran and heparin similarly improve biocompatibility and pharmacokinetic profiles [102].

NPs designed for pharmaceutical use, and thus for human administration, are typically composed of biocompatible and biodegradable materials, including natural polymers (e.g., gelatin, albumin), synthetic polymers (e.g., polylactic acid, polycyanoacrylates), solid lipids, or inorganic substances (Figure 1.3. [103]). Once administered, these NPs release their APIs *via* mechanisms such as diffusion, matrix swelling, erosion, or enzymatic degradation, enabling controlled, targeted, and sustained drug delivery [104].

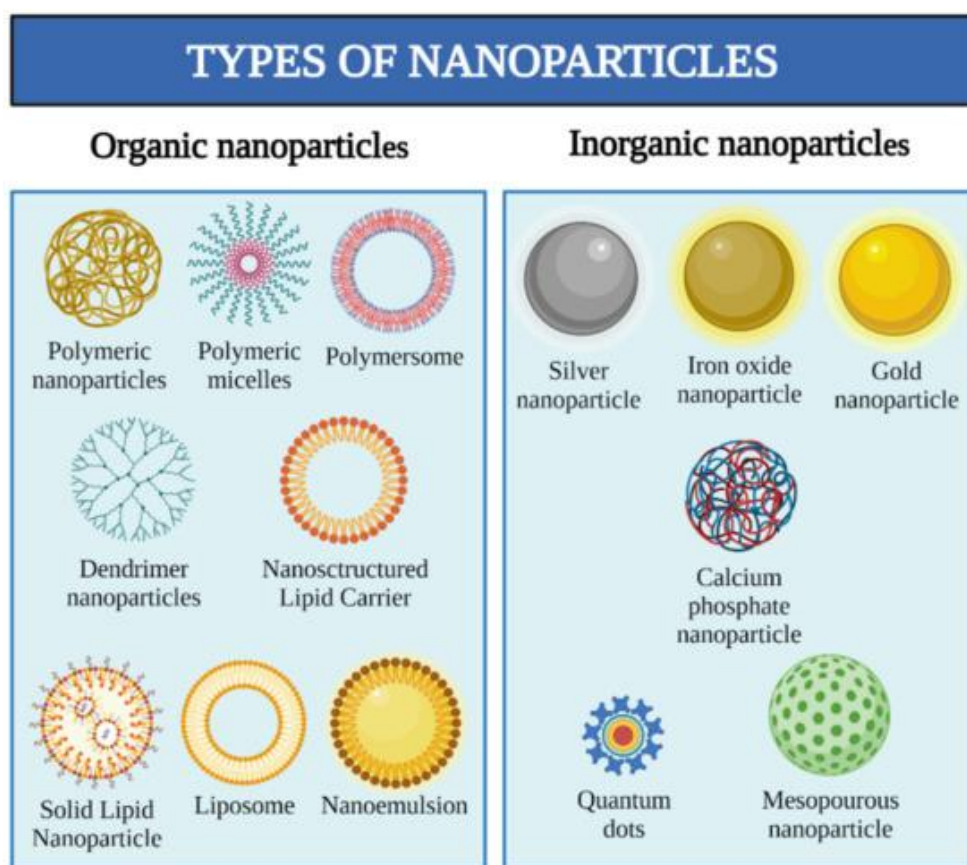


Figure 1.3. Schematic representation of the main classes of nanoparticles employed in nanomedicine.

The most commonly employed types of NPs in pharmaceutical applications, along with their respective preparation methods, will be discussed in the following sections.

1.4.1. Polymeric nanoparticles

The introduction of polymeric NPs into the pharmaceutical field dates back to the late 1970s. Over the past few decades, they have undergone significant development, emerging as highly versatile and effective DDs. Polymeric NPs are solid colloidal particles composed of biodegradable and biocompatible polymers capable of encapsulating both hydrophilic and hydrophobic drugs, as well as macromolecules such as nucleic acids and proteins [105]. The API can be physically dispersed, molecularly dissolved, entrapped, encapsulated, or bound within the polymeric matrix [106,107].

Depending on the preparation technique, polymeric NPs are generally categorized into nanospheres or nanocapsules [108] (Figure 1.4. [109]). Nanospheres are matrix systems in which the drug is homogeneously distributed throughout the polymer. In contrast, nanocapsules consist of a reservoir system where the drug is confined within a core surrounded by a polymeric membrane, which may consist of one or more polymeric layers. This membrane acts as a diffusion barrier, allowing for a controlled and sustained release of the encapsulated compound over time [106–108].

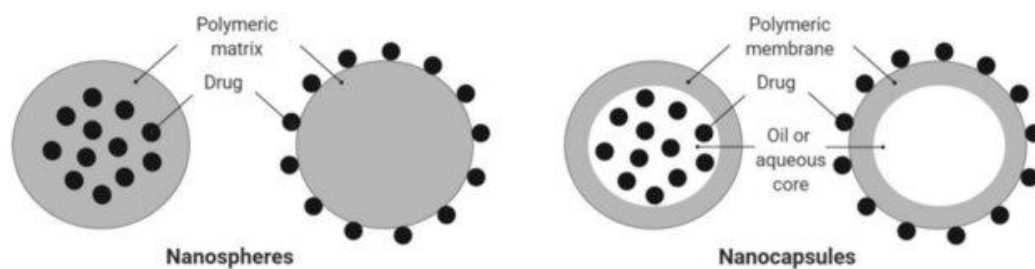


Figure 1.4. Structural illustration of nanospheres and nanocapsules, highlighting differences in drug distribution and architecture.

Drug release from polymeric NPs typically follows two main mechanisms: (a) surface erosion of the polymer matrix and subsequent diffusion of the drug, or (b) hydration of the polymer followed by drug diffusion through the swollen network [110]. In the case of nanospheres, drug release often follows a biphasic profile, characterized by an initial burst due to the presence of loosely bound drug on the particle surface, followed by a slower, sustained release from the matrix interior. Nanocapsules, on the other hand, generally exhibit a more gradual and prolonged release, governed by the diffusional properties and degradation kinetics of the surrounding polymeric membrane [111].

Polymeric NPs can be synthesized using a wide range of natural or synthetic polymers. These macromolecules are composed of repeating monomeric units covalently linked together and can be classified according to their chemical structure (e.g., block, random, graft copolymers) or composition (e.g., homopolymers or copolymers). Key factors must be carefully evaluated when selecting an appropriate polymer for NP formulation, as these significantly influence not only the physicochemical characteristics of the final system but also the pharmacokinetic and pharmacodynamic behavior of the encapsulated drug [112]. One of the primary aspects to consider is solubility, which is governed by

the nature of the functional groups present along the polymer backbone. Hydrophilic groups, such as hydroxyl or carboxyl moieties, typically confer water solubility, whereas hydrophobic characteristics are imparted by alkyl chains or aromatic rings. The aqueous solubility of the monomeric units plays a critical role in determining the *in vitro* and *in vivo* clearance of the resulting polymer. For example, polymers synthesized from water-soluble monomers are usually eliminated from the organism within 7 to 14 days, whereas those derived from poorly soluble monomers can persist in the body for up to eight weeks [113]. In addition to solubility, biocompatibility and systemic safety represent indispensable criteria. The selected polymer must not induce cytotoxic, immunogenic, or inflammatory responses upon administration and should be well-tolerated by the organism over the entire duration of its presence in the body [110,114]. Another critical parameter is the potential for functionalization. The polymer should possess, or be amenable to modification to introduce, reactive groups such as primary amines, carboxyls, or thiols. These groups enable the covalent attachment of targeting moieties capable of imparting organotropism or cellular specificity to the NP system [115]. Finally, biodegradability is a desirable feature in polymer selection. The degradation process depends on the presence of labile chemical bonds, such as esters, amides, or anhydrides, which can be hydrolyzed or enzymatically cleaved under physiological conditions [116]. Non-biodegradable polymers, due to their potential for long-term accumulation in tissues, are generally avoided unless the system is designed for short-term use or is physically removed after drug delivery, as in the case of subcutaneous implants or orally administered systems with limited residence time in the gastrointestinal tract [117].

Among synthetic polymers, those most frequently used in drug delivery include poly(D,L-lactic acid) (PLA), poly(D,L-glycolic acid) (PGA), and their copolymer poly(lactic-co-glycolic acid) (PLGA). Originally developed as resorbable surgical sutures, these polymers have become central in NP-based therapeutics due to their excellent biodegradability and biocompatibility [118,119]. Their degradation rate and drug release kinetics can be finely tuned by adjusting the molecular weight and the lactic/glycolic acid ratio, thus enabling controlled and prolonged release profiles over weeks or even months [120].

In addition to synthetic materials, a wide range of natural polymers has also been explored for NPs fabrication. These include proteins (e.g., gelatin, albumin, collagen), polysaccharides (e.g., starch, cellulose, dextran, HA), and polyesters (e.g., polyhydroxyalkanoates). Owing to their natural origin and physiological compatibility, these materials are abundant, cost-effective, and generally recognized as safe (GRAS) by regulatory authorities [121].

Among natural polymers, chitosan (CS, Figure 1.5. [122]) stands out as one of the most promising materials for pharmaceutical and biomedical applications due to its remarkable versatility, biocompatibility, and multifunctionality [123]. Derived from the partial deacetylation of chitin, a polysaccharide found in the exoskeletons of crustaceans, insects, and the cell walls of fungi, CS is composed of repeating units of D-glucosamine and *N*-acetyl-D-glucosamine linked through $\beta(1\rightarrow4)$ glycosidic bonds [122]. It is approved by the U.S. Food and Drug Administration (FDA) and classified as GRAS, confirming its excellent safety profile [124].

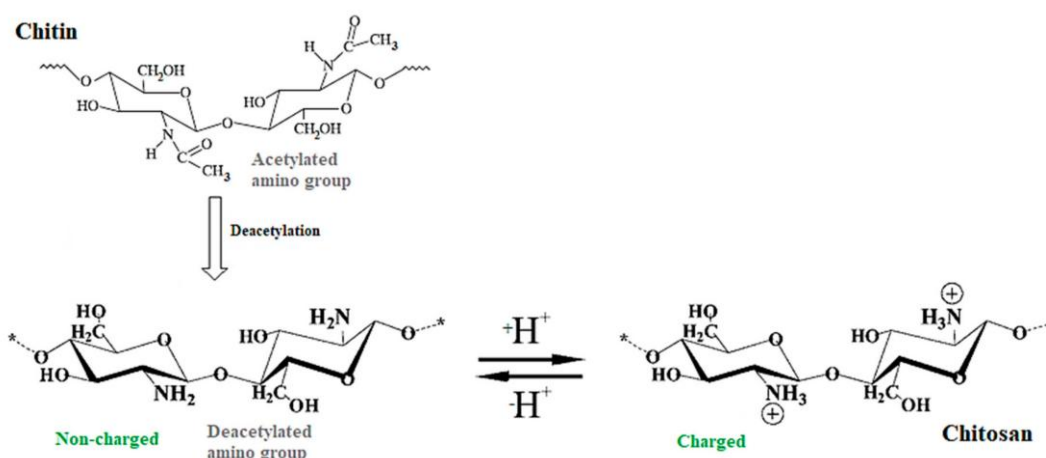


Figure 1.5. Chemical structures of chitin and chitosan. Chitin consists of *N*-acetyl-D-glucosamine units linked via $\beta(1\rightarrow4)$ glycosidic bonds. Partial deacetylation of chitin yields chitosan, composed of D-glucosamine and *N*-acetyl-D-glucosamine units. Also shown are the protonated and non-protonated forms of chitosan, which depend on environmental pH. Protonation of the amino groups at acidic pH ($pK_a \sim 6.3$) imparts cationic character and solubility in aqueous acidic media.

The physicochemical properties of CS, particularly its solubility, degradability, and biological activity, are governed by two critical parameters: the degree of deacetylation (DD), which must exceed 60% for biomedical applications, and the molecular weight, typically ranging from 300 to 1000 kDa [125–127]. A higher DD and molecular weight confer greater structural stability and slower enzymatic degradation, making CS particularly suitable for applications that require sustained release or long-term structural integrity, such as tissue engineering [128].

CS is soluble in mildly acidic aqueous media such as acetic acid, where the protonation of its amine groups ($pK_a \sim 6.3$) imparts a positive charge to the polymer [129]. This cationic nature grants CS excellent mucoadhesive properties, as it can form strong electrostatic interactions with the negatively charged sialic acid residues and mucins found on epithelial surfaces. These interactions enhance drug

retention at mucosal sites and improve both absorption and therapeutic efficacy [130].

One of the earliest reported applications demonstrating this property was the intranasal delivery of insulin using CS-based formulations. Illum *et al.* (1994) demonstrated that CS could significantly enhance the systemic absorption of insulin when administered intranasally. This was attributed to its ability to transiently open tight junctions in the nasal epithelium, facilitating paracellular transport of the peptide across the mucosa [131].

Beyond its role as a drug carrier, CS is also recognized as a bioactive polymer. It exhibits a wide range of biological functions including hemostatic [132], immunomodulatory [133], and antimicrobial [125] activities. Its antimicrobial action is thought to arise primarily from electrostatic interactions between its protonated amine groups and the negatively charged bacterial membranes, leading to membrane disruption, cytoplasmic leakage, and ultimately cell death. This effect is pH-dependent and significantly reduced at neutral pH due to deprotonation and decreased solubility [134]. Additional mechanisms have been proposed, including suppression of bacterial mRNA transcription [135], chelation of essential metal ions such as calcium and magnesium [136], and nutrient blockade by forming a physical barrier around microorganisms [137].

CS's activity depends on its physicochemical characteristics. A lower DD enhances its antimicrobial potency but also leads to faster degradation and potentially greater inflammatory responses. Conversely, higher DD correlates with reduced degradability and lower immunogenicity [138]. These parameters must therefore be tailored according to the intended application.

To overcome limitations such as poor solubility at physiological pH and to enhance its mucoadhesive potential, various semi-synthetic derivatives have been developed. Trimethyl CS, for instance, exhibits permanent positive charge across a wider pH range and improved solubility [139], while N-acetylcysteine CS derivatives have shown promising efficacy in ophthalmic treatments such as Lacrimera®, a medicated eye drop for dry eye syndrome [140].

CS is particularly advantageous in the field of nanomedicine. NPs based on CS are most commonly obtained through ionotropic gelation, a mild and solvent-free process that enables the formation of nanoscale carriers under aqueous conditions [141]. The details of this preparation method will be discussed in the following section.

Among the various biomedical applications of CS NPs, anticancer drug delivery has emerged as one of the most extensively studied areas [142]. For example, in a study by Jain *et al.* (2008), CS NPs loaded with 5-FU were engineered via ionotropic gelation using tripolyphosphate as a cross-linker. To enhance tumor specificity, the NPs were surface functionalized with HA, targeting CD44 receptors that are overexpressed in colon cancer cells. The resulting HA-conjugated CS NPs, with an average size of 150 ± 3.4 nm and positive surface charge, exhibited 7.9-fold higher cellular uptake in HT-29 colon cancer cells and a 2.6-fold increase in cytotoxicity compared to free 5-FU ($IC_{50} = 2.31$ μ g/mL vs. 6.02 μ g/mL). In addition to their enhanced therapeutic efficacy, CS NPs demonstrated excellent mucoadhesive properties and improved permeation across intestinal epithelium *in vitro*. The release profile of 5-FU from the NPs showed a sustained and pH-dependent pattern, with faster drug release observed at acidic pH, simulating the tumor microenvironment [143].

These findings highlight the potential of CS-based nanocarriers, particularly when combined with active targeting ligands, to achieve site-specific drug delivery, prolonged circulation time, enhanced therapeutic efficacy, and reduced systemic toxicity [142]. Thanks to its ability to be chemically modified, its compatibility with various administration routes, and its potential for integration with other carriers such as CDs, CS represents an ideal platform for the development of next-generation DDSs for the treatment of numerous diseases, especially those of neoplastic origin [142,144].

1.4.2. Preparation methods of polymeric nanoparticles

The selection of a suitable preparation method for drug-loaded polymeric NPs depends on several critical parameters, such as the physicochemical characteristics of the active compound, the specific therapeutic target, the nature of interactions between the drug and the polymeric matrix, and the desired colloidal profile of the final system. Since the same polymer may result in NPs with significantly different morphological and functional properties depending on the production technique (such as particle size, surface potential, and drug release behavior) it is essential to define the intended purpose of the formulation before selecting the fabrication method [141].

Generally, two main strategies are employed in the synthesis of polymeric NPs: (1) methods based on the *in situ* polymerization of monomeric units, and (2) methods that utilize preformed polymers which are subsequently assembled into nanoscale structures [145].

In the case of methods involving polymerization of monomers, the main techniques include:

- **Emulsion polymerization** (Figure 1.6. [141]): This technique involves the dispersion of a hydrophobic monomer in an aqueous medium containing a surfactant and a polymerization initiator. When the monomer encounters the initiator polymerization is initiated. The solid particles can be formed during the polymerization or after it. This method is applicable to the synthesis of various NPs, such as poly(methyl methacrylate) (PMMA) and poly[styrene/(dimethylamino)ethyl methacrylate] [141].

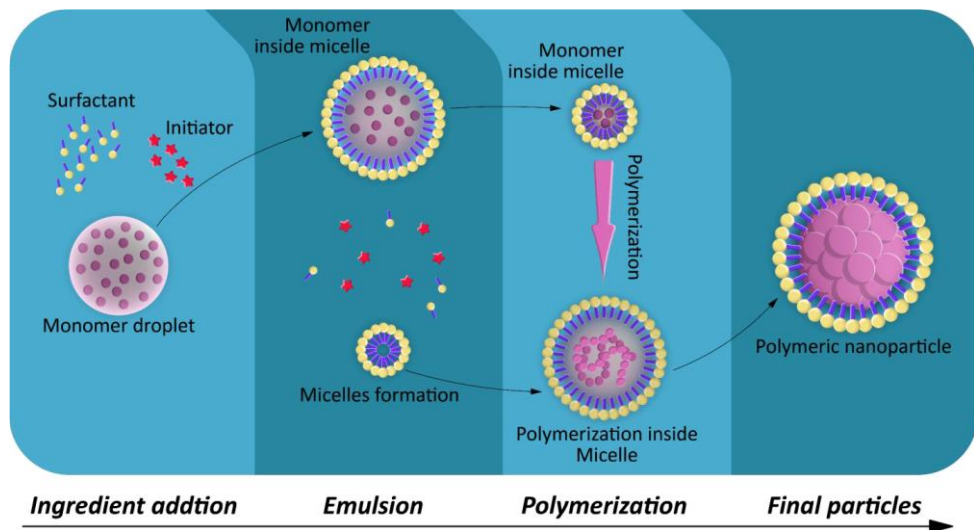


Figure 1.6. Emulsion polymerization. A water-immiscible monomer is dispersed in an aqueous medium containing a surfactant and an initiator; polymerization occurs upon interaction with free radicals or ionic initiators, leading to nanoparticle formation.

- **Miniemulsion polymerization** (Figure 1.7. [141]): This method involves the preparation of two distinct phases: an oil phase containing the monomer and an aqueous phase containing a surfactant and a water-soluble initiator. High-shear devices such as homogenizers or ultrasonication systems are used to produce monomer droplets (typically 50-500 nm) stabilized within

the aqueous medium. Only small amounts of surfactant are needed due to the stabilization provided by co-stabilizers. Polymerization occurs within these droplets via a chain-growth mechanism [146]

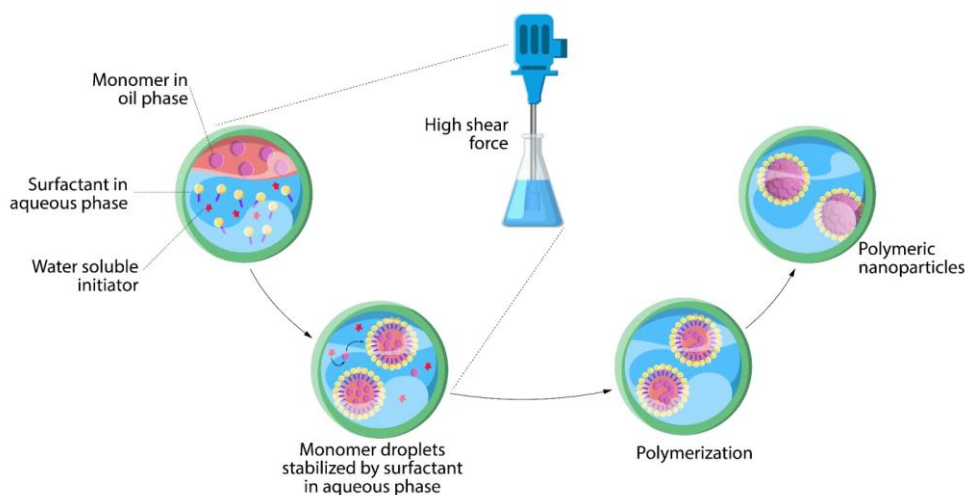


Figure 1.7. Miniemulsion polymerization. An oil phase containing the monomer is emulsified in water with a surfactant and a co-stabilizer under high shear; stable nanodroplets undergo polymerization to yield monodisperse nanoparticles.

In the case of methods based on the dispersion of preformed polymers, the following approaches are commonly used:

- **Single and double emulsion-solvent evaporation** (Figure 1.8. [141]):

These are the most widely used techniques for formulating NPs from hydrophobic polymers and were among the first to be developed both at the lab and industrial scale due to their reproducibility. In the single emulsion method, a polymer is dissolved in a volatile organic solvent and emulsified into an aqueous phase containing a stabilizer. The organic solvent is then evaporated, leading to the formation of solid NPs. Depending on the

solubility of the drug, it can be loaded into either phase, making this approach suitable for both hydrophilic and hydrophobic compounds. Typical polymers include PLA, PLGA, and poly(ϵ -caprolactone) (PCL), dissolved in solvents such as ethyl acetate, dichloromethane, or chloroform. The resulting droplets typically range from 20 to 200 nm [141,146,147]. In the double emulsion method (e.g., w/o/w), an internal aqueous phase is emulsified into a polymer-containing organic phase, and this is further emulsified into a second aqueous phase. This approach is particularly beneficial for encapsulating hydrophilic molecules, which are prone to low entrapment in standard o/w systems [141].

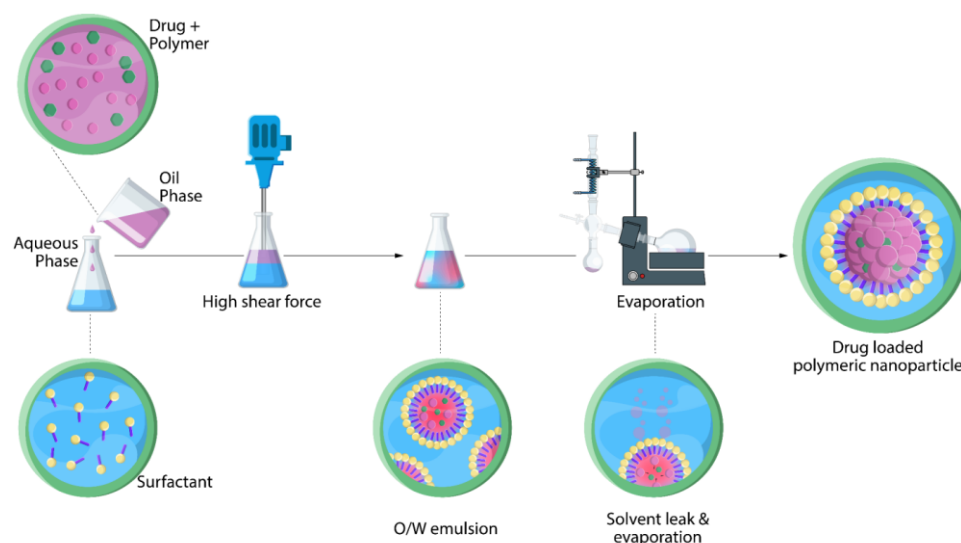


Figure 1.8. Single emulsion-solvent evaporation. In this method, a polymer is dissolved in a volatile organic solvent to form the dispersed phase, which is then emulsified into an aqueous phase containing a stabilizer. Upon evaporation of the solvent under stirring or reduced pressure, the polymer precipitates, leading to the formation of solid nanoparticles.

- Emulsion-diffusion** (Figure 1.9. [141]): This method offers several advantages, including the use of pharmaceutically acceptable solvents, high encapsulation efficiency (EE), reproducibility, and suitability for scale-up. It is based on the formation of an o/w emulsion using a partially water-miscible organic solvent containing the polymer and drug, which is then emulsified into an aqueous phase with a surfactant. Upon dilution with water, the organic solvent diffuses into the external phase, causing the precipitation of NPs. The solvent is subsequently removed via evaporation under reduced pressure. Ethyl acetate is commonly used due to its partial water miscibility [148]. This method generally yields nanospheres, but nanocapsules can be produced by incorporating small amounts of oil into the organic phase [149].

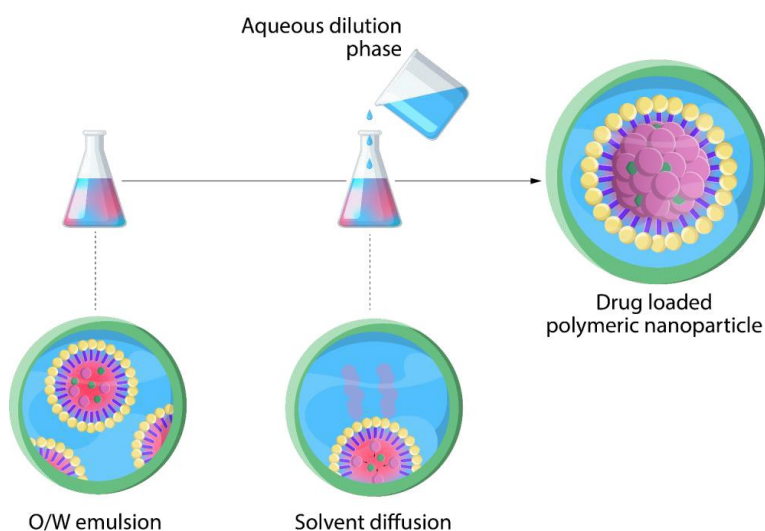


Figure 1.9. Emulsion-diffusion. An emulsion is prepared using a partially water-miscible solvent; upon dilution with excess water, the solvent diffuses into the aqueous phase, inducing nanoparticles precipitation.

- **Nanoprecipitation** (solvent displacement, Figure 1.10. [141]): First described by Fessi *et al.* [150], this technique is widely known for its simplicity and rapid execution. It involves dissolving both the polymer and the drug in a water-miscible organic solvent, which is subsequently added to an aqueous phase where the polymer is not soluble. As the solvent's ability to keep the polymer in solution diminishes, due to dilution or solvent diffusion, the polymer rapidly precipitates, resulting in the formation of NPs. The remaining solvent is then eliminated by evaporation or filtration [151]. Although the method is relatively simple and energy-efficient, the ratio between solvent and non-solvent, stirring speed, and ionic strength must be carefully optimized to obtain NPs with the desired physicochemical properties [152].

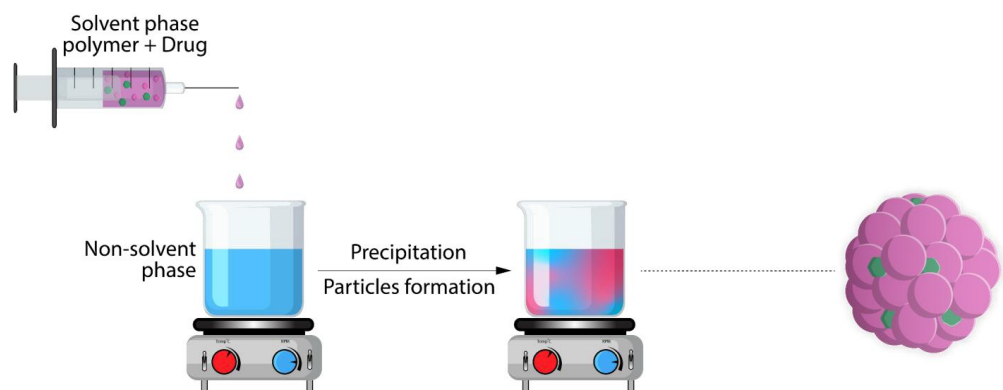


Figure 1.10. Nanoprecipitation (solvent displacement). A polymer is dissolved in a water-miscible organic solvent and added to water, where it precipitates due to reduced solvent quality, forming nanoparticles rapidly and under mild conditions.

- **Salting-out** (Figure 1.11. [141]): This method eliminates the use of surfactants and chlorinated solvents by exploiting the reduced miscibility

between an organic solvent and water in the presence of a high concentration of salt. The polymer is dissolved in a water-miscible organic solvent, while the aqueous phase is saturated with salting-out agents such as magnesium chloride, calcium chloride, or sucrose. After emulsification, dilution of the system decreases ionic strength, causing the solvent to migrate into the aqueous phase and resulting in NPs formation. The salt can then be removed through extensive washing [141]. The size of the resulting NPs typically ranges from 100 to 900 nm, depending on polymer concentration and aqueous phase volume [153].

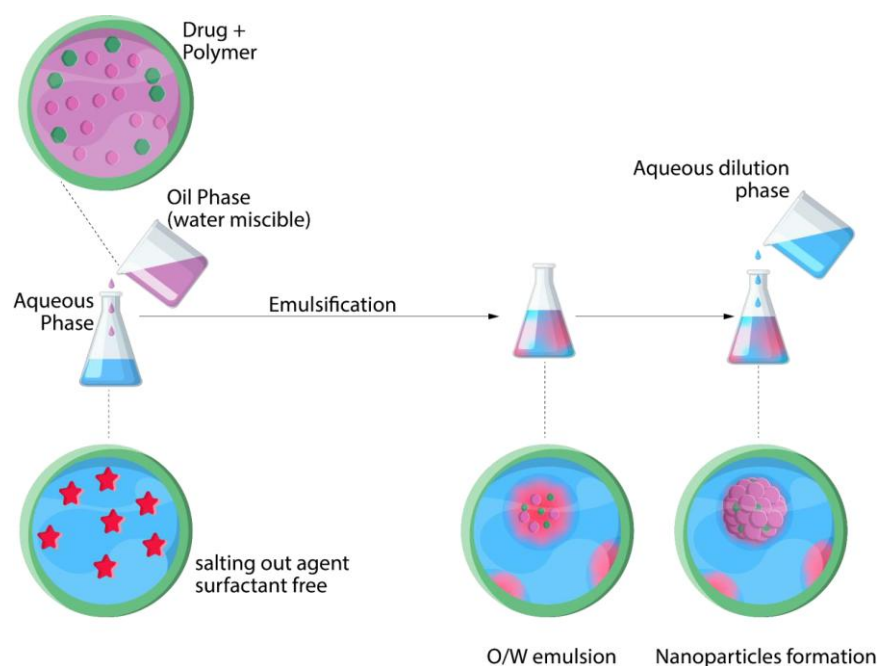


Figure 1.11. Salting-out. Emulsification occurs between an organic polymer solution and an aqueous phase saturated with a salting-out agent; dilution induces phase separation and particle formation without the need for surfactants or halogenated solvents.

- **Ionic gelation** (Figure 1.12. [141]): This technique is predominantly applied for the formulation of polysaccharide-based NPs, especially CS. It relies on the ionic interaction between positively charged amino groups of CS and negatively charged polyanions, including tripolyphosphate (TPP), dextran sulfate, sodium hyaluronate, or negatively charged CDs such as sulfobutylether- β -cyclodextrin (SBE- β -CD). Its simplicity and the absence of covalent crosslinking agents reduce toxicity risks and make it suitable for sensitive therapeutic applications. CS is typically dissolved in dilute acetic acid and added dropwise to the polyanion solution under continuous stirring, resulting in NPs formation via ionic gelation. This approach allows for the encapsulation of both small hydrophilic molecules and large macromolecules [154].

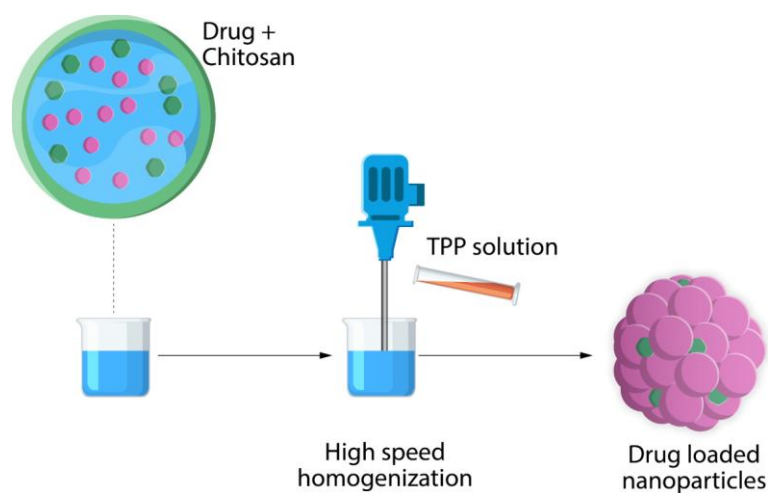


Figure 1.12. Ionic gelation. Nanoparticles are formed through ionic crosslinking between cationic polymers (e.g., chitosan) and polyanionic species (e.g., tripolyphosphate), in aqueous media and without the need for chemical crosslinkers.

1.4.3. Lipid-based nanoparticles

Lipid-based NPs represent a versatile and biocompatible platform for drug delivery, typically ranging in size from 30 to 1000 nm. These systems are composed of physiological or synthetic lipids, including fatty acids (such as palmitic, myristic, and stearic acids), mono- and diglycerides (e.g., glyceryl monostearate and glyceryl caprate), complex mixtures of triglycerides, or even waxes. Owing to their excellent biodegradability and minimal toxicity, these lipidic carriers are particularly well suited for biomedical applications. Their amphiphilic nature allows them to encapsulate both hydrophilic and lipophilic therapeutic agents [155].

Lipid NPs are generally categorized into two main classes based on the composition and structural organization of their lipid matrix: **Solid Lipid Nanoparticles** (SLNs), considered the first generation, and **Nanostructured Lipid Carriers** (NLCs), developed as a second-generation improvement [156].

SLNs were first introduced in the early 1990s as promising alternatives to conventional DDs such as emulsions, liposomes, and the previously described polymeric NPs. These colloidal carriers are formed by solid lipids at both room and body temperature, emulsified in an aqueous phase containing surfactants. Their formulation typically involves lipids classified as GRAS, contributing to their low toxicity and excellent biocompatibility. In addition to safety, SLNs offer several advantages, including good physical stability, scalability, low production cost, and the ability to protect encapsulated drugs from degradation. Lipid content in these systems generally varies from 0.1% to 30%, while surfactant concentrations typically range between 0.5% and 5%. Common surfactants include poloxamers,

polysorbates, and natural phospholipids such as lecithin derived from soy or egg sources [157].

SLNs can encapsulate active compounds by trapping them within their solid lipid matrix, which adopts a highly ordered crystalline structure. According to Mehnert and collaborators [158], drug distribution within the NP matrix can follow one of three models (Figure 1.13. [159]):

- **Homogeneous matrix model:** the drug is uniformly dispersed within the lipid matrix.
- **Drug-enriched shell model:** the active compound localizes preferentially at the particle surface.
- **Drug-enriched core model:** the drug is primarily concentrated within the lipid core of the NP.

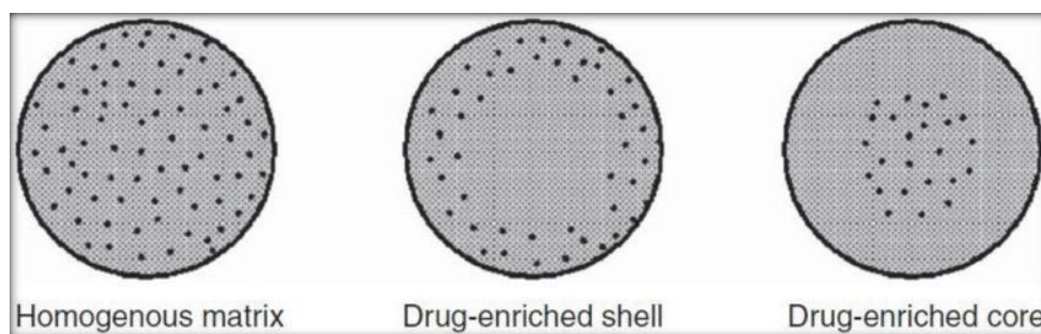


Figure 1.13. Models of drug distribution in Solid Lipid Nanoparticles: (1) homogeneous matrix, (2) drug-enriched shell, (3) drug-enriched core.

Despite their advantages, SLNs present some limitations, particularly related to the high degree of crystallinity of the lipid matrix, which can restrict drug loading capacity and promote drug expulsion over time [160].

To address these drawbacks, NLCs were developed. These second-generation systems consist of a blend of solid and liquid lipids, emulsified in the presence of surfactants and water. The inclusion of a liquid lipid disrupts the perfect crystalline arrangement of the solid matrix, resulting in a less ordered, more flexible internal structure. This architectural modification improves drug solubility and retention by increasing the number of spaces within the matrix, thus reducing the likelihood of drug expulsion during storage [161].

Liquid lipids typically employed in NLC formulations include medium-chain triglycerides (e.g., Mygliol), paraffin oil, 2-octyldodecanol, Labrafac® (propylene glycol dicaprylocaprate), isopropyl myristate, and squalene. Depending on the ratio and nature of the lipid components, NLCs can adopt three main structural types (Figure 1.14. [162]):

- **Type I (Imperfect matrix model):** prepared by mixing solid lipids with a small proportion of liquid lipids, leading to a more disordered matrix that allows higher drug incorporation.
- **Type II (Amorphous model):** prepared by using special lipids that prevent recrystallization (e.g., hydroxyoctacosanyl hydroxystearate, isopropyl myristate), thus creating a non-crystalline matrix that limits drug leakage.
- **Type III (Multiple oil-in-fat model):** contains a higher proportion of liquid lipids, forming nanocompartments within the solid matrix, which is especially useful for drugs that show higher solubility in oils. This structure favors both high loading efficiency and sustained drug release [161].

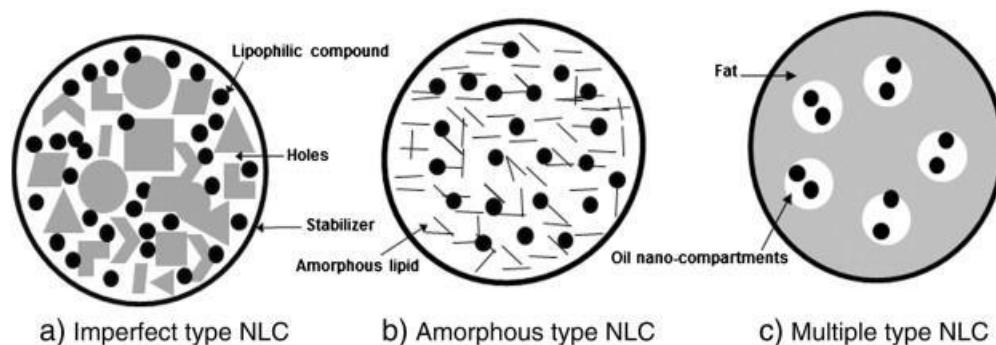


Figure 1.14. Types of Nanostructured Lipid Carriers: imperfect (type I), amorphous (type II), and multiple (type III), each with distinct lipid compositions affecting drug loading capacity and release profiles.

Through these innovations, NLCs overcome the limitations of SLNs and offer a more flexible, efficient, and stable nanocarrier system for controlled drug delivery [161].

1.4.4. Preparation methods of lipid-based nanoparticles

Since their introduction in the 1990s, a variety of methods have been developed for the preparation of lipid-based NPs [158]. These approaches are typically categorized into two main groups based on the amount of energy required during formulation: high-energy techniques, which rely on mechanical forces to generate NPs, and low-energy techniques, which exploit physicochemical mechanisms such as phase inversion or solvent diffusion. The choice of method is crucial, as it significantly influences the physical characteristics and performance of the final NPs. As with polymeric NPs, selection depends on factors such as the physicochemical properties of the drug, its solubility profile, and the desired NP size and morphology [163].

In the case of methods which require a high energy approach, the following techniques are commonly used:

- **High-pressure homogenization** (Figure 1.15. [164]): This technique is among the most commonly used for large-scale production of lipid-based NPs. It begins with the preparation of a pre-emulsion, typically obtained by dispersing the drug-loaded melted lipid phase into a hot aqueous surfactant solution under high-speed stirring. This pre-emulsion is then passed through a narrow gap at high pressures (100-2000 bar), where intense shear forces and cavitation reduce the droplet size to the nanometer scale. The final cooling step leads to lipid recrystallization and solid NP formation [165].

Two variations are used:

- *Hot homogenization*: the drug is first incorporated into melted lipids, and the resulting mixture is emulsified in a heated aqueous surfactant solution. The pre-emulsion is homogenized under high pressure and subsequently cooled to induce lipid solidification and NPs formation. This technique is suitable for lipophilic and thermally stable drugs [166].
- *Cold homogenization*: the melted mixture of drug and lipids is rapidly cooled (e.g., using liquid nitrogen), solidified, and milled into NPs. These are then dispersed in a cold aqueous surfactant solution and subjected to high-pressure homogenization. This method is preferred for thermolabile or hydrophilic drugs, although it typically produces larger and more polydisperse particles [167].

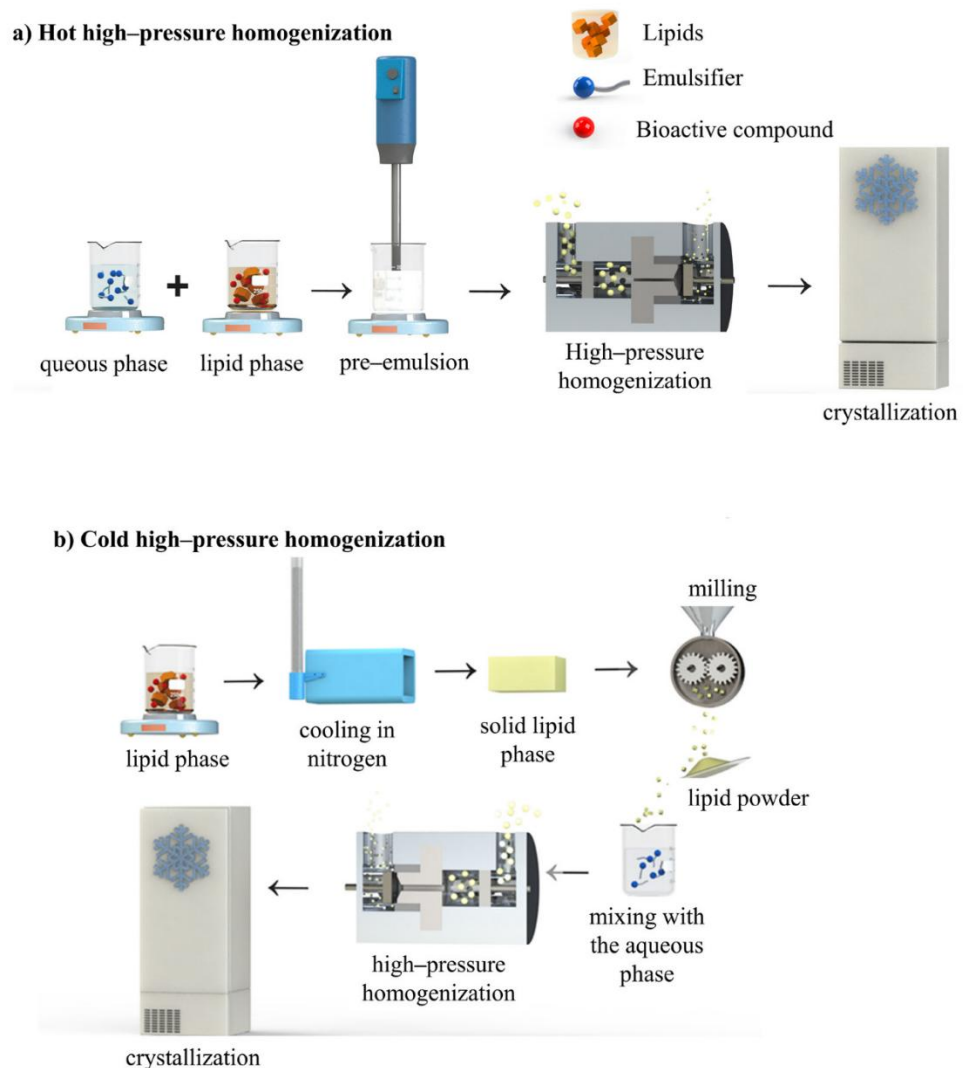


Figure 1.15. High-pressure homogenization. Lipid nanoparticles are obtained by forcing a lipid-aqueous mixture through a high-pressure gap, where shear stress and cavitation reduce droplet size to the nanometer scale, followed by cooling-induced lipid crystallization, either from the hot or cold process.

- **High shear homogenization and ultrasonication** (Figure 1.16. [164]): This method involves the dispersion of melted lipids into a hot aqueous surfactant solution using high-speed stirring (high shear homogenization), followed by ultrasonication to reduce droplet size. Ultrasonic cavitation creates tiny bubbles that collapse and release energy, helping NPs form

when the mixture cools. Although relatively simple, this technique can result in lower NP yield and potential metal contamination from probe erosion. Moreover, careful optimization of surfactant concentration, sonication duration, and intensity is essential [168].

Ultrasonic homogenization

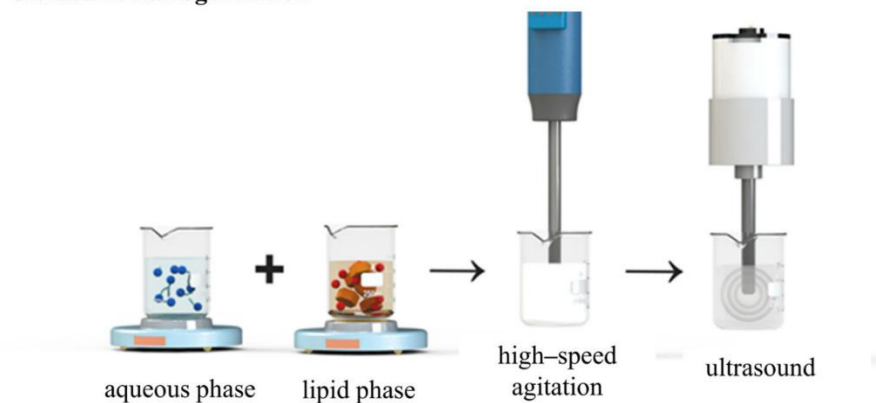


Figure 1.16. High shear homogenization and ultrasonication. Melted lipids are dispersed into a hot aqueous surfactant solution under high-speed stirring, then subjected to ultrasonic cavitation which reduces particle size; nanoparticles form upon cooling.

- **Double emulsion method:** Primarily used for the encapsulation of hydrophilic drugs [169], this technique involves the formation of a primary water-in-oil (w/o) emulsion, where the aqueous drug solution is dispersed in melted lipid. This is then emulsified into a second aqueous phase containing surfactants, yielding a water-in-oil-in-water (w/o/w) emulsion. Ultrasonication or high-shear stirring is then applied to stabilize the system. The NPs are formed upon cooling and removal of residual solvent. The method requires careful optimization to prevent the drug from escaping into the external aqueous phase [170].

In the case of methods which do not require a high energy approach, the following techniques are commonly used:

- **Solvent injection** (or solvent displacement, Figure 1.17. [164]): This method is suitable for thermosensitive drugs and involves dissolving the lipids and active compound in a water-miscible organic solvent (e.g., ethanol or acetone). The solution is injected into an aqueous surfactant medium under stirring, where rapid diffusion of the solvent into the aqueous phase causes lipid precipitation and NPs formation. The technique is easy to perform under mild conditions, though residual solvents must be eliminated [171].

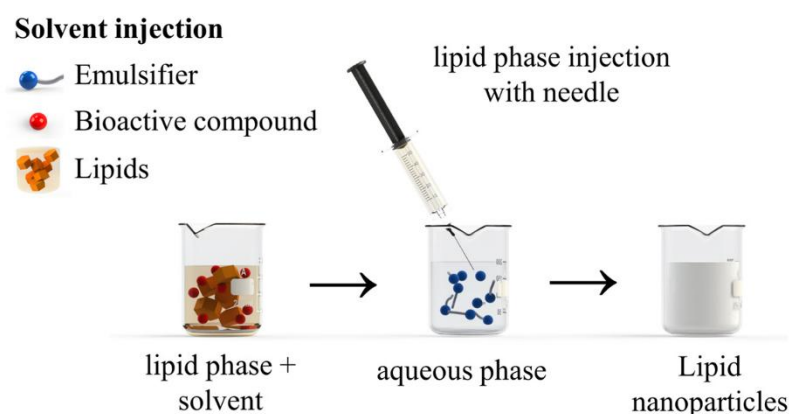


Figure 1.17. Solvent injection. Lipids and drug are dissolved in a water-miscible solvent and rapidly injected into an aqueous surfactant solution, inducing lipid precipitation and nanoparticle formation without the use of high energy.

- **Solvent evaporation or diffusion from emulsions** (Figure 1.18. [164]): The lipids and the drug are dissolved in an organic solvent that is either immiscible (e.g., chloroform) or partially miscible (e.g., ethyl acetate) with

water. This organic phase is emulsified in an aqueous surfactant solution. NPs form as the solvent is either evaporated (solvent evaporation technique) or diluted in water (solvent diffusion technique), leading to lipid solidification and drug entrapment [172,173].

Solvent diffusion

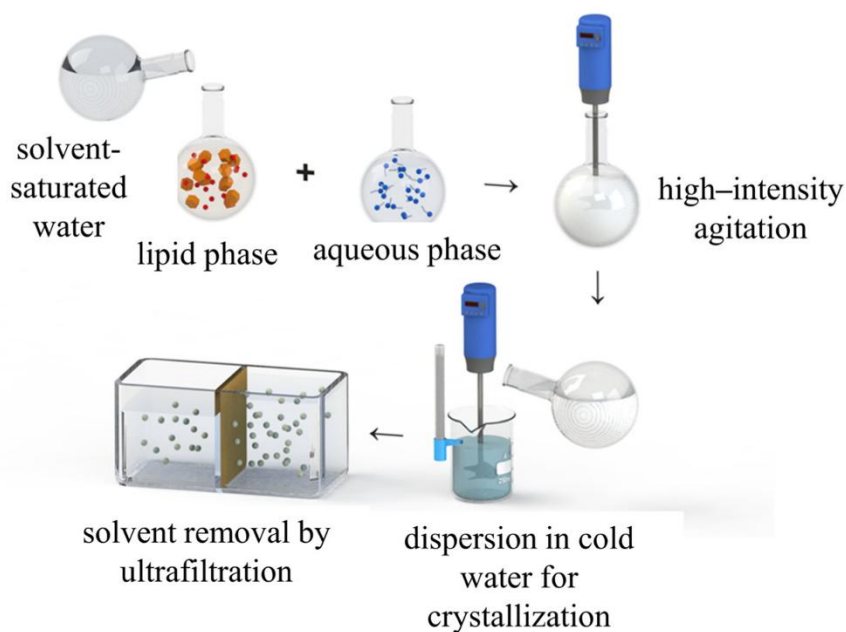


Figure 1.18. Solvent evaporation/diffusion. Lipids and drug dissolved in an organic solvent form an oil-in-water emulsion in aqueous surfactant; removal of solvent by evaporation or dilution results in nanoparticle formation.

- **Phase inversion temperature (PIT) method** (Figure 1.19. [164]): This technique exploits the temperature dependent hydrophilic-lipophilic balance of non-ionic surfactants. The drug is first dissolved or dispersed in the lipid phase, which is then emulsified with water at high temperature to form a water-in-oil (w/o) emulsion. As the system is gradually cooled below the PIT, phase inversion occurs, converting the emulsion into an oil-in-

water (o/w) nanoemulsion. Subsequent cooling leads to lipid solidification, encapsulating the drug within stable NPs. This solvent-free method produces well defined nanostructures, often smaller than 25 nm [174,175].

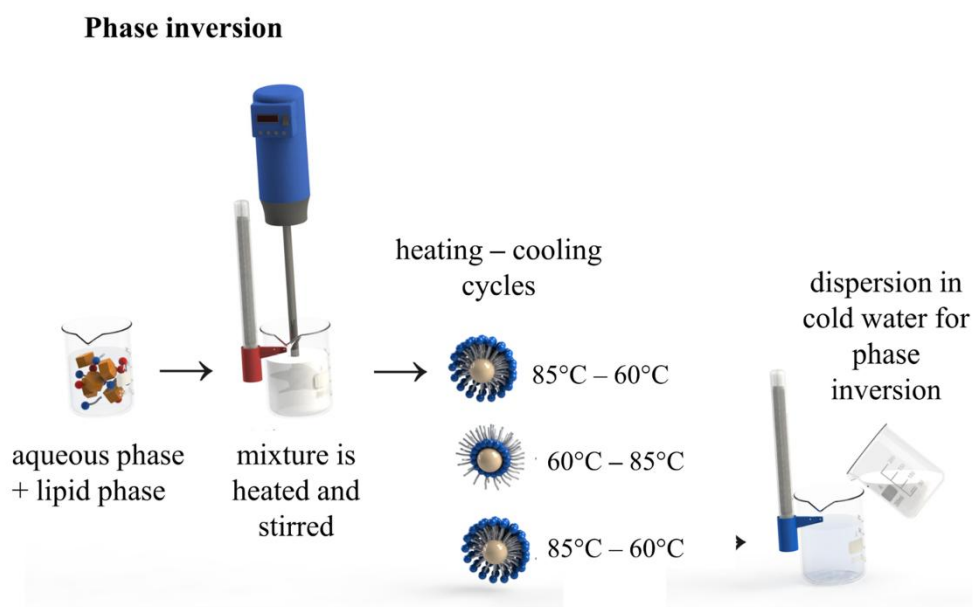


Figure 1.19. Phase inversion temperature method. This solvent-free method exploits temperature-induced changes in surfactant affinity to invert emulsion phases, forming oil-in-water nanoemulsions that solidify into stable lipid nanoparticles upon cooling.

- **Microemulsion method** (Figure 1.20. [164]): In this approach, a microemulsion is prepared by adding an aqueous surfactant/co-surfactant mixture to melted lipids containing the drug. The resulting hot microemulsion is rapidly dispersed into cold water (2-4 °C) under mechanical stirring. The sudden temperature drop causes rapid lipid crystallization and NPs formation. Parameters such as the choice of surfactant, temperature gradient, and mixing conditions are critical for reproducibility and product stability [176].

Microemulsion

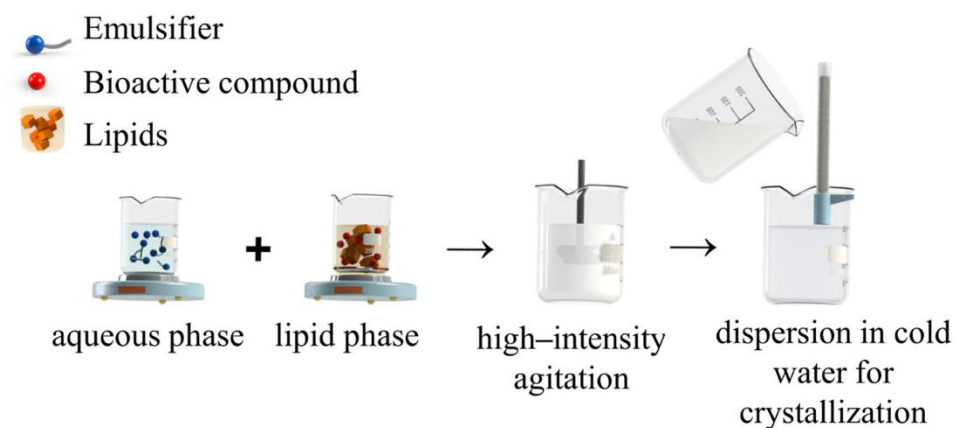


Figure 1.20. Microemulsion method. A hot microemulsion of lipids and drug is rapidly dispersed into cold aqueous phase, causing fast lipid crystallization and formation of stable nanoparticles.

1.5. Cyclodextrins

CDs (Figure 1.21. [177]) are cyclic oligosaccharides composed of glucopyranose units linked through α -(1 $\rightarrow$ 4) glycosidic bonds. Also referred to as cycloamyloses, cyclomaltoses, or Schardinger dextrins, they originate as enzymatic degradation products of starch [178]. Their biosynthesis is catalyzed by cyclodextrin glucanotransferase (CGTase), an enzyme that cleaves internal α -1,4-glycosidic bonds in starch-derived α -glucans, forming a covalent glycosyl-enzyme intermediate. This intermediate is then transferred to the 4-hydroxyl group of the nonreducing end of the same sugar chain, resulting in the formation of a new α -1,4-glycosidic bond and the production of cyclic oligosaccharides containing six to twelve glucose units [179].

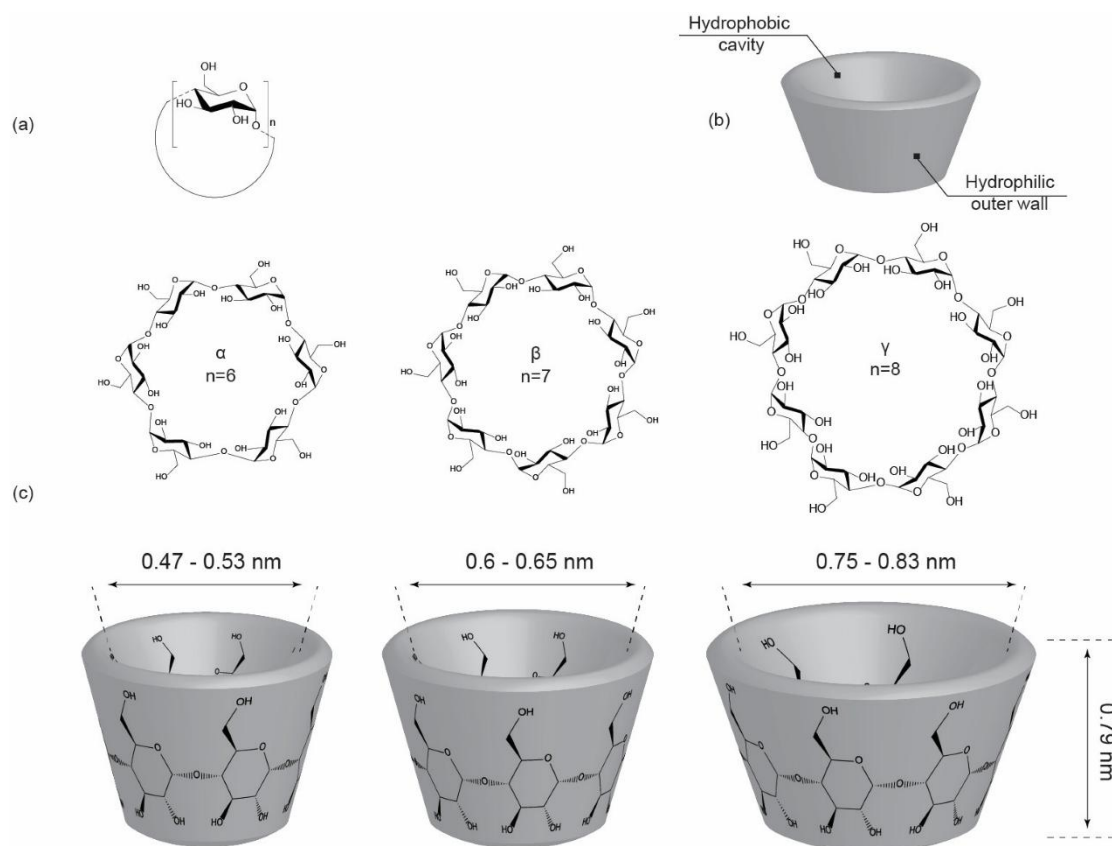


Figure 1.21. Schematic overview of cyclodextrins: **(a)** the repeating glucose unit forming the cyclic structure; **(b)** three-dimensional model illustrating the hydrophobic inner cavity and hydrophilic exterior; **(c)** chemical ring structures and dimensional comparison of α -, β -, and γ -cyclodextrins, composed of 6, 7, and 8 glucose units, respectively.

Originally discovered in the late 19th century during studies on potato starch digestion, CDs gained pharmaceutical relevance only in the mid-20th century due to their ability to enhance aqueous solubility, chemical stability, and systemic bioavailability of therapeutic agents. Additional pharmaceutical benefits include the mitigation of drug incompatibilities, conversion of liquid active substances into stable microcrystalline solids, reduction of gastrointestinal side effects, and masking of unpleasant taste or odor [179–182].

Moreover, extensive research has focused on the combined use of CDs with polymeric nano- and microparticles. CDs serve as important modulators of drug release from the polymeric matrix and enable the encapsulation of lipophilic drugs even when only aqueous solvents are employed during production [144]. A notable example is PLGA, a widely used polymer for producing nano- and microparticles due to its high biodegradability and lack of toxicity. Although PLGA achieves very high EE for lipophilic drugs, the strong affinity between the drug and polymer matrix can result in very slow or even incomplete drug release. Incorporation of CDs alters the apparent solubility of the lipophilic drug, thereby improving its release profile from the particulate system [183].

For example, in a study conducted by Cannavà *et al.* (2013), PLGA microspheres containing a celecoxib-dimethyl- β -CD complex were developed as a strategy to enhance the intra-articular delivery of celecoxib, a selective COX-2 inhibitor widely used in the treatment of osteoarthritis and rheumatoid arthritis. The study demonstrated that the incorporation of CDs not only facilitated drug encapsulation in aqueous processing conditions, but also allowed fine modulation of celecoxib release kinetics: increasing the concentration of dimethyl- β -CD led to a more pronounced burst effect and faster overall release, attributed to changes in matrix porosity and improved drug solubilization through complex formation. In addition, *ex vivo* experiments on human chondrocyte cultures confirmed that the microspheres, particularly those containing CD, enhanced the anti-inflammatory efficacy of celecoxib compared to the free drug, likely due to both improved release and increased cellular uptake. This concentration-dependent modulation of drug release underscores the potential of CDs as functional excipients in the design of advanced DDs [184].

Beyond pharmaceutical applications, CDs have demonstrated versatility across fields such as food technology, cosmetics, environmental remediation, and textile engineering, where they function as carriers for volatile compounds, stabilizers of sensitive ingredients, pollutant adsorbents, and fragrance delivery systems [185,186].

Structurally, native CDs, also known as first-generation CDs, are classified according to the number of glucose units they contain, specifically six in α -CD, seven in β -CD, and eight in γ -CD [180]. Thermodynamic and steric constraints prevent the formation of stable rings with fewer than six glucose units, while larger homologues are rarely used due to poor complexation efficiency [187]. The chair conformation adopted by each glucopyranose imparts a truncated cone shape, characterized by a hydrophobic cavity and a hydrophilic outer surface [188]. Although cavity depth remains constant at approximately 0.79 nm, the internal diameter increases with ring size: from 0.47-0.53 nm in α -CD to 0.75-0.83 nm in γ -CD [177,187]. The diameters are reported as ranges rather than fixed values to reflect the intrinsic flexibility and dynamic nature of the CD cavity [189]. The external hydrophilicity is conferred by hydroxyl groups, with primary hydroxyls located on the narrower rim and secondary hydroxyls on the wider rim [177]. In contrast, the interior cavity is considered relatively hydrophobic due to the inward-facing C-H bonds and glycosidic oxygen atoms. While the C-H groups contribute to the nonpolar environment, the glycosidic oxygens, through their lone electron pairs, introduce a mild Lewis basicity that can favor specific non-covalent interactions with guest molecules [186,190].

The extensive application of CDs in pharmaceutical technology stems from their ability to form non-covalent inclusion complexes with poorly soluble or unstable

compounds, including natural products and synthetic drugs. Through encapsulation, the guest molecule is stabilized at physiological pH and protected against oxidative degradation, hydrolysis, photolysis, and thermal stress [182,191]. The complexation process relies on van der Waals interactions, hydrogen bonding, electrostatic forces, conformational changes, and charge-transfer mechanisms. In aqueous environments, inclusion complexes exist in dynamic equilibrium with their free components, and the stoichiometry depends on the size and geometry of the guest molecule, typically adopting 1:1, 1:2, 2:1, or 2:2 ratios [191] (Figure 1.22.).

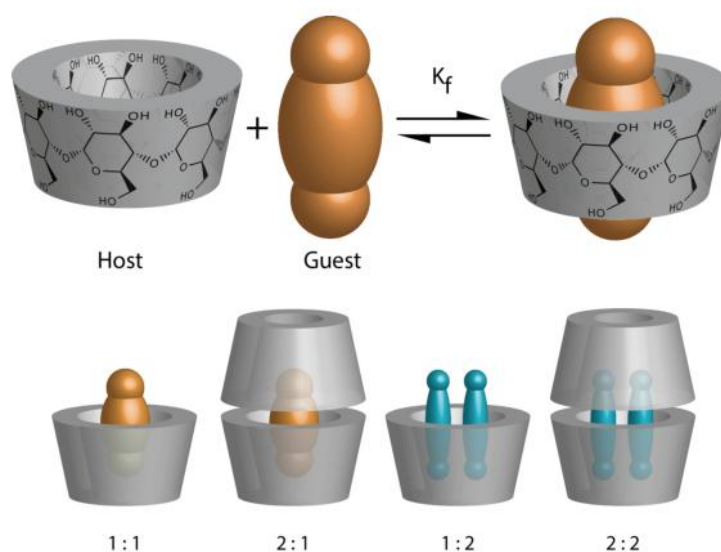
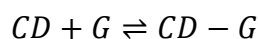


Figure 1.22. Schematic representation of host-guest inclusion complex formation between cyclodextrins and guest molecules. Various binding stoichiometries are shown, including 1:1, 2:1, 1:2, and 2:2 complexes.

A key advantage of CD inclusion complexation is the significant improvement of aqueous solubility for hydrophobic drugs, achieved through mechanisms distinct from those of surfactants or co-solvents such as PEG or ethanol. Unlike co-

solubilization systems, which often lead to drug precipitation upon dilution, CD-based complexes, particularly 1:1 systems, maintain solubility even after significant aqueous dilution [181,192]. Their nanometric dimensions further support the development of advanced pharmaceutical dosage forms, including nanocarriers and targeted delivery systems [193].

The strength of interaction between a CD and a guest molecule (G) is defined by the stability constant (K_s), which quantifies the equilibrium between free and complexed species in solution. This equilibrium can be represented as:



with the stability constant expressed as:

$$K_s = \frac{[CD - G]}{[CD][G]}$$

where the square brackets indicate the equilibrium concentrations (in mol·L⁻¹) of the free CD, the free G (such as a drug), and the resulting inclusion complex, respectively [194]. Typical K_s values range from 200 to 20,000 M⁻¹, depending on the specific CD-guest combination. Suboptimal values can compromise therapeutic performance: a low K_s means the complex is unstable and may release the drug too early, whereas an excessively high K_s may prevent the drug from being adequately released, ultimately reducing its bioavailability [195].

Among the native CDs, β -CD is the most commonly used due to its cavity size, which suits many drug molecules. However, its low aqueous solubility (18.5 g/L at 25°C), mainly attributed to intramolecular hydrogen bonding between secondary hydroxyls, presents a significant limitation. By contrast, α -CD and γ -CD offer superior solubility (130 g/L and 249 g/L at 25°C, respectively), although their cavity sizes may not accommodate as broad a range of compounds [196].

To overcome solubility limitations and subsequently enhance complexation performance, numerous chemical modifications have been developed, particularly targeting β -CD through substitution at the primary and/or secondary hydroxyl groups (Figure 1.23. [197]).

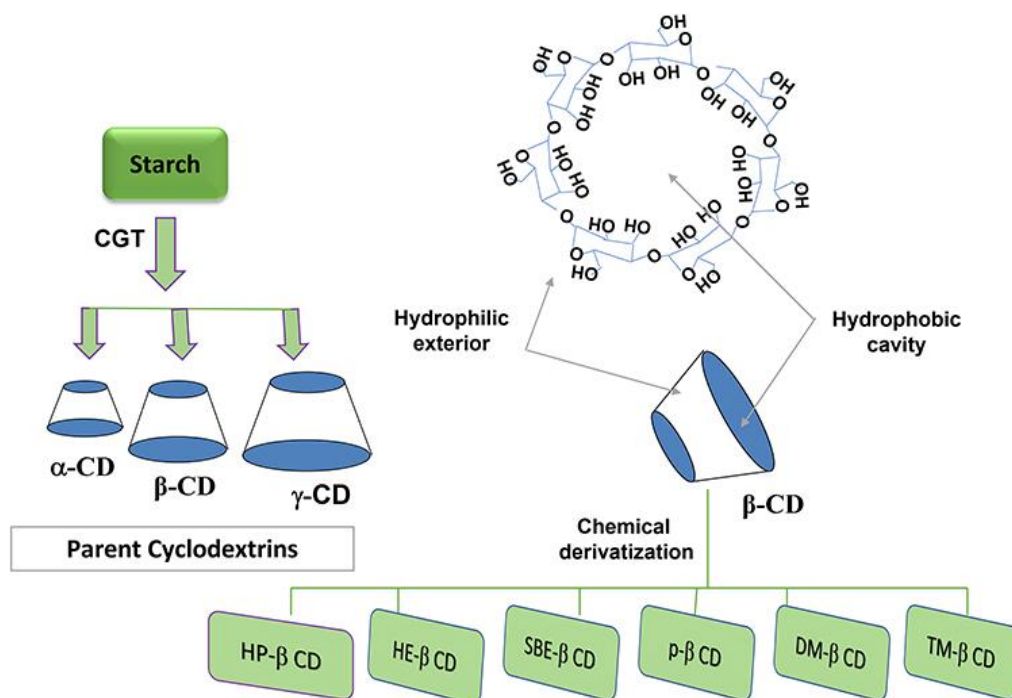


Figure 1.23. Schematic overview of cyclodextrin production and derivatization. Native CDs (α -, β -, and γ -CD) are enzymatically produced from starch via cyclodextrin glycosyltransferase. β -CD, with a hydrophobic cavity and hydrophilic exterior, can be chemically modified to yield derivatives (e.g., HP- β -CD, SBE- β -CD) with enhanced solubility and complexation abilities.

Alkylated derivatives such as methyl- β -CD and hydroxypropyl- β -CD (HP- β -CD) demonstrate markedly improved water solubility, often exceeding 500 mg/mL, while maintaining or enhancing complexation capacity, even for lipophilic drugs. Derivatives can be site-specific, producing well-characterized crystalline

compounds, or randomly substituted, where only the average degree of substitution is controlled.

Branching typically enhances solubility and reduces the hemolytic activity of native β -CD, although it may compromise complex stability due to steric hindrance. Polymeric β -CDs, which are generally poorly soluble, are predominantly used in sustained-release DDSs. In contrast, dimeric β -CDs offer an expanded cavity size, making them suitable for encapsulating bulkier drug molecules.

Other functionalized derivatives, such as carboxymethyl-, acetyl-, amino-, and polymer-conjugated β -CDs, provide additional functionalities. For instance, carboxymethyl- β -CD enables pH-sensitive drug release, while polymer-bound β -CD systems facilitate targeted delivery by incorporating ligands or antibodies onto the carrier surface [177].

Chemical modifications of CDs profoundly influence not only their physicochemical properties, but also their metabolic fate and safety profiles. Native CDs, including α -, β -, and γ -CD, are not systemically absorbed when administered orally. Instead, they pass unaltered through the upper gastrointestinal tract and are fermented in the colon by the gut microbiota into smaller carbohydrate units, which are subsequently absorbed and metabolized like dietary sugars [198]. In contrast, chemically modified CDs, especially those bearing bulky substituents, often resist enzymatic degradation due to steric hindrance. These derivatives are typically not metabolized and are excreted unchanged in the feces [199].

Importantly, the route of administration plays a critical role in determining the toxicological behavior of CDs [177]. While native β -CD is generally safe when ingested, parenteral administration in animal models has revealed nephrotoxic

effects. These adverse outcomes are attributed to its poor aqueous solubility and tendency to crystallize within renal tubules, leading to cellular injury characterized by vacuole formation, lysosomal swelling, and membrane destabilization, likely caused by cholesterol extraction from cell membranes [200].

Similarly, methylated CDs, such as methyl- β -CD, have demonstrated hemolytic activity *in vitro* and *in vivo*. This is thought to arise from their interactions with erythrocyte membrane lipids, particularly cholesterol, potentially disrupting the lipid bilayers and inducing cell lysis.

Given these concerns, attention has shifted toward safer alternatives that not only demonstrate minimal toxicity across all administration routes but also offer improved solubility and complexation characteristics: HP- β -CD and SBE- β -CD [195].

HP- β -CD is obtained by substituting hydroxyl groups on the CD ring with hydroxypropyl chains, typically at the 2-, 3-, and/or 6-positions. These substitutions improve aqueous solubility and reduce the likelihood of crystal formation. Despite the chemical modifications, HP- β -CD retains a hydrophobic cavity similar in size to that of native β -CD, making it particularly effective at forming inclusion complexes with neutral or weakly polar drug molecules [177]. Toxicological assessments conducted in multiple species, including mice, rats, dogs, and primates, indicate low systemic toxicity following both oral and intravenous administration. Observed effects, when present, tend to be mild and reversible. In human clinical use, HP- β -CD is well tolerated, with mild diarrhea being the most commonly reported side effect. Importantly, no renal impairment has been observed in healthy subjects or in patients receiving therapeutic doses [201].

SBE- β -CD is an anionic derivative generated by the random substitution of hydroxyl groups with sulfobutylether moieties. This functionalization not only significantly enhances solubility, but also introduces electrostatic properties that influence complexation behavior. The inclusion efficiency of SBE- β -CD is highest with neutral molecules, although positively charged drugs can also be encapsulated via favorable ionic interactions. Conversely, negatively charged compounds exhibit lower affinity due to electrostatic repulsion [177,202]. Despite its ionic nature, SBE- β -CD displays an excellent safety profile. It is eliminated primarily via the kidneys, undergoing glomerular filtration without evidence of tubular reabsorption or cellular toxicity. The presence of a negative charge is thought to prevent membrane-disruptive interactions, especially with cholesterol, thereby minimizing cytotoxicity [195].

Despite their favorable safety profiles, parenteral administration of these CDs must be carefully monitored, particularly in terms of concentration and volume. Regulatory agencies have established maximum acceptable daily intakes (16 mg/kg for HP- β -CD and 250 mg/kg for SBE- β -CD in parenteral formulations). Nevertheless, long-term or high-dose administration, especially in individuals with impaired renal function, remains a concern and warrants continued pharmacovigilance [177].

Several marketed drugs use HP- β -CD or SBE- β -CD to improve their solubility and stability. For instance, HP- β -CD is included in the intravenous formulation of itraconazole (Sporanox® IV), an antifungal used to treat serious fungal infections [203]. On the other hand, SBE- β -CD is present in voriconazole (Vfend® IV), another antifungal for severe infections, ziprasidone (Geodon® IM), an antipsychotic injection for acute agitation, and remdesivir (Veklury®), an antiviral

used to treat COVID-19 [177,204]. These examples highlight how CD derivatives help formulate drugs that otherwise have poor water solubility, enabling effective and safe administration.

1.5.1. Cyclodextrin production methods

As introduced in the previous paragraph, CDs are enzymatically synthesized through the action of CGTase, an extracellular enzyme that catalyzes their production from starch and related α -1,4-glucans [205]. This enzyme is predominantly obtained from bacterial strains, mainly from the genus *Bacillus*, with *B. macerans* and *B. circulans* being notable examples [206,207].

Two primary industrial processes are employed to produce CDs: the non-solvent process and the solvent process, each with distinct operational principles and product outcomes [208].

In the non-solvent process, starch undergoes enzymatic conversion without the addition of any complexing agents. This method results in a mixture of α -, β -, and γ -CDs. Initially, starch is subjected to partial hydrolysis by α -amylase to reduce molecular weight and viscosity, producing liquefied starch. This step is critical, as the presence of glucose or low molecular weight oligosaccharides can negatively impact the final yield of CDs [208,209]. After liquefaction, the solution is cooled, and CGTase is added to catalyze cyclization, producing a mixture of CDs [208]. Subsequently, the reaction mixture is acidified, and glucoamylase is introduced to hydrolyze interfering substances such as residual starch, dextrans, and maltose. The solution is then purified through ion-exchange resins and activated carbon treatment, followed by filtration and vacuum evaporation to concentrate the solids to approximately 45%. Cooling induces crystallization of CDs, which are then

washed, centrifuged, and dried to obtain the final crystalline product. While this method operates at lower starch concentrations (~15%), it generally tends to give lower yields, demands higher energy input, and may lead to the formation of unwanted side products [209].

Conversely, the solvent process utilizes organic complexing agents to selectively precipitate individual CD types, enhancing both purity and yield. Commonly employed solvents include toluene, ethanol, acetone, decanol, α -naphthol, and methyl ethyl ketone. For example, toluene selectively precipitates β -CD, decanol favors α -CD isolation, and cyclic compounds like α -naphthol or methyl ethyl ketone enable γ -CD recovery. In this approach, starch liquefaction and enzymatic conversion occur similarly to the non-solvent method, but during the CGTase-mediated reaction, a complexing agent is introduced. This agent forms insoluble inclusion complexes with specific CDs, facilitating their selective precipitation. The precipitate is separated by filtration or centrifugation, washed with water, and subjected to solvent recovery via distillation. The precipitated complexes are then redispersed in water and heated to release the solvent through distillation or liquid-liquid extraction, yielding a purified CD solution. After treatment with activated carbon and cooling, the CDs crystallize, and the crystals are washed, filtered, and dried. The solvent method typically employs higher starch concentrations (~30%), leading to significantly improved yields compared to the non-solvent process [209,210].

1.5.2. Methods for the preparation of cyclodextrin inclusion complexes

The formation of CD inclusion complexes plays a key role in improving the solubility, stability, and bioavailability of poorly soluble drug molecules. A variety

of complexation techniques exist, each relying on distinct mechanisms to promote host-guest interactions. However, these methods are not universally applicable. The choice of protocol must be tailored to the physicochemical properties of the drug candidate as well as scalability and desired final dosage form [211].

The methods that can be employed for the preparation of CD inclusion complexes are the following:

- **Co-precipitation:** Suitable for drugs with poor aqueous solubility, this method involves dissolving the guest molecule in a water-miscible organic solvent (e.g., ethanol or isopropanol) and adding it dropwise to an aqueous solution of CD under vigorous stirring. The process is typically carried out at 40-60 °C to enhance solubilization kinetics. Subsequent cooling induces precipitation of the solid inclusion complex, which is recovered via filtration or centrifugation and then dried. Complexation time may range from 24 to 48 hours, depending on the specific system. This technique is operationally simple but can be limited by slow kinetics and low yields for certain molecules [211].
- **Kneading:** Both the CD and the API are intimately combined in a mortar at 1:1 or 1:2 molar ratios. A minimal volume of purified water or hydroalcoholic solution is incorporated to form a cohesive paste, which undergoes continuous mechanical kneading for approximately one hour. This shearing action facilitates molecular diffusion and complexation. The resultant slurry is dried, sieved using an 80-mesh screen, and stored in a desiccator. Industrial-scale adaptation employs high-shear granulators, achieving analogous complexation within 15-60 minutes under controlled humidity (40-50%) and temperature modulation [212].

- **Supercritical CO₂ method:** Using scCO₂ as a clean solvent, both the CD and the API are placed into a high-pressure vessel under precisely controlled thermobaric conditions. Complexation proceeds within the supercritical phase. Subsequent rapid depressurization induces instantaneous CO₂ volatilization, yielding the purified solid inclusion complex devoid of organic residues. This solvent-free technique offers exceptional efficiency for thermolabile or oxidation-sensitive compounds while achieving high EE [213].
- **Grinding:** A precisely weighed solid mixture of CD and API undergoes intensive mechanical trituration, employing either mortar and pestle or ball-mill apparatus. The imparted mechanical energy generates localized amorphization, reduces crystalline domain size, and increases specific surface area, thereby promoting solid-state complexation. The resultant powder is sieved for uniformity and stored at controlled temperature and humidity. This eco-friendly and rapid method is scalable and solvent-free [214].
- **Spray-Drying:** A homogenous feed solution containing CD and dissolved/suspended API is atomized into a fine droplet dispersion within a heated air chamber. Rapid solvent evaporation yields free-flowing powder particles (nano- to micrometer range). This continuous process affords high throughput and excellent yield but is contraindicated for thermally labile or highly volatile compounds. Meticulous parameter control (e.g., inlet/outlet temperatures, feed rate, atomization pressure) is essential to prevent nozzle occlusion and ensure product consistency [180,211,215].

- **Freeze-Drying (Lyophilization):** The API (if sufficiently water-soluble) or an organic solution thereof is combined with a saturated aqueous CD solution under continuous agitation until thermodynamic equilibrium is reached. The resulting solution is rapidly frozen and then lyophilized: first through primary drying (sublimation under reduced pressure), followed by secondary drying (removal of bound water at constant temperature). The resulting amorphous, porous solid offers excellent molecular dispersion and long-term stability, although the process requires high energy input and may stress thermosensitive compounds [216,217].
- **Slurry Complexation:** Suitable for oily or liquid guests, this method involves creating a thick aqueous slurry of CD (50-60% solids) into which the drug is added. The mixture is stirred, often for 24-36 hours, until inclusion complexes precipitate or crystallize. These are then filtered, washed, and dried. Slurry processing reduces solvent use and operates at ambient conditions, making it practical and scalable [180,212,216].
- **Microwave-Assisted Preparation:** A precisely wetted solid mixture of CD and API (using minimal ethanol-water) is subjected to controlled microwave irradiation. Dielectric heating rapidly accelerates molecular mobility and interaction kinetics, promoting efficient complexation within minutes. The resultant moist solid is sieved and dried to yield the inclusion complex powder. The feasibility of this method relies on the API's tolerance to microwave dielectric heating without compromising its stability [211].
- **Neutralization/Precipitation Method:** An ionizable API is dissolved in an alkaline medium (e.g., NaOH solution) and combined with an aqueous CD solution under agitation. Controlled acidification (e.g., using dilute HCl) to

the stoichiometric equivalence point induces concurrent protonation and supersaturation, leading to precipitation of the inclusion complex as a defined solid. The precipitate is isolated by filtration, purified by washing, and dried. This method allows controlled formation of complexes but requires careful pH control and consideration of the API's acid-base stability profile [211].

1.5. Potential of nanotechnologies in cancer theranostics

As discussed in Section 1.3, nanotechnologies represent a highly promising tool in the therapeutic field, particularly in oncology. They enable the redesign of already known molecules toward more efficient, selective, and better-tolerated delivery strategies, improving both pharmacokinetic and pharmacodynamic profiles. Several nanostructured formulations, such as the aforementioned liposomal DOX [70] and nanoparticle-bound PTX [71], are already approved for clinical use, demonstrating that these approaches are no longer purely theoretical or experimental concepts but have already translated into tangible clinical benefits.

Beyond optimizing existing drugs, nanotechnology addresses critical pharmaceutical limitations commonly associated with anticancer candidates, including poor aqueous solubility, rapid systemic clearance, chemical instability, and non-specific biodistribution (limitations extensively discussed throughout this chapter in relation to conventional chemotherapy) [82,84].

In parallel with the evolution of advanced drug delivery systems, oncology has progressively moved toward integrated strategies capable not only of treating disease but also of monitoring therapeutic responses. Within this context, *theranostics*, a term derived from the combination of *therapy* and *diagnostics*,

refers to an integrated medical approach in which diagnostic imaging and therapeutic intervention are combined within the same molecular or technological platform.

This approach allows the same agent or system to be used for tumor detection, treatment guidance, and post-therapy evaluation, linking diagnosis directly to therapeutic decision-making. By integrating imaging and therapy into a unified framework, theranostics reduces the traditional separation between disease identification and treatment, enabling a more personalized and dynamically adjustable clinical management of cancer [218].

Historically, theranostics originated in nuclear medicine as an evolution of targeted radiotherapy, where radionuclides are selectively delivered to tumors through ligands recognizing specific molecular markers [219]. A well-established example is [^{177}Lu]DOTATATE, used for the treatment of neuroendocrine tumors. This radiopharmaceutical consists of a somatostatin analogue conjugated with lutetium-177, which selectively binds somatostatin receptor subtype 2 (SSTR2). Following receptor-mediated internalization, β^- emission induces DNA damage and tumor cell death. Concurrent γ emission allows single-photon emission computed tomography (SPECT) imaging for biodistribution assessment and therapy monitoring [219,220]. This dual functionality exemplifies the theranostic principle, whereby diagnosis and therapy rely on the same molecular targeting mechanism.

Additional theranostic applications include metaiodobenzylguanidine (MIBG), employed in pheochromocytoma, paraganglioma, and neuroblastoma. Due to its structural similarity to norepinephrine, MIBG is selectively internalized via the norepinephrine transporter (NET). Labeling with iodine-123 enables diagnostic imaging, whereas iodine-131 provides therapeutic β^- radiation, representing a

classical isotope pair sharing identical biological targeting properties. Similarly, radioactive iodine therapy in differentiated thyroid cancer exploits iodide uptake mediated by the sodium-iodide symporter (NIS), enabling both therapeutic irradiation and post-therapy whole-body scintigraphic imaging to identify residual or metastatic disease [219,221].

However, traditional nuclear theranostics is often constrained by reliance on specific receptor expression and limited modularity in integrating multiple components. Nanotechnology extends this concept into nanotheranostics, a more flexible and modular evolution of theranostic [221].

Nanotheranostic platforms are multifunctional nanoscale carriers that incorporate therapeutic agents, targeting moieties, and imaging probes within a unified structure. Unlike conventional radiopharmaceuticals, NPs provide exceptional design flexibility, allowing simultaneous loading of drugs, contrast agents (e.g., optical, magnetic, or nuclear), and ligands for active targeting. Their nanoscale dimensions facilitate passive tumor accumulation through the EPR effect, while active targeting strategies enhance cellular internalization and local drug concentration, thereby reducing off-target toxicity. A major advantage of nanotheranostics lies in the development of stimuli-responsive systems, capable of releasing therapeutic payloads in response to tumor-specific internal or external triggers. These include acidic pH, redox gradients, enzyme overexpression, temperature variations, or externally applied light and magnetic fields. Such responsiveness enables spatially and temporally controlled drug activation, improving therapeutic precision. Simultaneously, embedded imaging components allow real-time visualization of nanoparticle biodistribution, drug release kinetics, and treatment efficacy, supporting adaptive therapeutic strategies aligned with

precision oncology principles. Furthermore, nanoparticle encapsulation protects labile compounds from premature degradation and enhances solubility and bioavailability, reinforcing the DDS concepts discussed earlier in this chapter [222].

Recent studies have demonstrated the versatility of diverse nanotheranostic platforms. Gold NPs, with their plasmonic properties and biocompatibility, serve as multimodal imaging agents and carriers for targeted drug delivery or photothermal ablation. Mesoporous silica NPs, characterized by high surface area and tunable pore architecture, allow co-loading of multiple drugs and imaging probes, supporting controlled release and non-invasive monitoring. Dendrimers, with their highly branched and monodisperse structure, provide precise control over functionalization density, biodistribution, and release kinetics. Lipid- and polymer-based nanostructures, including liposomes and polymeric micelles, further enable image-guided therapeutic strategies compatible with clinically established formulations [222]. Emerging biomaterials, such as CS NPs functionalized with plant-derived compounds, exemplify biocompatible systems that integrate optical imaging with anticancer activity, further bridging natural products and nanotechnology as explored in this thesis [222,223].

In conclusion, nanotheranostics evolves traditional therapies into intelligent, multifunctional systems for selective delivery, controlled release, and diagnostic integration. By directly addressing the limitations of traditional anticancer therapies discussed throughout this chapter, nanotheranostic approaches provide a conceptual and technological foundation for the nanosystems developed and investigated in the following sections of this thesis.

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CHAPTER 2. DESIGN, SYNTHESIS AND NANOFORMULATION OF CHALCONES WITH POTENTIAL CYTOTOXICITY FOR ANTITUMOR APPLICATIONS

2.1. Chalcones

Chalcone compounds (1,3-diaryl-2-propenones, Figure 2.1.) are pivotal intermediates in the biosynthetic pathway leading to the formation of flavonoids, characterized by a C6-C3-C6 framework. As such, they are ubiquitous throughout the plant kingdom, with a wide distribution in vegetables, fruits, tea, and various non-edible plants, including those possessing potential medicinal applications [1]. The term "chalcone" was first introduced by Kostanecki and Tambor, who synthesized a series of chromophoric compounds featuring an α,β -unsaturated carbonyl bridge [2], and originates from the Greek word *chalcos*, meaning "bronze," in reference to the distinctive color frequently exhibited by these compounds [3]. In recent years, chalcone-based scaffolds have garnered significant interest in medicinal chemistry due to their broad spectrum of biological activities. The therapeutic application of chalcones has historical roots, as plant- and herb-derived chalcone-containing extracts have extensively been employed in traditional medicine for treating various ailments [1], including cancer [4], inflammation [5], and diabetes [6]. Several synthetic chalcone derivatives have reached clinical application; for instance, metochalcone was formerly used for its choleretic (bile-stimulating) properties [3], while sofalcone was employed as an antiulcer and gastroprotective drug, also showing activity against *Helicobacter pylori* [7]. Last but not least, two additional chalcones worth mentioning are hesperidin methyl chalcone and hesperidin trimethyl chalcone, which have demonstrated clinical

efficacy in alleviating symptoms associated with venous and lymphatic disorders (vasoprotective activity), supported by a favorable safety profile and satisfactory systemic bioavailability (Figure 2.1.) [8–10].

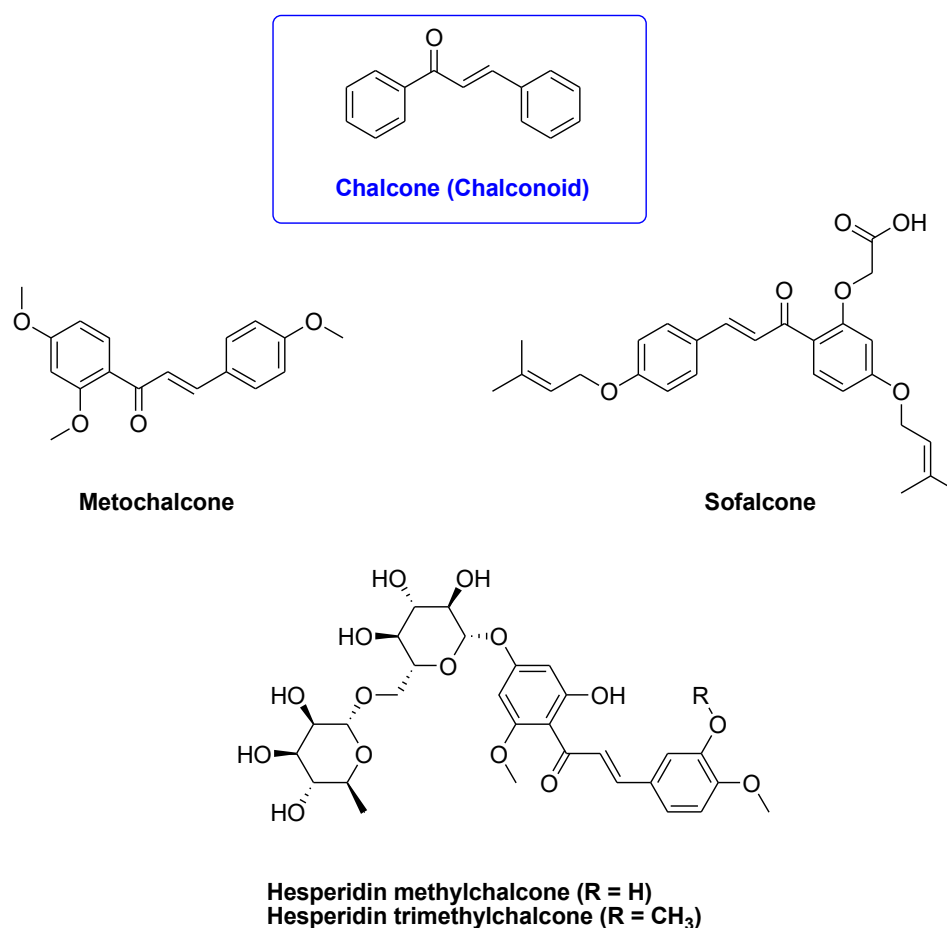


Figure 2.1. Chemical structures of chalcone (1,3-diaryl-2-propenone) and of three chalcone derivatives that have reached clinical application.

2.1.1. Chemistry of chalcones

Chalcones are a class of α,β -unsaturated ketones characterized by the presence of a central enone moiety ($-\text{CO}-\text{CH}=\text{CH}-$) that links two aromatic rings, commonly referred to as rings A (the one attached to the carbonyl group) and B (Figure 2.2.). This structural feature imparts to chalcones a characteristic linear C6-C3-C6

backbone, which plays a crucial role in determining their reactivity and biological activity. Typically, chalcones are small, non-chiral molecules with molecular weights ranging from 300 to 600 g/mol. They exhibit a high degree of lipophilicity, as indicated by their partition coefficient ($\log P$), which generally falls between 5 and 7, depending on the nature of the substituents. The conjugation between the carbonyl group and the olefinic double bond gives rise to an extended π -system, responsible for their characteristic coloration and their ability to absorb electromagnetic radiations in the in the ultraviolet (UV)-visible range [11].

Chalcones can exist as a conformational equilibrium between *trans-s-cis* and *trans-s-trans* isomers; this latter is thermodynamically more stable and predominantly present in natural sources [12].

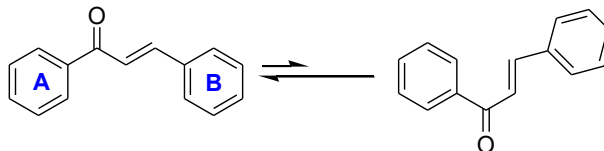


Figure 2.2. Comparison between the two isomers of chalcone.

Many naturally occurring chalcones are polyhydroxylated and often contain additional functional groups such as methoxy or prenyl substituents, which further influence their solubility, chemical reactivity and biological activity [1]. Their simple yet versatile scaffold makes chalcones valuable templates for the development of diverse compounds in both medicinal and synthetic organic chemistry.

2.1.2. Natural sources and biosynthesis

Chalcones are widespread natural compounds, with plants representing their primary biological source. This is largely due to their role as key biosynthetic precursors for a variety of polyphenolic classes of natural products, including flavanones, flavonols, flavanols, dihydroflavonols, flavones, isoflavones, isoflavonoids, aurones, and anthocyanidins [13]. While they are commonly found in medicinal plants, chalcones are particularly abundant in edible plant species [14]. An extensive body of scientific literature documents the discovery, isolation, and structural characterization of chalcones from edible plants, which exhibit remarkable structural diversity. For instance, several chalcones have been identified in tomatoes (*Solanum lycopersicum*), with concentrations varying depending on the ripening stage of the fruit. In particular, the highest levels of naringenin chalcone, together with other polyphenols such as quercetin, kaempferol, RTN, and certain dihydroxychalcones, have been reported in ripe tomatoes (Table 2.1.), and have been observed at the early stages of ripening, with a sharp decline as ripening progresses. Therefore, to maximize the health benefits associated with tomato consumption, such as antihistaminic, cardioprotective, chemopreventive, and hypoglycemic effects, it may be preferable to consume less mature tomatoes, when chalcones content is at its peak [15].

Table 2.1. Major naturally occurring chalcones in alphabetical order, their structure and their main biological activities.

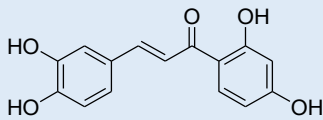
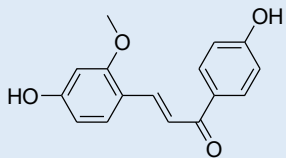
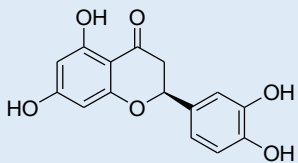
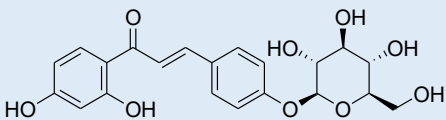
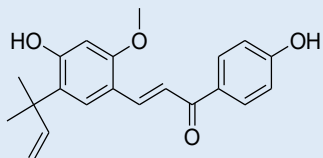
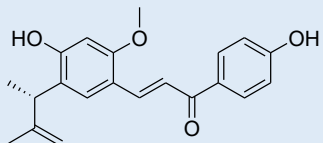
<i>Name</i>	Chemical structure	Biological activities
<i>Butein</i>		<i>Anticancer, antimicrobial, antioxidant, antiphlogistic, neuroprotective properties [16]</i>
<i>Echinatin</i>		<i>Anticancer, antioxidant, antiphlogistic properties [17]</i>
<i>Eriodictyol</i>		<i>Anticancer, antidiabetic, antioxidant, antiphlogistic, cardioprotective, hepatoprotective, neuroprotective properties [18]</i>
<i>Isoliquiritin</i>		<i>Antimicrobial, antioxidant, antiphlogistic, neuroprotective properties [19]</i>
<i>Licochalcone A</i>		<i>Anticancer, antioxidant, antiparasitic, antiphlogistic properties [20]</i>
<i>Licochalcone E</i>		<i>Anticancer, antimicrobial, antioxidant, antiphlogistic properties [21]</i>

Table 2.1. (continued)

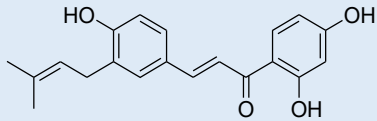
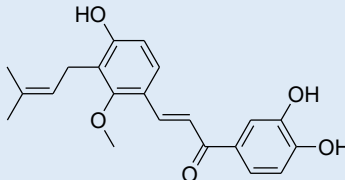
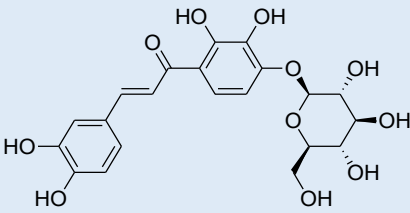
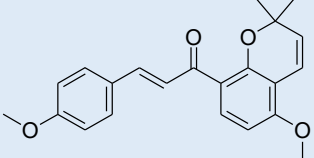
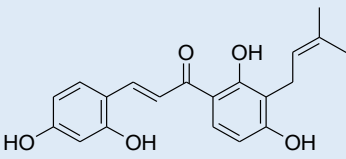
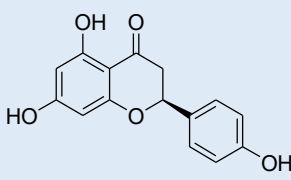
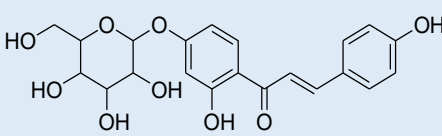
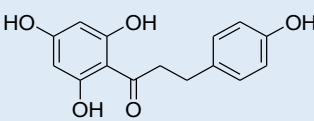
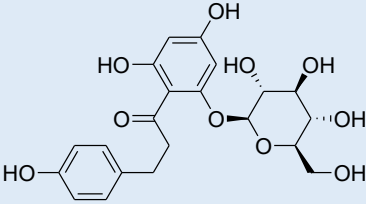
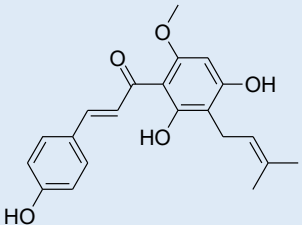
<i>Licoagrochalcone B</i>		NR
<i>Licoagrochalcone C</i>		<i>Antioxidant [22], antiphlogistic properties [23]</i>
<i>Marein</i>		<i>Anticancer, antiphlogistic, [24], antidiabetic [25], antioxidant [26], properties</i>
<i>Millepachine</i>		<i>Anticancer properties [27]</i>
<i>Morachalcone A</i>		<i>Antimalarial [28], neuroprotective [29], tyrosinase inhibitory [30] properties</i>
<i>Naringenin</i>		<i>Anticancer, antidiabetic, antioxidant, antiphlogistic, cardioprotective, neuroprotective properties [31]</i>
<i>Neoisoliquiritin</i>		<i>Antidepressive, antiphlogistic, chemopreventive properties [32]</i>
<i>Phloretin</i>		<i>Anticancer, antioxidant, antiphlogistic properties [20]</i>

Table 2.1. (continued)

<i>Phloridizin</i>		See above
<i>Xanthohumol</i>		Antiphlogistic, chemopreventive, phytoestrogenic properties [33].

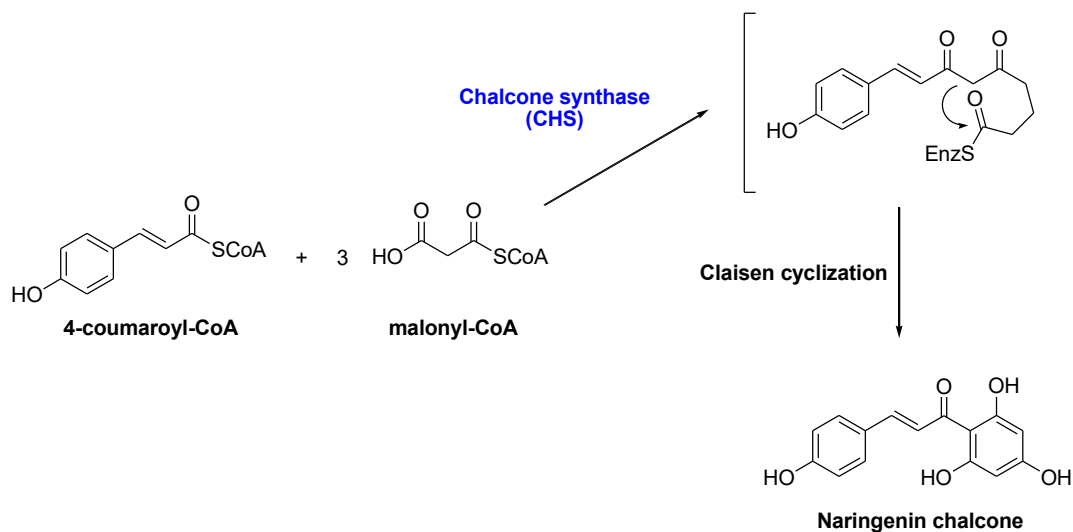
Note: NR = not reported.

Another food notably rich in chalcones and related polyphenols is the apple (*Malus domestica*). Apples have been found to contain chalcone derivatives (dihydrochalcones), such as phloretin and its conjugate phloridzin (also known as phlorizin) (Table 2.1.) [15], which exhibit a broad spectrum of biological activities, including antibacterial [34], anti-inflammatory [35] and anticancer [36] effects. Unlike tomatoes, where chalcones are primarily restricted to the skin, in apples these compounds are nearly ubiquitous throughout the fruit, with concentrations varying significantly depending on the specific cultivar [15].

However, the richest known natural source of chalcones is *Glycyrrhiza glabra* (licorice), with more than 50 distinct compounds isolated to date. This exceptional chemical diversity has made licorice a key component of traditional Chinese medicine, where it has been used to treat a wide range of conditions, from sore throat and respiratory infections like pneumonia to gastrointestinal disorders such as peptic ulcers [15]. Among the most prominent chalcones identified in licorice are isoliquiritin, neoisoliquiritin, and licochalcone A, along with several prenylated derivatives including licoagrochalcones B and C, echinatin, and morachalcone A

(Table 2.1.). In addition, more structurally complex chalcones have been found, such as tetrahydroxy- and pentahydroxy-prenylated forms and their methoxylated variants, all contributing to the broad structural and functional diversity of licorice-derived metabolites [1].

As for their biosynthesis, chalcones are produced through the catalytic action of the enzyme chalcone synthase (CHS), which was first identified in the 1970s and is widely distributed across both higher and lower plant species [37,38]. The molecular mechanism of CHS was elucidated following its crystallization from *Medicago sativa* by Noel and collaborators [39]. Structurally, CHS is a homodimeric enzyme, with each monomer containing an active site that includes four highly conserved amino acid residues (Cys164, Phe215, His303, and Asn336) essential for catalytic activity across CHS and CHS-like enzymes [11]. The biosynthetic reaction begins with the transfer of a coumaroyl group from 4-coumaroyl-CoA to the thiol group of Cys164. This is followed by three sequential condensations with malonyl-CoA units, yielding a linear tetraketide intermediate. This intermediate then undergoes a regioselective intramolecular Claisen-type cyclization, resulting in the formation of a chalcone scaffold, specifically naringenin chalcone. This product serves as the central intermediate in the biosynthesis of the previously mentioned polyphenolic families (Scheme 2.1.) [11].



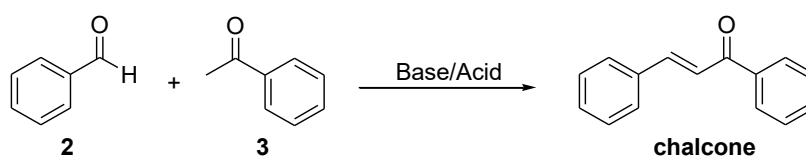
Scheme 2.1. Biosynthetic pathway leading to the synthesis of chalcones.

2.1.3. Synthetic approaches for the preparation of chalcones

As already highlighted in the previous sections, chalcones are a structurally simple yet highly versatile class of compounds. Their simplicity, along with the presence of multiple reactive hydrogen atoms and the subsequent potential to generate a wide range of biologically active derivatives, has made them valuable synthetic intermediates and privileged scaffolds in drug discovery [40]. Over the years, a variety of synthetic methodologies have been developed for the preparation of chalcone derivatives, each offering specific advantages in terms of yield, regioselectivity, reaction conditions, functional group compatibility and environmental friendliness. Nevertheless, the most common and widely adopted approach remains the Claisen-Schmidt condensation, which offers a simple yet versatile synthetic route for the preparation of structurally diverse chalcones [11].

2.1.3.1. Claisen-Schmidt Condensation

Due to its practical simplicity and broad applicability, the most commonly employed method for the synthesis of chalcones is the Claisen-Schmidt condensation. Also known in literature as crossed aldol condensation, this reaction involves the condensation of benzaldehyde **2** with methyl ketone **3** (typically acetophenone), under catalytic conditions (Scheme 2.2.). The Claisen-Schmidt condensation is named in honor of its original developers, R. L. Claisen and J. G. Schmidt [41,42].

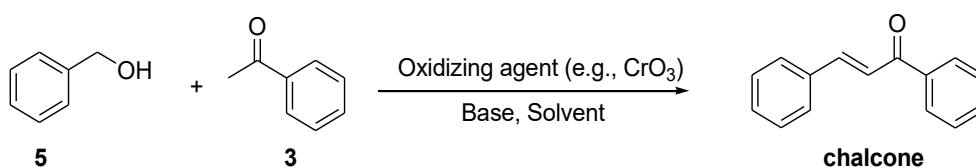


Scheme 2.2. Application of crossed aldol condensation within chalcone synthesis.

Within this specific context, two main categories of catalysts can be used [43]:

- Strong bases (e.g., sodium hydroxide [44], potassium hydroxide [45], or piperazine [46] in a hydroalcoholic mixture). In this case, the reaction proceeds through the formation of an enolate intermediate, which undergoes aldol addition followed by dehydration to yield the final chalcone product [11].
- Acidic catalysts (e.g., hydrochloric acid [47], dry hydrogen chloride gas [48], aluminum chloride [49], thionyl chloride [50], or *p*-toluenesulfonic acid [51]). In the acid-catalyzed version, the reaction typically proceeds via an enol intermediate rather than an enolate, but still follows a similar condensation pathway [11].

Despite its relatively high experimental simplicity, the aldol condensation method can sometimes lead to the formation of several side products, making the purification of the final compound more challenging. Moreover, reaction yields often show significant variation, ranging from as low as 10% to nearly quantitative, depending on the nature of the starting materials as well as the reaction conditions [43,52]. Additionally, the method relies on aldehydes, which are often volatile, reactive, and in many cases toxic, posing handling risks for operators. To overcome this last crucial limitation and expand the applicability of the reaction, recent efforts have been focused on one-pot protocols starting directly from benzyl alcohols **5** and ketones **3**. In these protocols, an appropriate catalyst, such as chromium(VI) oxide, is employed to oxidize the benzyl alcohol *in situ* to the corresponding aldehyde, which then immediately reacts with the ketone present in the reaction mixture to form the chalcone (Scheme 2.3.). By avoiding direct manipulation of toxic aldehydes and integrating oxidation with condensation, this method offers a safer and more efficient synthetic route.



Scheme 2.3. One-pot synthesis of chalcones from alcohol and ketone.

2.1.3.2. Solvent-free approaches

In order to synthesize chalcones, solvent-free approaches have also been explored as environmentally friendly alternatives to conventional methods. These include either grinding benzaldehyde and the appropriate ketone together with a base

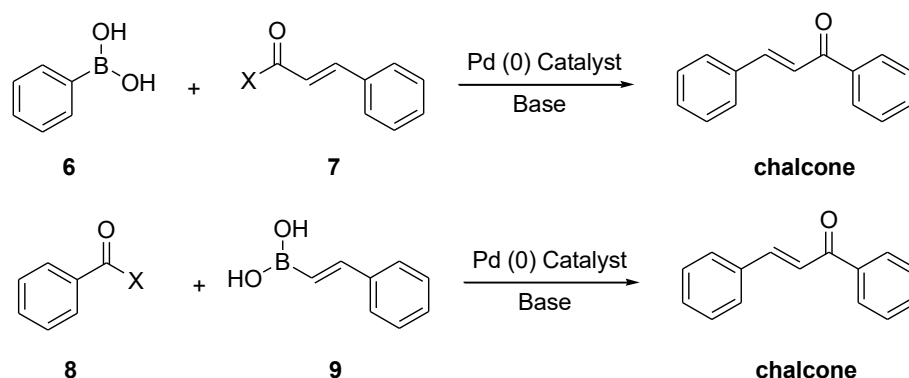
catalyst [53] (such as sodium hydroxide [53] or potassium fluoride supported on alumina [54]) to promote product formation through mechanical activation, or using microwave irradiation to achieve the same goal in a more energy-efficient and solvent-free manner [55]. For instance, Kulkarni *et al.* developed a protocol to synthesize fifteen chalcones, bearing various substituents on both aromatic rings A and B, using a simple solvent-free reaction with calcium oxide, which acts as a base catalyst [56].

Microwave-assisted synthesis offers several benefits including avoiding solvent effects, lowering side product formation, increasing reaction adaptability, drastically cutting down reaction times, and often boosting product yields [11].

2.1.3.3. Suzuki-Miyaura coupling

An alternative approach for chalcone synthesis involves cross-coupling reactions, notably the Suzuki reaction, first reported in 1979. Its significance in organic chemistry was recognized in 2010 when A. Suzuki, together with R. F. Heck and E. Negishi, was awarded the Nobel Prize in Chemistry. Also known as Suzuki-Miyaura coupling, this reaction enables the selective formation of carbon-carbon (C-C) bonds between two molecular fragments by coupling an aryl or vinyl halide with an aryl or vinyl boronic acid, in the presence of a palladium(0)-based catalyst and a suitable base (Scheme 2.4.) [57,58].

In the specific context of chalcone synthesis, two main strategies have been successfully applied, yielding promising results regardless of the electronic nature of the substituents on aromatic rings A and B: the coupling of cinnamoyl halide **7** with phenylboronic acid **6**, or alternatively, the coupling of benzoyl halide **8** with phenylvinylboronic acid **9** [59].



Scheme 2.4. Application of Suzuki coupling in the contest of chalcone synthesis.

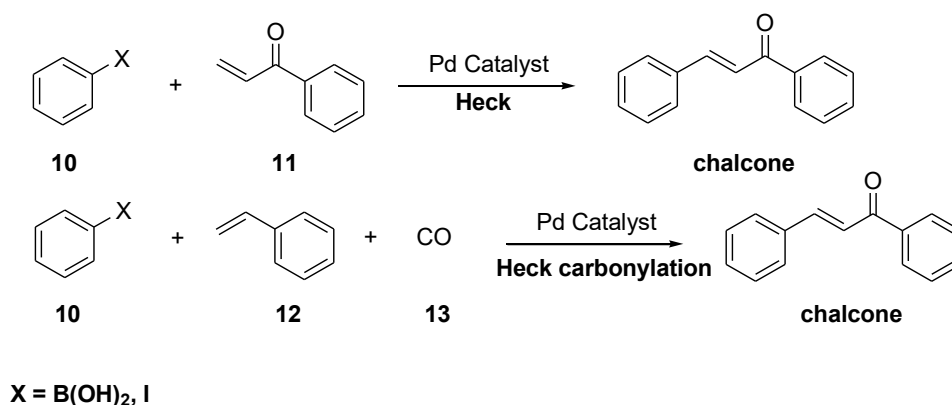
Reaction conditions are crucial to the efficiency of Suzuki-Miyaura coupling. In order to achieve high yields (typically ranging from 50% to 90%), optimal conditions include the use of an anhydrous solvent such as toluene, tetrakis(triphenylphosphine)palladium(0) as the catalyst, and a cerium-based base, such as cerium carbonate [60].

2.1.3.4. Heck coupling

Alongside Suzuki coupling, another valuable cross-coupling strategy applicable to the synthesis of chalcones is the Mizoroki-Heck reaction. First introduced by Tsutomu Mizoroki and later refined by Richard F. Heck, who was awarded the Nobel Prize in Chemistry in 2010, this palladium-catalyzed reaction is widely recognized as a cornerstone of modern organic synthesis. Traditionally employed for the preparation of stilbenes, the Mizoroki-Heck reaction enables the formation of C-C bonds between aryl or vinyl halides (or their boronic acid counterparts) and alkenes in the presence of a suitable base [61,62]. Due to the structural resemblance between stilbenes and chalcones, both containing conjugated π -systems linking two

aromatic rings, the Heck reaction has been effectively adapted for chalcone synthesis. In this modified context, the alkene component is replaced with an α,β -unsaturated ketone, which serves as the electrophilic coupling partner (Scheme 2.5.). Under palladium catalysis, this strategy enables the formation of the enone scaffold characteristic of chalcones through direct C-C bond formation [11].

A key study by Cavarischia *et al.* demonstrated the feasibility of this approach, reporting the synthesis of chalcones *via* the Heck reaction between various aryl halides **10** and α,β -unsaturated ketones **11** in the presence of palladium(II) acetate [Pd(OAc)₂], triphenylphosphine (PPh₃), triethylamine (TEA), and acetonitrile (CH₃CN) as the reaction solvent. The method allowed for good functional group tolerance and moderate to excellent yields, highlighting its synthetic utility in constructing diversely substituted chalcone derivatives under relatively mild conditions [60].



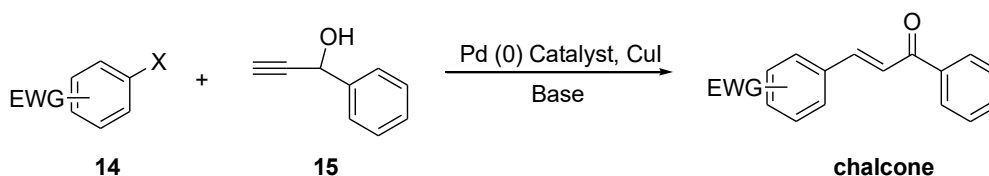
Scheme 2.5. Application of Heck coupling and Heck carbonylation in the context of chalcone synthesis.

The main drawback of this method lies in the limited availability of aryl vinyl ketones, which can restrict its broader application. To overcome this limitation, an alternative strategy involves the application of Heck carbonylation, a variant of the

traditional Heck reaction that introduces carbon monoxide (CO) into the reaction environment (Scheme 2.5.) [11]. In this process, aryl or vinyl halides (or their boronic acid or triflate analogs) **10** react with alkenes **12** in the presence of CO **13**, enabling the formation of α,β -unsaturated carbonyl compounds, including chalcones. This approach has been shown to afford yields ranging from approximately 70% to over 90% under the following reaction conditions: [(cinnamyl)PdCl]₂, 1,3-bis(diphenylphosphino)propane (dppp), triethylamine (NEt₃), toluene, 100 °C, and a reaction time of 20 hours [63–65].

2.1.3.5. Sonogashira isomerization coupling reaction

Among the various metal catalyzed cross-coupling strategies that can be exploited for chalcone synthesis, a particularly innovative and successful approach is the Sonogashira isomerization coupling reaction. First reported by Braun *et al.* in 2006, this methodology integrates the classical Sonogashira reaction, which forms C(sp²)-C(sp) bonds between aryl or vinyl halides and terminal alkynes in the presence of palladium and copper catalysts [66], with a subsequent base-catalyzed isomerization step [67].



Scheme 2.6. Application of Sonogashira isomerization coupling reaction in the contest of chalcone synthesis.

Also referred to as a Coupling-Isomerization Reaction (CIR), this process enables the one-pot transformation of propargylic alcohols **15** and aryl halides **14** into α,β -unsaturated ketones (Scheme 2.6.). Mechanistically, the Sonogashira coupling first produces an internal propargylic alcohol intermediate, which then undergoes a base-mediated isomerization, commonly catalyzed by triethylamine, via a formal 1,3-hydrogen shift to furnish the enone product. The reaction is generally carried out under reflux conditions in polar solvents such as THF, ethanol, or acetonitrile, in the presence of catalytic amounts of $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ and CuI . A key requirement for the success of the CIR is the presence of an electron-withdrawing group on the aryl halide. This not only facilitates oxidative addition to the palladium catalyst during the coupling step, but also stabilizes the resulting π -conjugated enone system, thereby enhancing the thermodynamic favorability of the isomerization. In contrast, electron-neutral or electron-rich halides often lead to poor conversions or stall the reaction sequence at the propargylic alcohol stage.

This strategy offers several advantages over classical methods such as aldol condensation: milder reaction conditions, broad functional group tolerance, and compatibility with heteroaromatic systems, making it a valuable approach for the synthesis of diversely substituted chalcones [67].

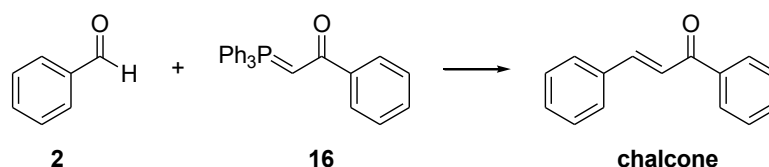
2.1.3.6. Wittig Reaction

Because chalcones contain an α,β -unsaturated carbonyl group and can thus be considered as alkenes, the Wittig reaction represents another valuable synthetic strategy for their preparation. This reaction involves the nucleophilic addition of a phosphorus ylide (commonly referred to as the *Wittig reagent*) to a carbonyl compound, resulting in the formation of an alkene and triphenylphosphine oxide as

a byproduct [68,69]. Like the previously mentioned coupling reactions, the Wittig reaction, first reported by the German chemist Georg Wittig in 1954, earned its discoverer the Nobel Prize in Chemistry in 1979.

Efforts to apply this methodology to chalcone synthesis have been explored over the years. An early example involved the reaction of benzaldehyde **2** with triphenylbenzoylmethylene phosphorane **16** in refluxing benzene for 30 hours, affording promising yields of around 70% (Scheme 2.7.) [70,71].

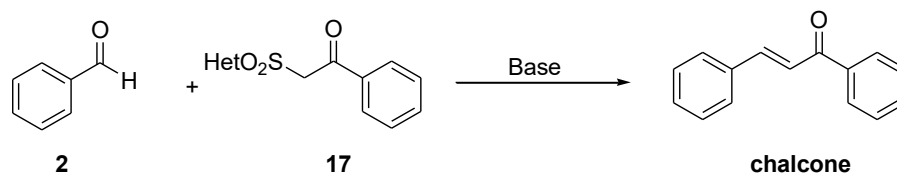
However, subsequent studies showed that microwave irradiation markedly improved the reaction outcome, boosting yields, often above 80%, while drastically reducing the reaction time from 30 hours to just 5-6 minutes, irrespective of the starting materials used [72].



Scheme 2.7. Application of Wittig reaction in the contest of chalcone synthesis.

2.1.3.7. Julia-Kocienski Olefination

Another synthetic approach commonly employed for the preparation of alkenes, and therefore applicable to chalcone synthesis, is the less widely known Julia-Kocienski olefination. This reaction enables the efficient formation of alkenes from heteroaryl sulfones (commonly referred to as *Julia reagents*) and aldehydes **2**, in a single step [73]. In the context of chalcone synthesis, the Julia reagent can be suitably modified to a heteroaryl sulfonyl phenyl ethanone derivative **17** to introduce the appropriate aryl framework (Scheme 2.8.) [11].



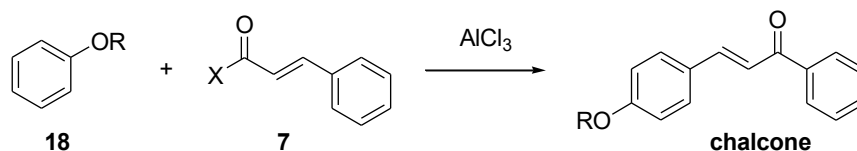
Scheme 2.8. Application of Julia-Kocienski olefination in the context of chalcone synthesis.

As with other olefination methods, reaction conditions play a critical role in determining the efficiency and outcome of the Julia-Kocienski reaction. Optimal results are typically achieved using nonpolar or moderately polar aprotic solvents, with tetrahydrofuran (THF) being the most suitable, followed by dichloromethane (DCM), chloroform (CHCl₃), and acetonitrile (CH₃CN). The use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a base, in combination with benzothiazole-derived sulfones, has been shown to significantly enhance both reactivity and selectivity. Notably, the reaction proceeds with high stereoselectivity, typically favoring the (*E*)-alkene isomer, which is desirable for the synthesis of chalcones [74].

2.1.3.8. Friedel-Crafts acylation reaction

As reported in a 1978 paper by Shotter *et al.*, another valuable method, yet less commonly explored, for the synthesis of chalcones is the Friedel-Crafts acylation reaction [75]. This classical electrophilic aromatic substitution involves the introduction of an acyl group (R-C=O) onto an activated aromatic ring, typically in the presence of a strong Lewis acid catalyst, such as aluminum chloride (AlCl₃) [76]. In the context of chalcone synthesis, the reaction can be adapted by employing

appropriately substituted aromatic ethers **18** as the aromatic substrate and cinnamoyl chloride **7** as the acylating agent (Scheme 2.9.) [75].



Scheme 2.9. Application of Friedel-Crafts acylation in the context of chalcone synthesis.

The key advantage of this strategy lies in its regioselectivity and the ability to introduce the α,β -unsaturated carbonyl system directly onto the aromatic nucleus. The electron-donating nature of alkoxy groups on the aromatic ring increases its reactivity towards electrophilic attack, thereby facilitating the acylation step. Shotter and coworkers demonstrated that this approach yields chalcone derivatives in fair yields, and under relatively mild conditions, provided that strict anhydrous conditions are maintained to preserve the activity of the Lewis acid catalyst [75].

2.1.4. Pharmacological profile and antitumor activity of chalcones

Chalcones represent a class of bioactive compounds characterized by a high pharmacological potential, as supported by an extensive body of literature documenting their broad spectrum of biological activities. Both natural and synthetic chalcone derivatives have been shown to exhibit multifaceted bioactivities, including anti-inflammatory [5], antigout [77], antihistaminic [78], antibacterial [79], antitubercular [80], antiviral [81] (notably against retroviruses such as HIV [82] and members of the *Coronaviridae* family [83], of particular relevance in the current epidemiological context), antiprotozoal [84], antioxidant

[85], antiobesity and hypolipidemic [86], antispasmodic [87], sedative/hypnoinductive [88], and neuroprotective [89] properties.

Of these diverse activities, the antitumor potential of chalcones stands out as particularly significant [4]. This is supported by numerous studies confirming their ability to inhibit tumor growth *in vitro* and *in vivo* [5,90–94], generating considerable interest especially given the well-documented limitations of conventional chemotherapeutics, which underscore the urgent need for novel therapeutic strategies [95]. The promising candidacy of chalcones for anticancer drug development stems primarily from their unique ability to modulate multiple molecular targets and signaling pathways involved in tumor initiation, progression, and metastasis, representing a key multitarget advantage [4].

This potential is exemplified by compounds like xanthohumol, a prenylated chalcone isolated from hop inflorescences (*Humulus lupulus* L., Cannabaceae) and consequently also found in beer. Together with its isomer 8-prenylnaringenin, xanthohumol exhibits potent antiproliferative and pro-apoptotic effects in various human cancer cell lines (e.g., breast, prostate, colorectal carcinomas) [96]. Another notable example is butein (Table 2.1.), a tetrahydroxychalcone found in medicinal plants such as *Dalbergia odorifera* and *Toxicodendron vernicifluum* [97]. Butein demonstrates significant anticancer properties, including inhibition of proliferation, induction of apoptosis, and suppression of metastasis and angiogenesis, effects attributed to its interference with key oncogenic signaling pathways [98].

Although the precise mechanisms underlying the broad biological activities of chalcones have not yet been fully elucidated, experimental evidence indicates that their antitumor effects result from a multitarget approach rather than a single specific mechanism [11].

2.1.4.1. Inhibition of the NF- κ B signaling pathway

One of the primary mechanisms through which chalcones exert their antiproliferative and anti-inflammatory effects involves the inhibition of the nuclear factor kappa B (NF- κ B) signaling pathway, a key transcription factor critically involved in inflammation, as well as in cell proliferation, apoptosis, and cancer progression [99]. NF- κ B is a protein complex composed of two subunits, p50 and p65, which, under resting conditions, are retained in the cytoplasm through association with an inhibitory protein known as inhibitor of κ B (I κ B). Upon inflammatory stimulation, such as the recognition of pathogens or damaged tissue by pattern recognition receptors (PRRs), I κ B undergoes phosphorylation mediated by I κ B kinases (IKKs). This post-translational modification triggers the dissociation of I κ B from the p50-p65 complex, its ubiquitination, and subsequent degradation by the 26S proteasome. The freed p50-p65 heterodimer then translocates to the nucleus, where it promotes the transcription of genes involved in inflammation, cell proliferation, apoptosis, and oncogenesis [100].

Chalcones are able to inhibit NF- κ B activation by covalently binding, *via* Michael addition, to the cysteine residue 179 (Cys179) of IKKs [99,101,102]. This interaction prevents I κ B phosphorylation, thereby stabilizing the p50-p65/I κ B complex in the cytoplasm and ultimately blocking NF- κ B nuclear translocation and transcriptional activity [11].

2.1.4.2. Inhibition of thioredoxin reductase activity

Thioredoxin reductase (TrxR) is a key enzyme of the thioredoxin system, one of the most important, though less well-known, cellular antioxidant defense mechanisms. To maintain redox homeostasis, TrxR uses NADPH to reduce

thioredoxin (Trx), a small redox-active protein that cycles between an oxidized (inactive) form and a reduced (active) state. Once reduced by TrxR, Trx can, in turn, reduce disulfide bonds in various cellular target proteins, thereby helping preserve their functional structure and activity in a reduced state. This redox-regulating system plays a crucial role in protecting cells from oxidative stress, which, if left unchecked, can damage DNA, lipids, proteins, and other vital cellular components [103,104]. Notably, the Trx/TrxR system is often found to be upregulated in a variety of human cancers, reflecting the increased demand for antioxidant protection in rapidly proliferating tumor cells. Due to their high metabolic rate and associated overproduction of reactive oxygen species (ROS), cancer cells rely heavily on Trx/TrxR activity to survive, evade apoptosis, and develop resistance to chemotherapeutic agents [105,106].

A key anticancer mechanism of chalcones involves interference with this redox-regulating system. Specifically, chalcones can inhibit TrxR by covalently binding, *via* Michael addition, to its catalytic selenocysteine residue. This interaction blocks the enzyme's activity, leading to the accumulation of intracellular ROS. This pro-oxidant shift disrupts the redox balance in tumor cells leading to a collapse of redox homeostasis and inducing apoptosis [107].

2.1.4.3. Antimitotic activity

Analogously to certain antineoplastic chemotherapeutic agents, such as vinca alkaloids and taxanes, several chalcones have demonstrated antiproliferative activity through interference with the process of cell division [108]. Specifically, their mechanism of action involves the disruption of mitotic spindle assembly, a structure essential for the accurate segregation of newly replicated DNA into

daughter cells during mitosis. Under physiological conditions, spindle fibers are formed through a tightly regulated and dynamic polymerization of α - and β -tubulin heterodimers. In the presence of mitotic inhibitors, this polymerization is disrupted, resulting in mitotic arrest and, frequently, apoptotic cell death [109].

A representative example is the natural chalcone millepachine (Table 2.1.), isolated from the seeds of the Chinese medicinal plant *Millettia pachycarpa*. This compound inhibits mitotic progression and induces programmed cell death in hepatocellular carcinoma cells, both *in vitro* and *in vivo*, exhibiting significantly higher potency than DOX while lacking its cardiotoxic effects [110]. Investigations of microtubule dynamics reveal that millepachine achieves this activity by suppressing tubulin polymerization via irreversible binding at the colchicine site on β -tubulin [27]. However, direct tubulin binding does not represent its sole mechanism of action. The antimitotic activity characteristic of chalcones frequently extends beyond immediate tubulin interaction to encompass well-documented indirect pathways. These include inhibition of CDK1 (cyclin-dependent kinase 1, essential for mitotic entry) [111], activation of CHK2 (checkpoint kinase 2, involved in DNA damage response and G2/M arrest) [112], and induction of G2/M cell cycle arrest through modulation of cdc25C (a phosphatase responsible for CDK1 activation) [113]. Further mechanism involves the activation of the mitochondrial apoptotic pathway, evidenced by an elevated Bax/Bcl-2 ratio, cytochrome c release, and caspases 9 and 3 activation [110].

2.1.4.4. Protein-kinase inhibition

An additional key mechanism of action attributed to chalcones is the inhibition of protein kinase activity [11]. These enzymes, such as the epidermal growth factor

receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR), are crucial mediators of intracellular signal transduction. Upon activation by their ligands (such as peptide hormones, cytokines, or growth factors), they initiate signaling cascades that regulate essential cellular processes like proliferation, apoptosis, immune responses, and tissue homeostasis. In cancer, however, these receptors are often overexpressed, resulting in persistent activation of proliferative pathways. By blocking kinase activity, chalcones interfere with these deregulated signals and thereby exert antitumor effects through the suppression of uncontrolled cell growth [114].

Among natural chalcones, butein, phloretin, and marein (Table 2.1.) demonstrate significant inhibitory effects on EGFR tyrosine kinase *in vitro*. Butein exhibits the strongest activity ($IC_{50} = 8 \mu M$), followed by marein ($IC_{50} = 19 \mu M$) and phloretin ($IC_{50} = 25 \mu M$). Molecular modelling indicates that all three compounds dock into the ATP-binding pocket of EGFR, with binding stabilized through a combination of hydrogen bonds and hydrophobic interactions involving key active site residues [24].

2.1.4.5. Activation of the NRF2 pathway

Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor that plays a fundamental cellular defense mechanism against oxidative stress and electrophilic damage [115,116]. Upon activation, NRF2 induces the expression of a broad range of cytoprotective genes, including phase II detoxification enzymes and antioxidant proteins, such as NAD(P)H-quinone oxidoreductase 1 (NQO1). These proteins collectively contribute to neutralizing ROS, maintaining redox homeostasis, detoxifying harmful compounds, and preserving the structural and

functional integrity of biomolecules [117]. Under physiological conditions, NRF2 is bound in the cytoplasm to Kelch-like ECH-associated protein 1 (Keap1), which targets it for ubiquitination and subsequent proteasomal degradation. In response to oxidative or electrophilic stimuli, specific cysteine residues within Keap1 undergo covalent modification, resulting in a conformational change that impairs Keap1's ability to repress NRF2 [115,116]. Freed from Keap1-mediated degradation, NRF2 translocates into the nucleus, where it binds to antioxidant response elements (AREs) in the promoters of its target genes, triggering a transcriptional program that enhances cellular resilience [115,118].

Recent studies have shown that certain chalcones (e.g., xanthohumol [119] and licochalcone E [120], Table 2.1.) can activate the NRF2 pathway through covalent modification of specific cysteine residues on Keap1 *via* Michael addition [121]. This activation enhances the expression of antioxidant and detoxifying enzymes, thereby strengthening cellular defenses against oxidative stress. Contrary to what might be expected, this protective mechanism correlates more closely with chemopreventive effects rather than direct anticancer activity in established tumors. By boosting antioxidant defenses in healthy cells, NRF2 activation helps prevent oxidative DNA damage and oncogenic mutations [118]. However, in established cancers, this same mechanism may paradoxically promote tumor cell survival [122], disease progression [123,124], and therapy resistance [125,126]. This occurs because NRF2 protects malignant cells from oxidative stress, which would otherwise impair their viability. This phenomenon is often referred to as “the dark side of NRF2”, highlighting how a system that normally safeguards healthy cells can be exploited by cancer cells for their own advantage [127].

2.1.4.6. Accessory mechanisms

In addition to the aforementioned mechanisms of action, chalcones exert their antitumor effects through several accessory pathways. One of the most studied among these is the inhibition of cyclooxygenase enzymes (COX-1 and COX-2), particularly COX-2, a gene transcriptionally regulated by NF- κ B and frequently overexpressed in various tumors. COX-2 is implicated in the promotion of inflammation, angiogenesis, and resistance to apoptosis, hallmarks of cancer. By downregulating COX-2 expression or enzymatic activity, chalcones can suppress pro-inflammatory tumor microenvironments and sensitize cancer cells to cell death [128].

Chalcones have also been reported to interfere with key signaling cascades such as the JAK/STAT [129], ROS/MAPK [130], and p38 MAPK pathways [131], all of which are involved in the regulation of cell proliferation, survival, and stress response.

Finally, it is important to mention their well-documented antioxidant activity, mainly attributed to the electronic delocalization provided by the conjugated system in their chemical structure [132]. While this feature is more commonly associated with chemopreventive effects rather than direct cytotoxicity, it still contributes to their overall antitumor potential by mitigating oxidative DNA damage and cellular stress.

2.1.5. Halogenated chalcones as promising anticancer agents

Chalcones are structurally simple yet highly versatile compounds, capable of interacting with a wide variety of biological targets. This multitarget potential is largely due to the presence of the α,β -unsaturated carbonyl system, which can act

as a Michael-type acceptor [11]. Although this broad reactivity complicates the establishment of clear structure-activity relationships (SAR), it has not limited the development of chalcone derivatives as lead compounds for diverse therapeutic areas, including diabetes [133], fungal [134] and bacterial [135] infections, among others.

Regarding their anticancer potential, a pioneering study by Cabrera *et al.* (2007) systematically compared the cytotoxicity of synthetic chalcones, flavanones, and flavones across several human cancer cell lines (renal TK-10, breast MCF-7, and colon HT-29). This work highlighted a key principle now widely recognized: the linear structure of chalcones, featuring the conjugated α,β -unsaturated carbonyl system, confers significantly higher cytotoxicity compared to their cyclic counterparts, suggesting a fundamental structural advantage for anticancer activity. Fourteen out of 53 compounds displayed strong cytotoxicity against at least two of the three tested cell lines, with chalcones being clearly more active. For instance, the chalcone (*E*)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (compound **19**, Figure 2.3.) achieved 4% survival in TK-10 cells at 100 μ M, while its cyclic flavone counterpart 2-(4-methoxyphenyl)-4H-chromen-4-one (compound **20**) showed 82% survival. Moreover, the essential role of the α,β -unsaturated system was confirmed by the reduced activity of saturated analogues lacking the conjugated double bond, further underscoring the importance of the enone moiety [136].

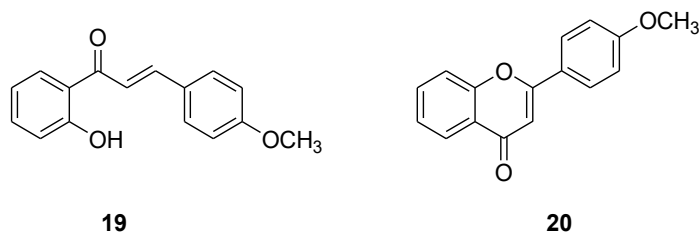
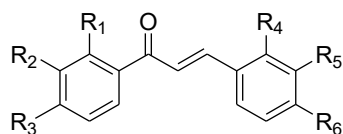


Figure 2.3. Chemical structure of compound **19** (*E*)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one) and its cyclic flavone counterpart **20**.

When it comes to anticancer activity, among the diverse structural modifications explored, halogenation has emerged as a particularly effective strategy to enhance the cytotoxic potential of chalcones. While the linear α,β -unsaturated framework provides a crucial foundation for biological activity, subsequent research has shown that the introduction of halogen atoms on the aromatic rings can significantly modulate both potency and selectivity. A fundamental experimental advance was reported by Ramalho *et al.* (2013), who identified compound **21** ((*E*)-3-(4-chlorophenyl)-1-phenylprop-2-en-1-one, Figure 2.4.) as a potent cytotoxic agent among a series of synthesized chalcone derivatives.



- 21:** R₆ = Cl
22: R₁ = OH; R₃ = OH; R₄ = Cl
23: R₁ = CH₃; R₆ = Cl
24: R₂ = Cl; R₆ = Br
25: R₅ = Cl
26: R₅ = Br
27: R₅ = Br; R₆ = OCH₃
28: R₃ = NO₂; R₅ = Cl

Figure 2.4. Chemical structure of halogenated chalcones described in the literature. Unless otherwise indicated, all substituents (R₁-R₆) are hydrogen atoms.

This derivative, bearing a *para*-chloro substituent on ring B, demonstrated significant cytotoxicity across three human cancer cell lines: melanoma (MDA-MB-435, $IC_{50} = 2.6 \mu\text{g/mL}$), colon carcinoma (HCT-8, $IC_{50} = 2.5 \mu\text{g/mL}$), and glioblastoma (SF-295, $IC_{50} = 1.9 \mu\text{g/mL}$). Its antiproliferative activity was attributed to the electron-withdrawing effect of the chlorine atom, which increases the electrophilicity of the enone system and promotes interaction with cellular targets. In addition to its cytotoxic effect, compound **21** moderately inhibited cathepsin K (86.7% inhibition at 125 μM ; $IC_{50} = 98.8 \mu\text{M}$), an enzyme implicated in tumor progression and bone resorption, while showing negligible activity against cathepsin B [137].

In the context of overcoming multidrug resistance (MDR), Juvalé *et al.* (2012) identified halogenated chalcones as selective inhibitors of the Breast cancer resistance protein (BCRP/ABCG2). Compound **22** (*(E)*-3-(2-chlorophenyl)-1-(2,4-dihydroxyphenyl)prop-2-en-1-one, Figure 2.4.), with a chlorine atom in the *ortho* position of ring B and hydroxyl groups at position 2' and 4' of ring A, exhibited significant inhibitory activity against BCRP. It achieved IC_{50} values of $3.21 \pm 0.69 \mu\text{M}$ in MCF-7 MX cells (human breast cancer cells overexpressing BCRP) and $3.28 \pm 0.50 \mu\text{M}$ in MDCK-BCRP cells (canine kidney cells transfected with human BCRP). Structural analysis confirmed that *ortho*-halogen substitution on ring B, combined with dihydroxy substitutions on ring A, optimizes interactions with the transporter. The study highlights halogenated chalcones as promising agents to circumvent BCRP/ABCG2-mediated drug resistance [138].

The dual role of halogenated chalcones in cytotoxicity and tumor suppressor modulation was further elucidated by Iftikhar *et al.* (2017). Again, compound **21** emerged as a key agent, demonstrating significant antiproliferative activity against

colon (HCT116) and breast (CAL-51) carcinoma cell lines, with GI_{50} values in the low micromolar range ($\sim 6 \mu\text{M}$). Notably, this compound induced rapid and dose-dependent accumulation of the tumor suppressor protein p53 in HCT116 cells within four hours of treatment. Cycloheximide chase assays revealed that this p53 stabilization occurred at the post-translational level via inhibition of proteasomal degradation pathways. The activity was strictly dependent on the conjugated enone system: the saturated analogue lacked both p53 stabilization ability and cytotoxic potency ($GI_{50} > 100 \mu\text{M}$), thereby confirming the structural necessity of the α,β -unsaturated carbonyl system [139].

In 2020, Sahin *et al.* expanded the investigation of halogenated chalcones to liver cancer. Among a series of synthetic chalcones tested on six hepatocellular carcinoma cell lines, compound **21** (again, with a *para*-chloro substitution on ring B) emerged as one of the most effective. It demonstrated marked cytotoxicity against aggressive, poorly differentiated lines (Mahlavu, FOCUS, SNU475), while showing minimal cytotoxicity toward non-transformed dental pulp stem cells (DPSC). Mechanistically, compound **21** induced SubG1 cell cycle arrest and apoptosis through inhibition of Akt phosphorylation, leading to increased p21 levels, Rb dephosphorylation, and reduced NF- κ B-p65 transcriptional activity, signaling events aligned with suppression of proliferation and activation of programmed cell death. These data emphasize the relevance of PI3K/Akt pathway targeting by halogenated chalcones, particularly in therapy-resistant liver cancers [140].

Further advancing the field, Brahmana *et al.* (2022) evaluated halogenated chalcones against MCF-7 breast cancer cells. Compound **23** ((*E*)-3-(4-chlorophenyl)-1-(*o*-tolyl)prop-2-en-1-one, Figure 2.4.), with a *para*-chloro

substituent on ring B and a methyl group on ring A, exhibited potent cytotoxicity ($IC_{50} = 3.66 \mu\text{g/mL}$). This activity was attributed to the electron-donating methyl group, which increases electron density in the aromatic ring, enhancing electrophilicity at the carbonyl carbon and facilitating interactions with cellular targets. A related hybrid derivative, compound **24** ((*E*)-3-(4-bromophenyl)-1-(3-chlorophenyl)prop-2-en-1-one), also showed significant activity ($IC_{50} = 21.62 \mu\text{g/mL}$), demonstrating the role of halogen substitution patterns and electronic effects in antitumor potency [141].

A more recent contribution was provided by Borlot et al. (2023), who studied halogenated chalcones as potential agents against gastric adenocarcinoma. Compounds **25** ((*E*)-3-(3-chlorophenyl)-1-phenylprop-2-en-1-one, Figure 2.4.) and **26** ((*E*)-3-(3-bromophenyl)-1-phenylprop-2-en-1-one), both featuring halogen atoms in the *meta* position of ring B, showed superior cytotoxicity compared to cisplatin, with CI_{50} values of 3.5 ± 0.51 and $3.88 \pm 0.90 \mu\text{g/mL}$, respectively, and high selectivity indices ($SI > 4$) against tumor versus normal cells. Molecular docking suggested that these effects are mediated via inhibition of inducible nitric oxide synthase (iNOS), a key mediator in tumor progression, with the *meta*-bromine capable of forming a covalent bond with Trp366. Additional hybrid structures synthesized in this study, such as compound **27** ((*E*)-3-(3-bromo-4-methoxyphenyl)-1-phenylprop-2-en-1-one) and the nitro-halogenated derivative **28** ((*E*)-3-(3-chlorophenyl)-1-(4-nitrophenyl)prop-2-en-1-one), also demonstrated notable cytotoxic and selectivity profiles, with SI values of 6.32 and 4.63, respectively [142].

2.2. Aim of the research

The data presented in the previous section clearly show that the of halogen substituents enhances the cytotoxic potency associated with chalcone compounds, likely by promoting electron delocalization across the enone moiety and by facilitating hydrophobic or covalent interactions with intracellular targets. Building on these findings, the work described in this chapter focused on the synthesis of 15 novel chalcones, each featuring diverse substitution patterns on the phenolic A-ring (Figure 2.5.). This compound library was designed to systematically investigate the influence of both the number and the nature of halogen substituents at the A-position on biochemical activity, while also exploring correlations with the well-established antitumor properties of phenyl chalcones.

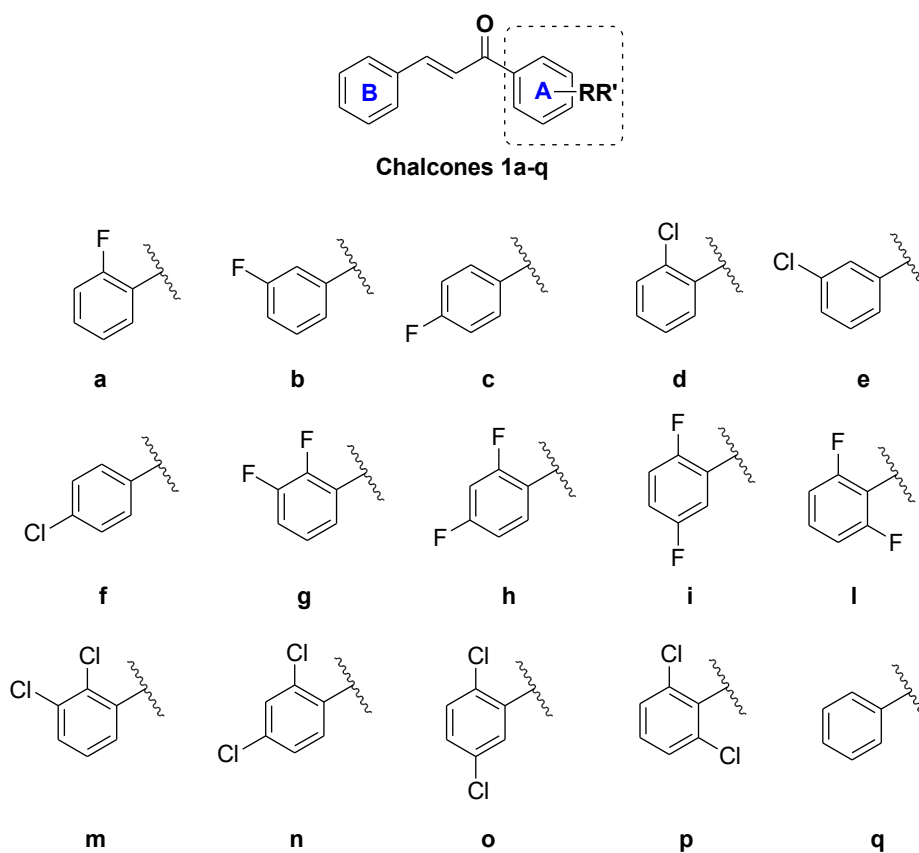


Figure 2.5. Design of the novel chalcones **1a-q**.

Beyond synthesis, the primary aim of this research was the comprehensive development and characterization of these halogenated chalcone derivatives as potential anticancer agents through a stepwise experimental approach. First, the compounds were synthesized *via* Claisen-Schmidt condensation, which enabled the efficient assembly of the chalcone scaffold under mild reaction conditions, yielding products with moderate to good yields and high purity. Subsequently, an in-depth evaluation of their physicochemical properties was performed, including solubility in various solvents, long-term stability under different environmental conditions, and photodegradation studies to assess their susceptibility to light exposure. All of these factors are crucial for practical pharmaceutical handling, formulation, and storage.

Given the high lipophilicity of these compounds, which often translates into poor aqueous solubility, limited bioavailability, and challenges in systemic administration, the study further investigated their incorporation into nanoscale drug-delivery systems. Specifically, PLGA NPs and NLCs were developed to enhance drug loading, improve EE, modulate release kinetics, and promote selective delivery to tumor tissues. This strategy aimed to increase therapeutic efficacy while minimizing off-target effects and systemic toxicity. In addition, nanoparticle formulations were coated with FITC-CS to enable potential theranostic applications by combining therapeutic activity with traceable imaging capabilities.

This work lays the groundwork for preclinical studies and the development of halogenated chalcones as potential anticancer compounds, elucidating the relationship between structural modifications and biological activity and offering a foundation for future optimization and therapeutic applications.

2.3. Materials and methods

2.3.1. Materials

Fluorescein isothiocyanate (FITC; formula: $C_{21}H_{11}NO_5S$; MW: 389.38 g/mol), low molecular weight chitosan (CS; formula: $(C_{12}H_{24}N_2O_9)_n$; degree of deacetylation 75-85%; MW range: 50,000-190,000 Da), poly(lactide-co-glycolide) (PLGA; lactide:glycolide ratio 50:50; MW range 5,000-10,000 Da), and Tween 80® (T80) were purchased from Sigma-Aldrich Chemie® (St. Louis, MO, USA). The solid lipid Compritol® 888 ATO (COM) and the liquid lipid Labrafac™ MC60 (LPG) were kindly gifted by Gattefossé SAS (Saint-Priest, France). The water used in this study was purified by double distillation, followed by deionization and final filtration through 0.22 µm Millipore® GSWP filters (Bedford, MA, USA). All other chemicals used in this work that are not listed above were of analytical grade.

2.3.2. Synthesis of chalcones 1a-q

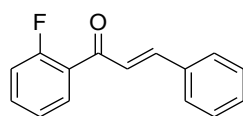
2.3.2.1 General information

Commercial reagents and solvents were used without further purification procedures. Anhydrous solvents were supplied by Sigma-Aldrich Chemie® (St. Louis, MO, USA). Elemental analyses (C, H, N) were performed using a Carlo Erba Model 1106 elemental analyzer, and the results were within $\pm 0.4\%$ of the theoretical values. The progress of reactions was monitored by thin-layer chromatography (TLC) using Merck F₂₅₄ aluminum-backed silica gel plates. Compounds were purified by flash column chromatography using Merck silica gel (200-400 mesh). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian 500 MHz spectrometer equipped with a ONE_NMR probe, operating at 499.74 MHz for ^1H and 125.73 MHz for ^{13}C , respectively. Residual

signals of deuterated solvents were used as internal references. Splitting patterns are reported as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m), or broad singlet (bs). Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (J) are reported in Hertz (Hz).

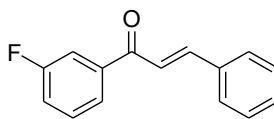
2.3.2.2 Experimental procedures and analytical data

Synthesis of (*E*)-1-(2-fluorophenyl)-3-phenylprop-2-en-1-one (**1a**)



Sodium hydroxide **4** (189.45 mg, 4.74 mmol) was dissolved in a hydroalcoholic solution (8 mL H₂O and 6.6 mL EtOH) in a 50 mL flask and cooled to 0 °C in an ice bath. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2-fluoroacetophenone **3a** (116.73 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature and monitored by TLC until the starting materials were consumed (normally 2-4 hours), using petroleum ether/ethyl acetate (9:1) as the eluent. After this time the solvents were removed under reduced pressure, the mixture was extracted with dichloromethane (DCM), washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (9:1) as the eluent to afford the pure product **1a** (90.8 mg, yield 42%). Consistency: yellow oil; R_f = 0.64 (petroleum ether/EtOAc 9:1); the spectroscopic data are in agreement with those found in literature [143]. Elemental analysis: calcd for C₁₅H₁₁FO: C, 79.63; H, 4.90; found: C 79.51, H 4.82.

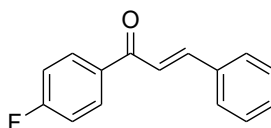
Synthesis of (*E*)-1-(3-fluorophenyl)-3-phenylprop-2-en-1-one (**1b**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 3-fluoroacetophenone **3b** (116.21 μ L, 0.95 mmol) were then added to the prepared solution, and the reaction mixture was stirred at room temperature, resulting in the precipitation of compound **1b** (124 mg, yield 58%). The solid was isolated by vacuum filtration using a Büchner funnel and a vacuum-compatible Erlenmeyer flask, washed with cold water, and used without further purification for scientific purposes.

Consistency: pale yellow powder; R_f = 0.62 (petroleum ether/EtOAc 95:5); the spectroscopic data are in agreement with those found in literature [143]. Elemental analysis: calcd for $C_{15}H_{11}FO$: C, 79.63; H, 4.90; found: C 79.76, H 4.83.

Synthesis of (*E*)-1-(4-fluorophenyl)-3-phenylprop-2-en-1-one (**1c**)

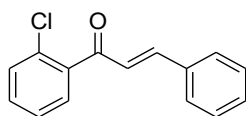


A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 4-fluoroacetophenone **3c** (114.98 μ L, 0.95 mmol) were then added to the prepared solution, and the reaction mixture was stirred at room temperature, resulting in the precipitation of compound **1c** (134 mg, yield 62%). The solid was isolated by vacuum filtration using a Büchner funnel and a vacuum-

compatible Erlenmeyer flask, washed with cold water, and used without further purification for scientific purposes.

Consistency: pale yellow powder; $R_f = 0.70$ (petroleum ether/EtOAc 9:1); the spectroscopic data are in agreement with those found in literature [143]. Elemental analysis: calcd for $C_{15}H_{11}FO$: C, 79.63; H, 4.90; found: C 79.56, H 4.98.

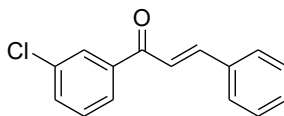
Synthesis of (*E*)-1-(2-chlorophenyl)-3-phenylprop-2-en-1-one (**1d**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2-chloroacetophenone **3d** (123.26 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (9:1) as the eluent to give the pure compound **1d** (123 mg, yield 53%).

Consistency: yellow oil; $R_f = 0.64$ (petroleum ether/EtOAc 9:1). The spectroscopic data are in agreement with those found in literature [144]. Elemental analysis: calcd for $C_{15}H_{11}ClO$: C, 74.23; H, 4.57; found: C 74.32, H 4.59.

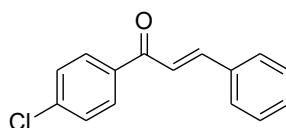
Synthesis of (*E*)-1-(3-chlorophenyl)-3-phenylprop-2-en-1-one (**1e**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 3-chloroacetophenone **3e** (122.95 μ L, 0.95 mmol) were then added to the prepared solution, and the reaction mixture was stirred at room temperature, resulting in the precipitation of compound **1e** (200 mg, yield 86%). The solid was isolated by vacuum filtration using a Büchner funnel and a vacuum-compatible Erlenmeyer flask, washed with cold water, and used without further purification for scientific purposes.

Consistency: pale yellow powder; R_f = 0.70 (petroleum ether/EtOAc 9:1). ^1H NMR (500 MHz, CDCl_3): δ : 7.41 – 7.49 (m, 5H), 7.57 – 7.54 (m, 1H), 7.63 – 7.68 (m, 2H), 7.83 (d, J = 15.7 Hz, 1H), 7.89 – 7.91 (m, 1H), 7.97 – 7.99 (m, 1H) ppm; ^{13}C NMR (125 MHz, CDCl_3): δ : 121.67, 126.69, 128.72, 128.73, 129.17, 130.11, 130.98, 132.84, 134.77, 135.09, 139.96, 145.85, 189.30. Elemental analysis: calcd for $\text{C}_{15}\text{H}_{11}\text{ClO}$: C, 74.23; H, 4.57; found: C 74.17, H 4.42.

Synthesis of (*E*)-1-(4-chlorophenyl)-3-phenylprop-2-en-1-one (**1f**)

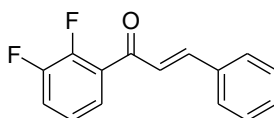


A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 4-chloroacetophenone **3f** (122.84 μ L, 0.95 mmol) were then added to the prepared solution, and the reaction mixture was stirred at room temperature, resulting in the precipitation of compound **1f** (138 mg, yield 60%). The solid was isolated by vacuum filtration using a Büchner funnel and a vacuum-

compatible Erlenmeyer flask, washed with cold water, and used without further purification for scientific purposes.

Consistency: pale yellow powder; $R_f = 0.72$ (petroleum ether/EtOAc 9:1). ^1H NMR (500 MHz, CDCl_3): δ : 7.40 – 7.45 (m, 3H), 7.46 – 7.51 (m, 3H), 7.62 – 7.67 (m, 2H), 7.82 (d, $J = 15.7$ Hz, 1H), 7.95 – 7.98 (m, 2H) ppm; ^{13}C NMR (125 MHz, CDCl_3): δ : 121.67, 128.66, 129.10, 129.16, 130.06, 130.89, 134.85, 136.66, 139.37, 145.51, 189.35. Elemental analysis: calcd for $\text{C}_{15}\text{H}_{11}\text{ClO}$: C, 74.23; H, 4.57; found: C 74.26, H 4.55.

Synthesis of (*E*)-1-(2,3-difluorophenyl)-3-phenylprop-2-en-1-one (**1g**)

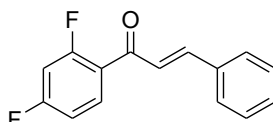


A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μL , 0.95 mmol) and 2,3-difluoroacetophenone **3g** (121.62 μL , 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (9:1) as the eluent to give the pure compound **1g** (125.5 mg, yield 54%).

Consistency: yellow oil; $R_f = 0.46$ (petroleum ether/EtOAc 9:1). ^1H NMR (500 MHz, CDCl_3): δ : 7.20 (td, $J = 8.0$ Hz and 4.3 Hz, 1H), 7.32 – 7.37 (m, 2H), 7.39 – 7.45 (m, 3H), 7.53 – 7.57 (m, 1H), 7.60 – 7.65 (m, 2H), 7.75 (d, $J = 15.8$ Hz, 1H) ppm; ^{13}C NMR (125 MHz, CDCl_3): δ : 120.67 (d, $J = 16.4$ Hz), 124.44 (dd, $J = 6.4$ Hz e 4.7 Hz), 125.09 (d, $J = 5.6$ Hz), 125.37, 126.55, 128.67, 129.00, 130.95,

134.41, 145.73, 149.09 (dd, $J = 174.8$ Hz e 15.9 Hz), 151.11 (dd, $J = 169.0$, 16.0 Hz), 187.89 (d, $J = 2.3$ Hz). Elemental analysis: calcd for $C_{15}H_{10}F_2O$: C, 73.76; H, 4.13; found: C 73.66, H 4.29.

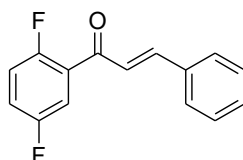
Synthesis of (E)-1-(2,4-difluorophenyl)-3-phenylprop-2-en-1-one (1h)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,4-difluoroacetophenone **3h** (119.85 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (9:1) as the eluent to give the pure compound **1h** (98.2 mg, yield 42%).

Consistency: yellow oil; $R_f = 0.65$ (petroleum ether/EtOAc 99:1); the spectroscopic data are in agreement with those found in literature [144]. Elemental analysis: calcd for $C_{15}H_{10}F_2O$: C, 73.76; H, 4.13; found: C 73.67, H 4.06.

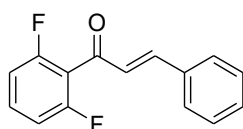
Synthesis of (E)-1-(2,5-difluorophenyl)-3-phenylprop-2-en-1-one (1i)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,5-difluoroacetophenone **3i** (119.95 μ L, 0.95 mmol) were

then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (99:1) as the eluent to give the pure compound **1i** (33.1 mg, yield 14%). Consistency: yellow oil; R_f = 0.52 (petroleum ether/EtOAc 99:1); the spectroscopic data are in agreement with those found in literature [144]. Elemental analysis: calcd for $C_{15}H_{10}F_2O$: C, 73.76; H, 4.13; found: C 73.71, H 4.16.

Synthesis of (*E*)-1-(2,6-difluorophenyl)-3-phenylprop-2-en-1-one (**1l**)

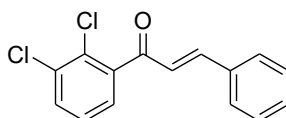


A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,6-difluoroacetophenone **3l** (123.76 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (95:5) as the eluent to give the pure compound **1l** (35.4 mg, yield 15%).

Consistency: white powder; R_f = 0.47 (petroleum ether/EtOAc 95:5). ^1H NMR (500 MHz, $CDCl_3$): δ : 6.96 – 7.02 (m, 2H), 7.06 (d, J = 16.1 Hz, 1H), 7.37 – 7.44 (m, 4H), 7.49 (d, J = 16.1 Hz, 1H), 7.55 – 7.57 (m, 2H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ : 111.99 (dd, J = 20.7 Hz and 4.7 Hz), 127.15, 128.68, 128.99, 131.10, 131.99 (t, J = 10.1 Hz), 134.11, 146.99, 159.91 (d, J = 252.6 Hz), 159.97 (d, J =

252.6 Hz), 187.63. Elemental analysis: calcd for C₁₅H₁₀F₂O: C, 73.76; H, 4.13; found: C 73.79, H 4.20.

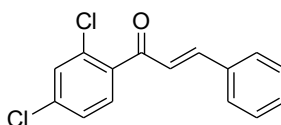
Synthesis of (*E*)-1-(2,3-dichlorophenyl)-3-phenylprop-2-en-1-one (**1m**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,3-dichloroacetophenone **3m** (138.49 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (9:1) as the eluent to give the pure compound **1m** (102 mg, yield 38%).

Consistency: yellow oil; R_f = 0.55 (petroleum ether/EtOAc 9:1). ¹H NMR (500 MHz, CDCl₃): δ : 7.05 (d, J = 16.1 Hz, 1H), 7.22 – 7.27 (m, 1H), 7.29 – 7.34 (m, 2H), 7.35 – 7.41 (m, 3H), 7.50 – 7.57 (m, 3H) ppm; ¹³C NMR (125 MHz, CDCl₃) = δ : 126.04, 126.82, 127.11, 127.69, 128.69, 129.07, 131.17, 131.91, 132.37, 133.99, 134.17, 147.08, 193.12. Elemental analysis: calcd for C₁₅H₁₀Cl₂O: C, 65.01; H, 3.64; found: C 65.10, H 3.70.

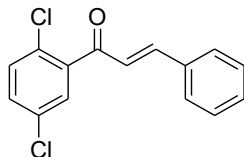
Synthesis of (*E*)-1-(2,4-dichlorophenyl)-3-phenylprop-2-en-1-one (**1n**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,4-dichloroacetophenone **3n** (135.66 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (9:1) as the eluent to give the pure compound **1n** (46.5 mg, yield 18%).

Consistency: yellow oil; R_f = 0.68 (petroleum ether/EtOAc 9:1); the spectroscopic data are in agreement with those found in literature [145]. Elemental analysis: calcd for $C_{15}H_{10}Cl_2O$: C, 65.01; H, 3.64; found: C 64.98, H 3.52.

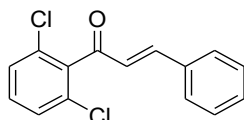
Synthesis of (*E*)-1-(2,5-dichlorophenyl)-3-phenylprop-2-en-1-one (**1o**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,5-dichloroacetophenone **3o** (179.08 mg, 0.95 mmol) were then added to the prepared solution, and the reaction mixture was stirred at room temperature, resulting in the precipitation of compound **1o** (234 mg, yield 89%). The solid was isolated by vacuum filtration using a Büchner funnel and a vacuum-compatible Erlenmeyer flask, washed with cold water, and used without further purification for scientific purposes.

Consistency: white powder; $R_f = 0.65$ (petroleum ether/EtOAc 99:1); the spectroscopic data are in agreement with those found in literature [146]. Elemental analysis: calcd for $C_{15}H_{10}Cl_2O$: C, 65.01; H, 3.64; found: C 65.08, H 3.58.

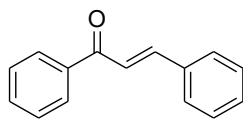
Synthesis of (*E*)-1-(2,6-dichlorophenyl)-3-phenylprop-2-en-1-one (**1p**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and 2,6-dichloroacetophenone **3p** (179.08 mg, 0.95 mmol) were then added to the prepared solution, and the reaction mixture was stirred at room temperature, resulting in the precipitation of compound **1p** (183.8 mg, yield 70%). The solid was isolated by vacuum filtration using a Büchner funnel and a vacuum-compatible Erlenmeyer flask, washed with cold water, and used without further purification for scientific purposes.

Consistency: white powder; $R_f = 0.49$ (petroleum ether/EtOAc 9:1). ^1H NMR (500 MHz, $CDCl_3$): δ : 6.96 (dd, $J = 16.4$ Hz, 1H), 7.24 – 7.28 (m, 1H), 7.30 – 7.34 (m, 1H), 7.36 – 7.43 (m, 5H), 7.56 – 7.53 (m, 2H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ : 126.44, 128.16, 128.70, 129.00, 130.59, 131.19, 131.84, 134.07, 137.91, 147.75, 192.70. Elemental analysis: calcd for $C_{15}H_{10}Cl_2O$: C, 65.01; H, 3.64; found: C 64.99, H 3.72.

Synthesis of (*E*)-1,3-diphenylprop-2-en-1-one (**1q**)



A hydroalcoholic solution of sodium hydroxide **4** (189.45 mg, 4.74 mmol) was prepared in a 50 mL flask as described for compound **1a**. Benzaldehyde **2** (96.15 μ L, 0.95 mmol) and acetophenone **3q** (110.50 μ L, 0.95 mmol) were then added to the prepared solution and the reaction mixture was stirred at room temperature, affording a crude residue which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (95:5) as the eluent to give the pure compound **1q** (61.2 mg, yield 54%).

Consistency: light yellow powder; R_f = 0.41 (petroleum ether/EtOAc 95:5); the spectroscopic data are in agreement with those found in literature [147]. Elemental analysis: calcd for $C_{15}H_{12}O$: C, 86.51; H, 5.81; found: C 86.44, H 5.92.

2.3.3. UV-Vis characterization of compound **1e**

The ultraviolet-visible (UV-Vis) spectroscopic investigation of compound **1e** and the determination of its molar extinction coefficient (ϵ) were carried out in three solvents: ethanol (EtOH), methanol (MeOH), and a MeOH/water mixture in a 5:1 (v/v) ratio, all of analytical grade. Although MeOH is not suitable for use in drug-delivery formulations due to its known toxicity and its use should be limited for operator safety, as it is considered neurotoxic, it was employed in this study exclusively for analytical purposes. Its low UV background absorbance and excellent solubilizing ability toward compound **1e**, which tends to precipitate even in the presence of small amounts of water, make MeOH the preferred co-solvent

for the UV-Vis spectroscopic characterization of the compound. For each solvent, a stock solution was prepared by accurately weighing 1 mg of compound **1e** and dissolving it in a 50 mL volumetric flask filled to the mark with solvent, thus obtaining a stock solution with a concentration of 0.020 mg/mL. Subsequently, a series of dilutions was prepared to obtain eight different concentrations, ranging from 0.020 mg/mL to 0.0004 mg/mL. This concentration range was selected to ensure that the maximum absorbance values recorded at the compound's absorption maximum ($\lambda_{\text{max}} \approx 313$ nm, depending on the solvent) remained below 2 absorbance units, thereby ensuring that the measurements fell within the linear dynamic range of the instrument and complied with the Beer-Lambert Law. UV-Vis absorbance spectra were acquired over the 200-800 nm spectral range at room temperature using a double-beam spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) equipped with 1 cm path length quartz cuvettes. All sample spectra were baseline-corrected by subtracting the absorbance of the corresponding solvent blank. For each solvent system, calibration curves were constructed by plotting the absorbance values as a function of the corresponding concentrations expressed in mg/mL. All measurements and calibration curves were performed in triplicate to ensure reproducibility and reliability of the results. The ϵ , expressed in $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, was calculated using the following equation:

$$\epsilon = \text{slope} \cdot \text{MW}$$

Where:

- Slope refers to the slope of the calibration curve obtained for each solvent;
- MW is the molecular weight of compound **1e** (242.7 g/mol);

2.3.4. Solubility studies of compound 1e

The solubility of compound **1e** was assessed in the same three analytical-grade solvents used for its UV-Vis characterization: EtOH, MeOH, and a MeOH/water mixture in a 5:1 (v/v) ratio. An excess amount of compound **1e** was added to 10 mL of each solvent to ensure saturation, and the suspensions were magnetically stirred at 25 ± 0.5 °C in the dark to prevent potential photodegradation. At predefined time intervals, aliquots of known volume were withdrawn, filtered through 0.22 µm nylon syringe filters (Millex™, Merck Millipore, Darmstadt, Germany), and the concentration of dissolved compound was determined by UV-Vis spectroscopy at the characteristic wavelength of compound **1e** ($\lambda_{\text{max}} \approx 313$ nm, depending on the solvent). Solubility equilibrium was considered to be reached when no significant change in concentration was observed between two consecutive time points (24 hours). After each sampling, the withdrawn volume was immediately replaced with an equal volume of fresh solvent, and all measured concentrations were corrected to account for cumulative dilution. All experiments were performed in triplicate.

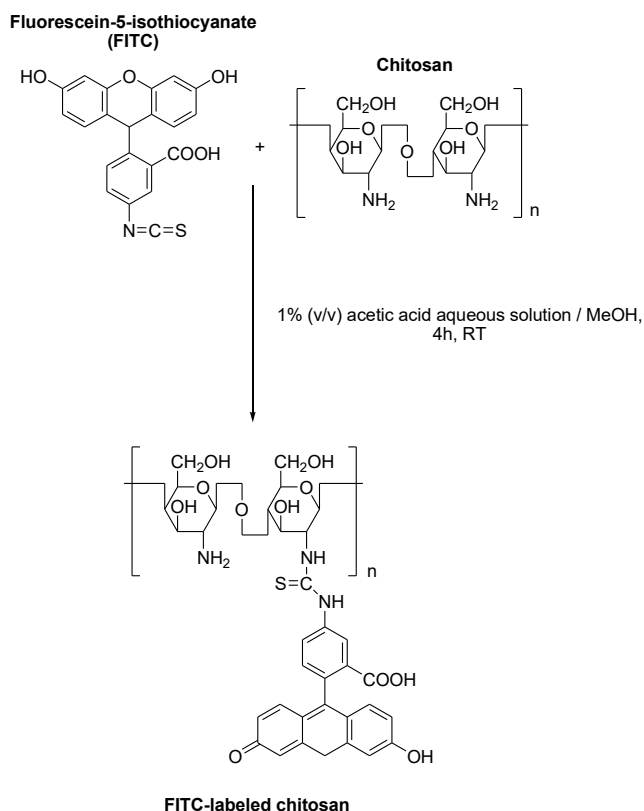
2.3.5. Stability studies of compound 1e

The stability of compound **1e** was assessed in the same three analytical-grade solvents used for its UV-Vis characterization: EtOH, MeOH, and a MeOH/water mixture in a 5:1 (v/v) ratio. Solutions of compound **1e** were prepared by dissolving 1 mg of compound in 100 mL of each solvent (4.13×10^{-5} M) and stored at 25 ± 0.5 °C under two different conditions: exposure to ambient light and protection from light (dark). Over a period of one month, samples were analyzed at predefined

time intervals using UV-Vis spectroscopy to monitor potential degradation or changes in the absorption profile. All experiments were conducted in triplicate.

2.3.6. Synthesis of fluorescent chitosan

The synthesis of FITC-CS was carried out by reacting CS with the fluorescent probe FITC. This reaction relies on the interaction between the isothiocyanate group of FITC and the primary amine groups of CS. The procedure was performed following the protocol described by Moussa *et al.* [148], with some modifications (Scheme 2.10.).



Scheme 2.10. Reaction between fluorescein isothiocyanate (FITC) and chitosan to form FITC-labeled chitosan via thiourea bond formation.

Briefly, a solution containing 1% w/v of CS in 20 mL of 1% (v/v) acetic acid aqueous solution was prepared and stirred overnight to ensure complete dissolution of CS. The following day, this solution was mixed with a FITC solution prepared by dissolving 20 mg of FITC in 20 mL of MeOH. The resulting mixture was allowed to react at room temperature (25.0 ± 0.5 °C) in the dark under continuous magnetic stirring for 4 hours, allowing the FITC to bind to the amine groups of CS. At the end of the reaction, the solution pH was adjusted to 10 by adding a few drops of 2M NaOH, inducing the precipitation of FITC-CS. To remove the excess unreacted FITC, the precipitate was subjected to successive centrifugation cycles (Heraeus Megafuge 16, Thermo Fisher Scientific, Waltham, MA, USA) at 8000 rpm for 10 minutes, which enabled the coarse elimination of free FITC, as monitored by the decreasing fluorescence intensity of the supernatant. The pellet was then redissolved in 20 mL of 1% (v/v) aqueous acetic acid and further purified by dialysis against 4 L of distilled water for 3 days at room temperature (25.0 ± 0.5 °C) in the dark, with daily replacement of the dialysis medium, to ensure the complete removal of any residual free FITC. Finally, the purified FITC-CS was lyophilized using an Alpha 1-4 LSCbasic freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

2.3.7. Preparation of blank and compound 1e-loaded PLGA nanoparticles

Both blank and compound **1e**-loaded PLGA NPs were synthesized using a nanoprecipitation technique, also known as solvent displacement or solvent diffusion method [149]. The procedure involved combining an organic phase, composed of 10 mg of PLGA dissolved in 5 mL of acetone, with an aqueous phase consisting of 5 mL of water containing 0.5% (w/v) Tween 80. For drug-loaded NPs,

predetermined amounts of compound **1e** (0.1-0.5 mg) were co-dissolved in the organic phase prior to addition. The organic phase was introduced dropwise into the aqueous phase under continuous magnetic stirring (600 rpm). The instantaneous diffusion of acetone into the aqueous phase induced polymer nanoprecipitation, yielding a crude NP dispersion that was subsequently stirred for 12 hours to ensure complete solvent evaporation. Purification was performed through five sequential centrifugation cycles (15,000 rpm, 1 h, 4 °C) using a refrigerated ultracentrifuge (Frontier™ 5000 Multi Pro FC5830R, OHAUS Corporation, USA). After each centrifugation, the supernatant was carefully separated for further processing, while the NP pellet was resuspended in 0.5 mL of ultrapure water by vortex mixing (Lab1st Variable Speed Vortex Mixer, Lab1st Scientific, USA). For blank NPs, the final supernatant obtained after the fifth centrifugation was discarded. In contrast, the corresponding supernatant from drug-loaded NPs was collected and analyzed *via* UV-Vis spectrophotometry to determine the EE %. After resuspension in water, all NP pellets were pooled, supplemented with trehalose as a cryoprotectant (2.5% w/v), and lyophilized. The resulting dry powder was stored in a desiccator under anhydrous conditions until further characterization.

2.3.8. Coating of PLGA nanoparticles with fluorescent chitosan

To impart fluorescence to both blank and compound **1e**-loaded PLGA NPs, a coating of FITC-CS was applied, exploiting the electrostatic interaction between the positively charged amino groups of CS and the negatively charged surface of the NPs. A 0.2% (w/v) FITC-CS solution was prepared in a 1% (v/v) acetic acid aqueous solution, and the solution was stirred overnight in the dark to ensure complete solubilization of the CS and to prevent FITC photodegradation. The

following day, the solution was filtered through a 0.22 μm syringe filter. Different amounts of the prepared FITC-CS solution (0.5, 0.25, 0.05, 0.045, 0.040) were then added dropwise to the PLGA NPs dispersion, both blank and compound **1e**-loaded, prepared as described in the previous section. To facilitate accurate dispensing of the smaller volumes, the respective amounts of FITC-CS solution were brought to a final volume of 1 mL with 1% (v/v) acetic acid aqueous solution. The mixture was stirred for 1 hour to ensure uniform coating of the NPs. After the coating process, a small aliquot of the resulting fluorescently labeled PLGA NPs was withdrawn and subjected to physicochemical characterization in the liquid state. Trehalose (2.5% w/v) was then added to the remaining suspension as a cryoprotectant, followed by freeze-drying for 72 hours. The resulting lyophilized powder was stored in a desiccator under dry conditions until further analysis.

2.3.9. Preparation of blank and compound 1e-loaded nanostructured lipid carriers

Both blank and compound **1e**-loaded NLCs were prepared using a high-pressure homogenization technique [150], following a protocol established by our research group. The formulation process involved combining a lipid phase, composed of COM and LPG in a 7:3 ratio (w/w) dissolved in 1 mL of EtOH, with an aqueous phase consisting of 9 mL of water containing 5% (w/w) Tween 80. Both phases were separately heated to 84 °C in a water bath to ensure complete solubilization of COM and to facilitate subsequent emulsification. The lipid phase was then gradually added dropwise into the aqueous phase under high-speed homogenization using an Ultra-Turrax T 25 (IKA-Werke, Staufen, Germany) operating at 11,000 rpm for 5 minutes. To further reduce particle size and improve

dispersion uniformity, the resulting pre-emulsion was subjected to ultrasonication for 15 minutes, maintaining the temperature at 84 °C using a Bandelin Sonorex RK 514 ultrasonic device (Berlin, Germany). The obtained NLC dispersion was then rapidly cooled in an ice bath to promote stabilization. Purification of the formulation was performed by transferring the obtained dispersion into Falcon tubes equipped with a 10 kDa MWCO filter (Thermo Scientific™, Waltham, Massachusetts, USA) and subjecting it to ultrafiltration at 1,000 rcf, 25 °C for 20 minutes. The permeate was discarded, and the retentate containing the purified NLCs was collected. Trehalose (5% w/v) was added as a cryoprotectant, and the formulation was freeze-dried for 72 hours. The lyophilized samples were stored in a desiccator until further analysis.

Compound **1e**-loaded NLCs were prepared following the same procedure, with the addition of compound **1e** (5, 10, 15, or 20 mg) to the lipid phase prior to homogenization. After ultrafiltration under the same conditions (1,000 rcf, 25 °C, 20 minutes), the permeate was collected for the indirect determination of EE, whereas the retentate was processed as described for the blank formulations. Trehalose (5% w/v) was again added to the retentate, and the loaded NLCs were freeze-dried for 72 hours.

Before proceeding with the preparation of compound **1e**-loaded NLCs, the stability of compound **1e** at the production temperature (84 °C) was verified by UV-Vis spectroscopy. These analyses confirmed that the compound remains stable only under light-protected conditions, whereas even minimal exposure induces slight photodegradation. Accordingly, all preparations were carried out under light protection.

2.3.10. Coating of nanostructured lipid carriers with fluorescent chitosan

To impart fluorescence to both blank and compound **1e**-loaded NLCs, a coating with FITC-CS was applied, using a method analogous to that employed for the PLGA NPs. A 0.2% (w/v) FITC-CS solution was prepared in a 1% (v/v) acetic acid aqueous solution, and the solution was stirred overnight in the dark to ensure complete solubilization of the CS and prevent FITC photodegradation. The following day, the solution was filtered through a 0.22 µm syringe filter. Different amounts of the prepared FITC-CS solution (1, 2, 2.3, and 2.5 mL) were then added dropwise to the NLC dispersion, both blank and compound **1e**-loaded, prepared as described in the previous section. The mixture was stirred for 1 hour to ensure proper and uniform coating of the NLCs. After the coating process, a small aliquot of the resulting fluorescently labeled NLCs was withdrawn and subjected to physicochemical characterization in the liquid state. Trehalose (5% w/v) was then added to the remaining suspension as a cryoprotectant, followed by freeze-drying. The resulting lyophilized powder was stored in a desiccator under dry conditions until further analysis.

2.3.11. Technological characterization of nanoparticulate systems

The production yield of the nanoparticulate systems, both PLGA NPs and NLCs, was determined gravimetrically and calculated according to the following equation:

$$\text{Yield \%} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \cdot 100$$

The amount of compound **1e** encapsulated within the nanoparticulate systems was determined using an indirect method, based on the difference between the initial amount of compound **1e** used for the preparation and the amount of compound **1e**

detected in the permeate (in the case of NLCs) or in the supernatant (in the case of PLGA NPs). The concentration of compound **1e** in these fractions was quantified by UV-Vis spectroscopy over the spectral range of 200-800 nm, at the characteristic wavelength of compound **1e** ($\lambda_{\text{max}} \approx 313$ nm). The EE % was calculated according to the following equation:

$$\text{EE \%} = \frac{\text{Initial compound } \mathbf{1e} \text{ content} - \text{Free compound } \mathbf{1e}}{\text{Initial compound } \mathbf{1e} \text{ content}} \cdot 100$$

The drug content (DC) % was then calculated according to the following equation, for both PLGA NPs and NLCs:

$$\text{DC \%} = \frac{\text{Initial compound } \mathbf{1e} \text{ content} - \text{Free compound } \mathbf{1e}}{\text{Weight of recovered NPs}} \cdot 100$$

The hydrodynamic diameter (D_H), polydispersity index (PDI), and ζ of the nanoparticulate systems were determined using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK), equipped with a 4 mW He-Ne laser ($\lambda = 633$ nm). Samples were diluted in ultrapure water and briefly sonicated prior to analysis. Particle size and PDI were measured by photon correlation spectroscopy (PCS) at 25 ± 0.5 °C and a fixed scattering angle of 173°. The PDI was used to assess size distribution uniformity, with values below 0.3 indicative of a narrow distribution and good colloidal stability. ζ was measured by electrophoretic light scattering (ELS) under similar conditions, after appropriate dilution to minimize multiple scattering effects. Data were analyzed using Zetasizer Software (Malvern Instruments Ltd.), and all measurements were performed in triplicate and reported as mean $\pm$ standard deviation (SD).

2.3.12. In vitro release studies of compound 1e-loaded nanostructured lipid carriers

The in vitro release profile of compound **1e** from NLCs was evaluated employing the dialysis bag method. In brief, lyophilized **1e**-loaded NLC formulations containing 1 mg of compound **1e** were reconstituted in 5 mL of phosphate-buffered saline (PBS, pH 7.4) containing 20% (v/v) MeOH and transferred into dialysis membranes (MWCO 3.5 kDa) that had been pre-hydrated in PBS for 24 hours prior to use. Once filled, the membranes were sealed and immersed in 150 mL of the same MeOH-containing PBS, with MeOH being necessary to ensure the solubility of the highly lipophilic compound **1e**. The experiments were conducted under constant magnetic stirring (150 rpm) at 37.0 ± 0.5 °C in a thermostatically controlled water bath (Telesystem 15.40 with Telemodul 40C, Thermo Scientific, Waltham, MA, USA) and in the dark to prevent photodegradation of compound **1e**. At predetermined time intervals, the entire volume of the external medium was withdrawn and immediately replaced with an equal volume of fresh medium to maintain sink conditions. The collected samples were evaporated to dryness under reduced pressure using a rotary evaporator (BUCHI Rotavapor®, R-200/205, Switzerland) and reconstituted in 3 mL of EtOH. Quantification of the released compound **1e** was then performed spectrophotometrically at its characteristic absorption wavelength ($\lambda_{\text{max}} \approx 313$ nm). For comparison, the release profile of free (non-encapsulated) compound **1e** was also evaluated under the same conditions. Drug release was expressed as the cumulative percentage of compound **1e** released over time.

2.3.13. Thermogravimetric analysis (TGA)

To evaluate the stability of the NLCs after lyophilization over a two-month period, thermal decomposition profiles were assessed by thermogravimetric analysis (TGA) using a Discovery SDT 650 system (TA Instruments-Waters LLC, Milford, MA, USA). Each month, for three consecutive measurements, aliquots of approximately 5-10 mg of each sample (free compound **1e**, blank NLCs, and compound **1e**-loaded NLCs, both uncoated and coated with FC) were accurately weighed and placed in alumina crucibles. To minimize sample oxidation, the analyses were carried out under a dynamic nitrogen atmosphere (flow rate: 100.0 mL/min), over a temperature range of 25-800 °C, with a controlled heating rate of 20.0 °C/min.

Thermal parameters were extracted from the TG curves using Origin (OriginLab, Northampton, MA, USA). $T_{\max}$ values were obtained from the first derivative of the TGA curve (DTG) by identifying the relevant local minima, corresponding to the maximum degradation rates. T_{onset} was determined by the tangent extrapolation method, in which a tangent line is drawn to the TGA curve at $T_{\max}$ and extended until it intersects with the corresponding baseline. The final residue was calculated as the normalized mass percentage remaining at 800 °C [151].

2.3.14. Biological activity

2.3.14.1. In vitro studies: cell cultures

Five different human cancer cell lines were used for the in vitro study. Each cell line was cultured in 75 cm² flasks with its respective growth medium, maintained at 37 °C in a humidified atmosphere with 5% CO₂. The culture medium was replaced twice a week, and cells were grown until reaching approximately 80%

confluence. The following human cancer cell lines were used in the *in vitro* experiments:

- Human glioblastoma cell line U-87 (U-87 ATCC HTB-14™; *Homo sapiens* grade IV cerebral glioblastoma), obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), was cultured in Dulbecco's Modified Eagle Medium (DMEM—Sigma-Aldrich, catalog no. D5030; St. Louis, MO, USA), supplemented with antibiotics (penicillin 1000 U/mL and streptomycin 0.1 mg/mL; Sigma-Aldrich, catalog no. P4333), L-glutamine (GlutaMAX™, ThermoFisher Scientific, catalog no. 35050061; Waltham, MA, USA), and 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, catalog no. 12103C).
- Human osteosarcoma cell line U2OS (ATCC HTB-96), obtained from ATCC, was cultured in McCoy's 5A Modified Medium (ATCC, catalog no. 30-2007), supplemented with 10% FBS and 1% penicillin-streptomycin.
- Human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065), obtained from ATCC, was cultured in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich, catalog no. M5650), supplemented with 10% FBS and 1% penicillin-streptomycin.
- Human follicular thyroid carcinoma cell line FTC-133, obtained from ATCC, was cultured in RPMI-1640 medium (Sigma-Aldrich, catalog no. R8758), supplemented with 10% FBS and 1% penicillin-streptomycin.
- Human anaplastic thyroid carcinoma cell line 8305C, obtained from ATCC, was cultured in RPMI-1640 medium (Sigma-Aldrich, catalog no. R8758), supplemented with 10% FBS and 1% penicillin-streptomycin.

2.3.14.2. Cell Treatments

Each cancer cell line was seeded in 96-well plates (Corning Cell Culture, Corning, NY, USA) at a density of 4×10^4 cells/well, with a final volume of 150 μ L per well. After 24 hours, treatments were carried out for 24 hours with the following test compounds:

- **1a** $\rightarrow$ U87, FTC-133, and 8305c cells were treated with increasing concentrations of **1a**: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, and 100 μ M.

HepG2 and U2OS cells were treated with **1a** at higher concentrations: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.

- **1c** $\rightarrow$ U87, FTC-133, 8305c, HepG2, and U2OS cells were treated with **1c** at concentrations of 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.
- **1d** $\rightarrow$ U87, 8305c, HepG2, and U2OS cells were treated with **1d** at concentrations of 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.

FTC-133 cells were treated with **1d** at higher concentrations: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, 500 μ M, 1 mM, and 10 mM.

- **1e** $\rightarrow$ U87, FTC-133, 8305c, HepG2, and U2OS cells were treated with **1e** at concentrations of 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.
- **1f** $\rightarrow$ 8305c cells were treated with increasing concentrations of **1f**: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.

- **1g** → 8305c cells were treated with increasing concentrations of **1g**: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.
- **1i** → U87, FTC-133, 8305c, HepG2, and U2OS cells were treated with **1i** at concentrations of 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, 100 μ M, 300 μ M, and 500 μ M.
- **1l** → U87, HepG2, and U2OS cells were treated with **1l** at concentrations of 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, and 100 μ M.
Higher concentrations of **1l** (300 μ M, 500 μ M, 1 mM, and 10 mM) were tested in FTC-133 and 8305c cell lines.
- **1m** → U87, FTC-133, HepG2, and U2OS cells were treated with increasing concentrations of **1m**: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, and 100 μ M.
Higher concentrations of **1m** (300 μ M and 500 μ M) were tested in the 8305c cell line.
- **1n** → U87, FTC-133, HepG2, and U2OS cells were treated with increasing concentrations of **1n**: 0.5 μ M, 1 μ M, 10 μ M, 30 μ M, 50 μ M, 80 μ M, and 100 μ M.
Higher concentrations of **1n** (300 μ M and 500 μ M) were tested in the 8305c cell line.
- **1o** → U87 and U2OS cells were treated with increasing concentrations of **1o**: 50 μ M, 100 μ M, 300 μ M, 500 μ M, 1 mM, and 10 mM.
- **1p** → U87 and U2OS cells were treated with increasing concentrations of **1p**: 50 μ M, 100 μ M, 300 μ M, 500 μ M, 1 mM, and 10 mM.

- **1q** → U87 and U2OS cells were treated with increasing concentrations of **1q**: 50 μ M, 100 μ M, 300 μ M, 500 μ M, 1 mM, and 10 mM.

2.3.14.3. Cell viability assay (MTT assay)

To investigate the cytotoxic effect of chalcones **1a-q**, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay) was performed. This assay is based on the ability of viable cells to reduce the yellow, water-soluble tetrazolium salt MTT into insoluble blue formazan crystals. This reaction occurs only in metabolically active cells, driven by mitochondrial dehydrogenases and involving NADH and NADPH cofactors. The determination of cell viability based on MTT reduction is highly accurate, as the absorbance is directly proportional to the amount of formazan produced and, consequently, to the number of viable cells. Specifically, each cancer cell line was seeded in 96-well plates at a density of 4×10^4 cells/well, in a final volume of 150 μ L. After 24 hours of incubation, cells were treated for an additional 24 hours with increasing concentrations of the compounds, as detailed in section 2.2.14.2. Following treatment, cells were incubated at 37 °C with MTT (0.2 mg/mL) for 1 hour. Subsequently, the MTT solution was removed, and the cells were lysed with 100 μ L of dimethyl sulfoxide (DMSO). The amount of reduced MTT (formazan) was quantified by measuring the optical density (OD) at 550 nm using a microplate reader [152].

2.3.15. Statistical Analysis

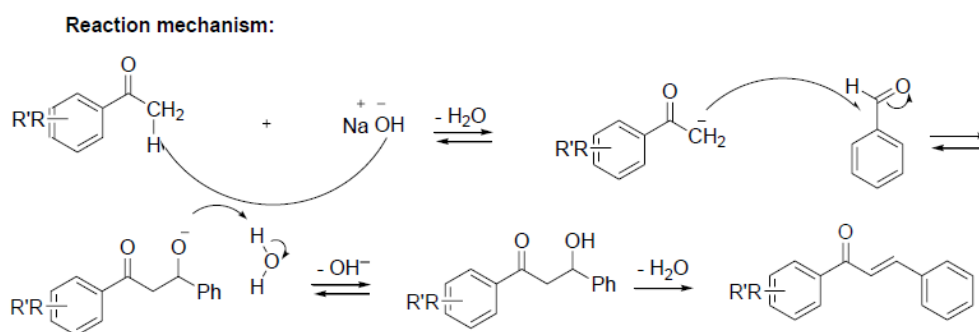
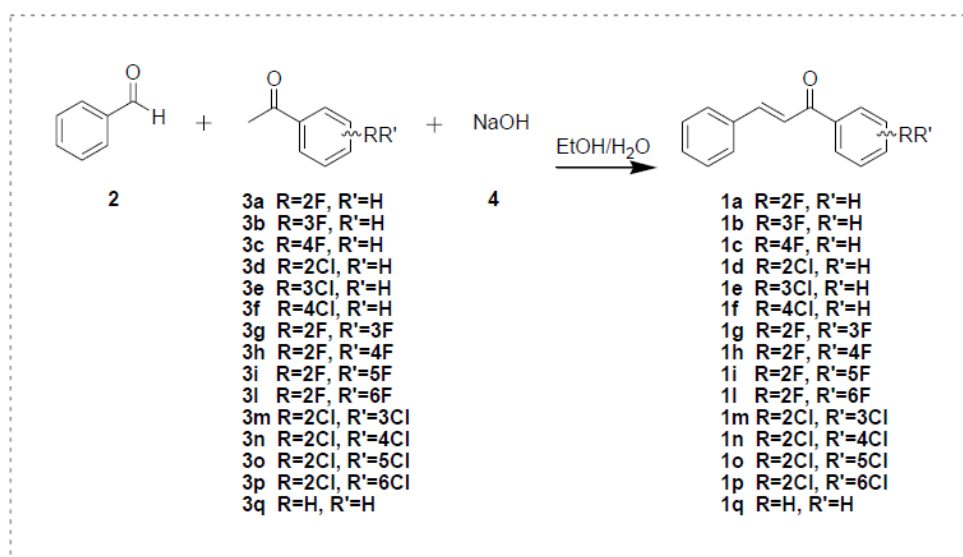
Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data from triplicate experiments are expressed as mean $\pm$ SD. Group differences were evaluated by one- or two-way ANOVA, followed by Bonferroni or Dunnett post-hoc tests. A p-value < 0.05 was considered statistically significant.

2.4. Results and Discussion

2.4.1. Synthesis of chalcones **1a-q**

Chalcones **1a-q** were synthesized via a crossed aldol condensation, commonly referred to as the Claisen-Schmidt reaction, between benzaldehyde **2** and halogen-substituted acetophenones **3a-q**, in the presence of a strong base such as sodium hydroxide **4**, in a hydroalcoholic solution (Scheme 2.11.).

In the reaction mechanism, deprotonation of the acidic α -hydrogen of acetophenones **3a-q** by base **4** generates the corresponding enolate anion. This nucleophile then attacks the electrophilic carbonyl carbon of benzaldehyde **2**, by forming a β -hydroxy ketone intermediate. Subsequent base-catalyzed dehydration of this intermediate yields the conjugated α,β -unsaturated ketone scaffold characteristic of chalcones. This approach efficiently produced chalcones **1a-q** in good to excellent yields, depending on the substitution pattern.



Scheme 2.11. Claisen-Schmidt condensation between benzaldehyde **2** and variously substituted acetophenones **3a-q**.

2.4.2. Biological activity of chalcones **1a-q**

The newly synthesized chalcones **1a-q** were evaluated for their cytotoxic activity against a panel of solid tumor cell lines, namely U87 (glioblastoma), HEPG2 (hepatocellular carcinoma), U2OS (osteosarcoma), FTC-133 (follicular thyroid carcinoma), and 8305C (anaplastic thyroid carcinoma).

Table 2.2. Cytotoxic activity of halogenated chalcones **1a-q** on U87 (glioblastoma), HEPG2 (hepatocellular carcinoma), U2OS (osteosarcoma), FTC-133 (follicular thyroid carcinoma), and 8305C (thyroid carcinoma) cell lines.

	U87	HEPG2	U2OS	FTC-133	8305C
1a	92.85 μ M	661.97 μ M	313.14 μ M	87.22 μ M	90.97 μ M
1b	u.s.	u.s.	u.s.	u.s.	u.s.
1c	u.s.	50.44 μ M	<50 μ M	50.44 μ M	437.08 μ M
1d	n.i.	n.i.	n.i.	n.i.	n.i.
1e	u.s.	47.04 μM	4.64 μM	37.26 μM	61.70 μM
1f	u.s.	u.s.	u.s.	u.s.	104.21 μ M
1g	u.s.	u.s.	u.s.	u.s.	69.08 μ M
1h	u.s.	u.s.	u.s.	u.s.	u.s.
1i	n.i.	n.i.	n.i.	n.i.	n.i.
1l	n.i.	n.i.	69.46 μ M	n.i.	775.34 μ M
1m	n.i.	n.i.	n.i.	n.i.	n.i.
1n	613.85 μ M	n.i.	n.i.	n.i.	n.i.
1o	<50 μ M	u.s.	<50 μ M	u.s.	u.s.
1p	<50 μ M	u.s.	<50 μ M	u.s.	u.s.
1q	171.27 μ M	u.s.	166.41 μ M	u.s.	u.s.

Notes: u.s. = under screening; n.i. = no inhibition.

Among the series, compound **1e** (Figure 2.6.), bearing a chlorine atom at the *meta* position of the aromatic ring A, showed the strongest activity. It was active across all tested cell lines, with EC₅₀ values ranging from 37 to 61 μ M. The best result was observed against osteosarcoma cells, where compound **1e** displayed an EC₅₀ value of 4.64 μ M.

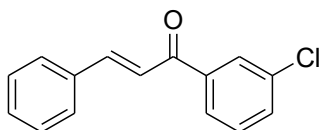


Figure 2.6. Chemical structure of chalcone **1e**, bearing a *meta*-chloro substituent on the aromatic ring A (the ring bearing the carbonyl).

Based on these promising results, compound **1e** was selected for further investigation. Specifically, UV-visible absorption, chemical stability, and solubility studies were conducted to identify the most suitable organic solvent or combination of solvents for its manipulation and future formulation. Given the known challenges associated with the poor solubility and bioavailability of chalcone derivatives [1], we also explored the incorporation of compound **1e** into nanocarrier systems. These platforms aim to enhance its aqueous solubility, protect it from chemical degradation, and promote its targeted delivery to cancer cells. Ultimately, these efforts are intended to support the development of compound **1e** as a potential agent for anticancer theranostics, combining diagnostic and therapeutic capabilities within a single system.

2.4.3. Physicochemical profiling of chalcone 1e for formulation development

The UV-Vis absorption spectrum of chalcone **1e**, recorded in EtOH, MeOH, and a MeOH/water mixture (5:1, v/v) over the spectral range of 200-800 nm, consistently exhibits two characteristic absorption bands, irrespective of the solvent employed (Figure 2.7.). The first, an intense band observed in the 220-230 nm region, is attributed to $\pi \rightarrow \pi^*$ electronic transitions within the aromatic rings. Although prominent, this band lacks structural specificity and is therefore not considered diagnostic. The second, more prominent absorption band is centered around 313

nm, with slight solvent-dependent variations (312 nm in organic solvents and 315 nm in the MeOH/water mixture). This band primarily arises from $\pi \rightarrow \pi^*$ transitions associated with the extended conjugated system of the chalcone scaffold, particularly involving the α,β -unsaturated carbonyl moiety. A minor contribution from $n \rightarrow \pi^*$ transitions of the carbonyl group in the same spectral region cannot be excluded, although it is generally weaker and masked by the dominant $\pi \rightarrow \pi^*$ absorption.

This spectral pattern aligns well with literature reports, which describe α,β -unsaturated carbonyl compounds as typically exhibiting two characteristic absorption bands corresponding to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions [153]. The presence of a chlorine atom in the *meta* position of ring B (the aromatic ring bearing the carbonyl group) exerts an electron-withdrawing inductive effect. Theoretically, such a substituent could alter the electronic distribution within the conjugated system [154]. However, due to its *meta* substitution, the chlorine atom does not participate in resonance with the π -system and therefore does not induce any significant bathochromic shift. As a result, the absorption profile of chalcone **1e** remains consistent with that of a moderately conjugated system.

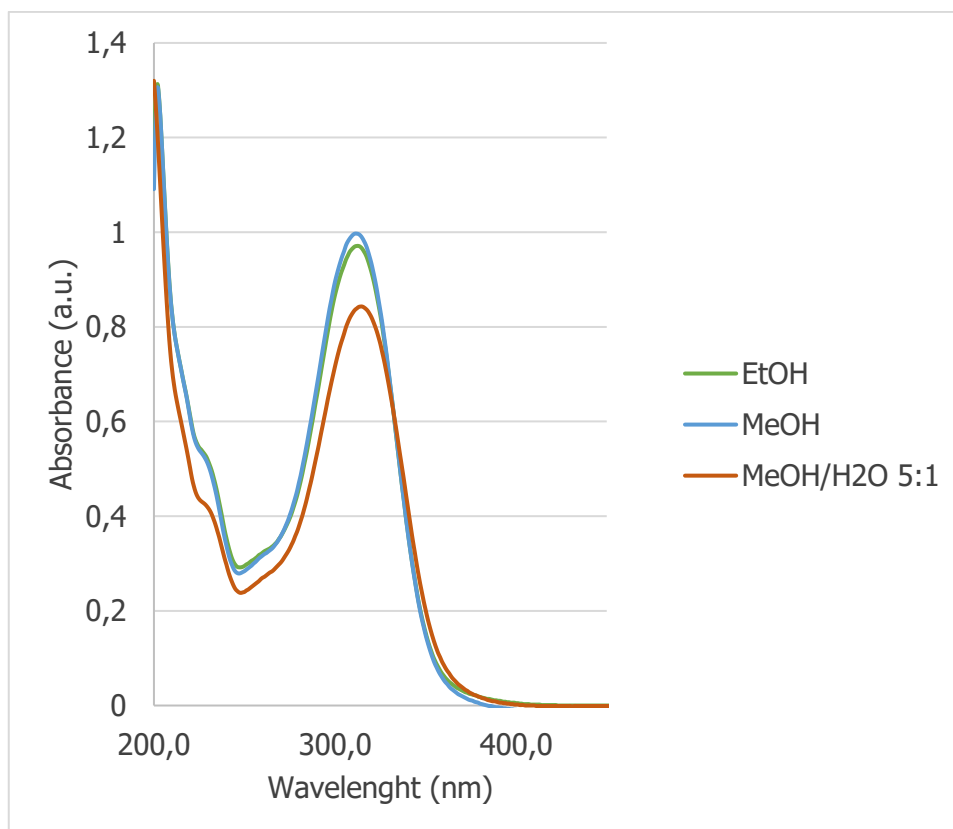


Figure 2.7. UV-Vis absorbance profile of chalcone **1e** in various solvents (ethanol, methanol, and methanol/water mixture, 5:1 v/v), recorded at a concentration of 6.2×10^{-5} M.

In the same solvents, three calibration curves were constructed in order to determine the ϵ , by applying the equation reported in Section 2.3.3. This parameter plays a fundamental role in UV-Vis spectrophotometry, as it quantifies the extent to which a chemical species absorbs light at a given wavelength. The ϵ is a constant that varies from molecule to molecule, depending on its electronic structure and the specific transition involved [155].

The experimentally determined ϵ for chalcone **1e** in EtOH, MeOH, and MeOH/water (5:1, v/v) were 2.06×10^4 , 2.11×10^4 , and 1.79×10^4 L·mol⁻¹·cm⁻¹, respectively. These results indicate that chalcone **1e** exhibits comparable molar absorptivity in EtOH and MeOH, with a slightly higher value observed in MeOH.

Conversely, a notable decrease in absorptivity was recorded in the MeOH/water mixture. This reduction can be rationalized by considering the increased polarity and hydrogen bonding capacity of the aqueous solvent system, which may alter solute-solvent interactions and affect the electronic distribution within the conjugated system of the molecule.

To further investigate the stability of chalcone **1e**, its UV-Vis absorbance at 313 nm was monitored over a one-month period under both light and dark conditions, in the same three solvents, to assess the compound's photostability and solvent-dependent behavior. Under dark conditions, no changes were observed in either the absorbance intensity or the spectral profile (i.e., no hypsochromic or bathochromic shifts), indicating a high intrinsic stability of chalcone **1e** in the absence of light, regardless of the solvent used.

In contrast, under continuous exposure to ambient light, the compound exhibited time-dependent degradation:

- The first graph (Figure 2.8.) illustrates the behavior of chalcone **1e** during the first 72 hours of light exposure. In MeOH, the compound displays excellent stability, with no variation in absorbance over time. In EtOH, a moderate initial decrease is observed, followed by a plateau, suggesting limited but measurable degradation. In sharp contrast, the MeOH/water (5:1 v/v) mixture shows a continuous and progressive decline in absorbance from the earliest time points. This behavior cannot be attributed to poor solubility alone, as no such decrease occurred under dark conditions. Rather, the combination of light and an aqueous environment likely promotes photodegradation, reducing the effective concentration of the compound in solution.

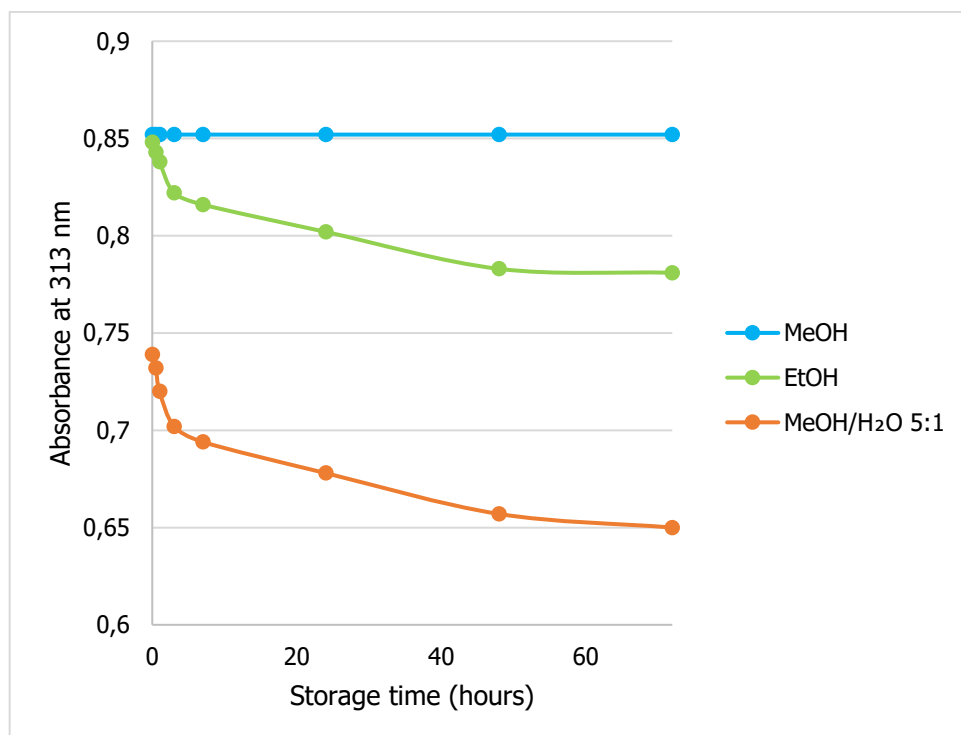


Figure 2.8. UV-Vis absorbance at 313 nm of chalcone **1e** (4.13×10^{-5} M, $25^\circ\text{C} \pm 0.5^\circ\text{C}$) over the first 72 hours under light. Monitoring in MeOH (blue), EtOH (green) and MeOH/H₂O 5:1 (orange) demonstrates excellent photostability in MeOH, moderate degradation in EtOH, and rapid absorbance decline in the aqueous mixture.

- The second graph (Figure 2.9.) presents the long-term behavior over four weeks of light exposure. All three solvents exhibit a decrease in absorbance, confirming the gradual photodegradation of chalcone **1e**. MeOH again emerges as the most effective solvent in slowing down this process, while EtOH shows a more pronounced decline. In the MeOH/water mixture, the absorbance decreases significantly, consistent with both reduced solubility and increased susceptibility to degradation.

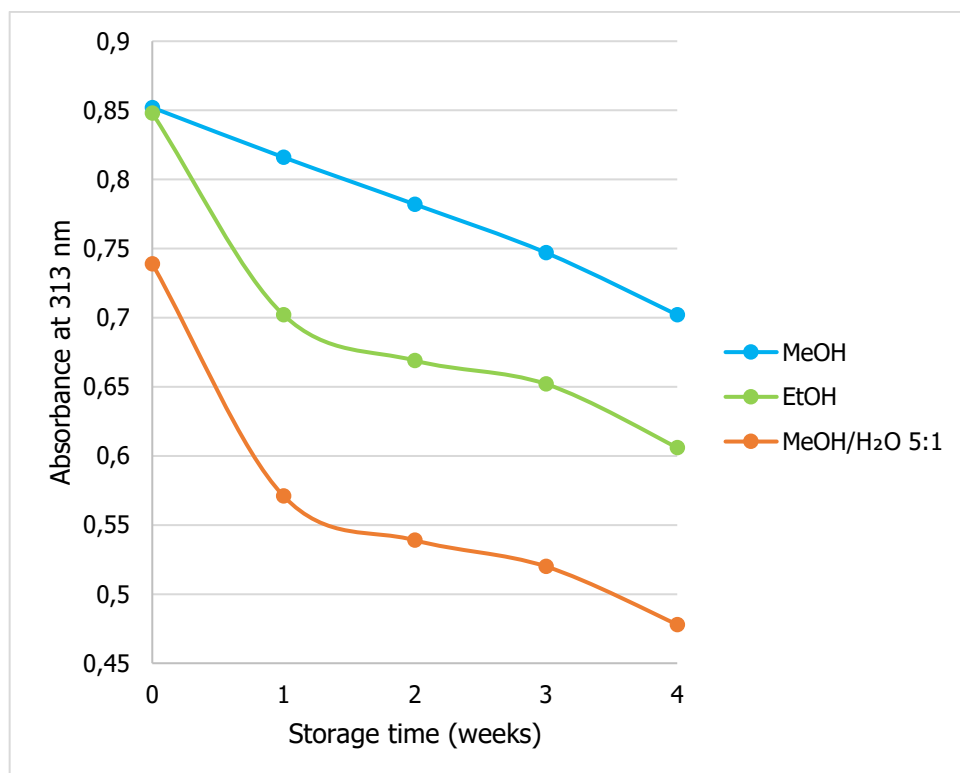


Figure 2.9. Long-term UV-Vis absorbance at 313 nm of chalcone **1e** (4.13×10^{-5} M, $25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$) over four weeks under light. Monitoring in MeOH (blue), EtOH (green) and MeOH/H₂O 5:1 (orange) reveals that photodegradation occurs gradually in all solvents, with MeOH providing the highest stability, and the aqueous mixture the least.

It is noteworthy that, in all samples exposed to light, no spectral shifts were observed, neither toward longer (bathochromic) nor shorter (hypsochromic) wavelengths. This indicates that the degradation process does not involve progressive modifications of the chromophore's electronic structure, but rather a net loss of the active compound, likely due to photodegradation or oxidation.

Finally, the solubility of chalcone **1e** was assessed in the same three solvents at $25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. The compound demonstrated a solubility of ~ 16 mg/mL in MeOH, ~ 11 mg/mL in EtOH, and only ~ 2.8 mg/mL in the MeOH/water mixture. These results indicate that chalcone **1e** displays high affinity for polar organic solvents,

such as MeOH and EtOH, while showing limited compatibility with aqueous environments. The pronounced decrease in solubility observed in the MeOH/water system suggests that the presence of water significantly compromises solvation of the compound, consistent with its lipophilic character. Even small amounts of water appear to act as an antisolvent, markedly reducing solubility. This behavior aligns with the photostability findings, where aqueous conditions were shown to accelerate degradation.

2.4.4. Encapsulation of compound 1e into PLGA-based nanoparticles

Compound **1e** exhibits such a high degree of hydrophobicity that its solubility in pure water is below the detection limit of UV-Vis spectrophotometry, unless water is mixed in a small proportion an organic co-solvent. This severely limited solubility translates into a poor drug-likeness profile, significantly restricting its potential for pharmaceutical development despite its promising biological activity. To overcome these challenges and enhance its bioavailability, compound **1e** was incorporated into nanoparticulate delivery systems designed for highly lipophilic compounds. Specifically, PLGA NPs were selected and prepared via the nanoprecipitation method. A series of formulations were developed incorporating increasing amounts of compound **1e** (from 0.1 mg to 0.5 mg), and were characterized in terms of EE %, yield, DC %, mean particle size, ζ , and PDI.

Table 2.3. Encapsulation efficiency (EE %), yield, and drug content (DC %) of PLGA NPs loaded with increasing amounts of compound **1e** (expressed in mg), with or without FITC-CS surface coating. Data are expressed as mean $\pm$ SD.

<i>Samples</i>	EE % $\pm$ SD	Yield % $\pm$ SD	DC % $\pm$ SD
Blank	N.A.	61.4 $\pm$ 4.85	N.A.
Blank (+ 0.045 mL FITC-CS)	N.A.	60.7 $\pm$ 6.32	N.A.
0.1 mg	43.0 $\pm$ 1.81	61.3 $\pm$ 3.89	0.20 $\pm$ 0.05
0.2 mg	38.5 $\pm$ 2.04	60.0 $\pm$ 7.12	0.37 $\pm$ 0.06
0.3 mg	43.0 $\pm$ 1.62	57.6 $\pm$ 6.32	0.64 $\pm$ 0.16
0.4 mg	59.8 $\pm$ 2.22	60.9 $\pm$ 4.78	1.12 $\pm$ 2.40
0.5 mg	n.r.	n.r.	n.r.
0.4 mg (+ 0.045 mL FITC-CS)	57.1 $\pm$ 2.85	61.4 $\pm$ 5.28	1.05 $\pm$ 1.58

Notes: N.A. = not applicable; n.r. = not retrievable.

Table 2.4. Hydrodynamic diameter (D_H), polydispersity index (PDI), and zeta potential (ζ) of PLGA NPs loaded with increasing amounts of compound **1e** (expressed in mg), with or without FITC-CS surface coating. Different volumes of FITC-CS solution were tested to evaluate their effect on nanoparticle surface properties. Data are expressed as mean $\pm$ SD.

Samples	Hydrodynamic diameter (D_H) $\pm$ SD (nm)	Polydispersity index (PDI) $\pm$ SD	Zeta potential (ζ) $\pm$ SD (mV)
Blank	67.78 $\pm$ 1.27	0.14 $\pm$ 0.05	-34.11 $\pm$ 1.24
Blank (+ 0.5 mL FITC-CS)	n.r.	n.r.	> 60
Blank (+ 0.25 mL FITC-CS)	n.r.	n.r.	> 60
Blank (+ 0.05 mL FITC-CS)	151.6 $\pm$ 1.48	0.24 $\pm$ 0.02	+37.02 $\pm$ 0.97
Blank (+ 0.045 mL FITC-CS)	142.3 $\pm$ 1.36	0.26 $\pm$ 0.01	+30.92 $\pm$ 1.08
Blank (+ 0.04 mL FITC-CS)	141.5 $\pm$ 0.18	0.28 $\pm$ 0.01	+24.26 $\pm$ 1.11
0.1 mg	72.53 $\pm$ 1.34	0.06 $\pm$ 0.01	-32.78 $\pm$ 0.06
0.2 mg	69.96 $\pm$ 2.26	0.14 $\pm$ 0.03	-33.14 $\pm$ 1.76
0.3 mg	74.07 $\pm$ 1.46	0.08 $\pm$ 0.02	-30.68 $\pm$ 0.88
0.4 mg	77.44 $\pm$ 1.63	0.11 $\pm$ 0.04	-31.89 $\pm$ 0.55
0.4 mg (+ 0.045 mL FITC-CS)	143.9 $\pm$ 1.63	0.23 $\pm$ 0.01	+30.17 $\pm$ 1.95

Notes: n.r. = not retrievable.

As shown in Table 2.4., increasing the drug loading resulted in a slight increase in particle size, while acceptable monodispersity was maintained ($PDI < 0.3$), along with satisfactory colloidal stability (consistently around -30 mV for uncoated samples). Attempts to formulate NPs with higher drug amounts (0.5 mg) were unsuccessful: all such preparations failed, likely due to the compound's strong hydrophobicity, causing precipitation in the acetone-water system during NPs preparation.

To obtain NPs suitable not only for therapeutic applications but also for diagnostic purposes, a FITC-CS coating was applied to the NP surface. Various volumes of coating solution (0.2% w/v FITC-CS in 1% v/v aqueous acetic acid solution) were tested to identify the optimal conditions that ensure coating efficiency without compromising NP stability. Higher volumes of coating solution led to the appearance of two NP populations in the size distribution analysis by Zetasizer, which could not be clearly distinguished, along with irregular ζ values (>60 mV), likely due to excess polymer accumulation. In contrast, intermediate volumes (particularly 0.045 mL, identified as optimal) produced a well-defined positive surface charge (approximately +30 mV for both blank and compound **1e**-loaded samples), indicating that minimal amounts of FITC-CS are sufficient to achieve effective surface functionalization.

Despite these encouraging findings in terms of colloidal stability and surface charge modulation, a critical limitation remained: the EE % was relatively low, ranging from 38.5% to 69.8% (Table 2.3.). This is particularly problematic given the very small starting quantities of compound **1e** used (maximum 0.4 mg), which resulted in extremely limited DC. These values are clearly insufficient for any therapeutically relevant application, especially in the context of systemic

administration, and underscore the need to explore alternative formulation strategies.

2.4.5. Encapsulation of compound **1e** into nanostructured lipid carriers

Given the unsuccessful results obtained with the encapsulation of compound **1e** into PLGA-based NPs, it was necessary to pursue an alternative formulation strategy. To this end, NLCs were selected as a more suitable delivery system for such a highly lipophilic drug. A series of NLC formulations containing increasing amounts of compound **1e** (from 5 mg to 20 mg) was developed and characterized in the same manner as the previous PLGA-based systems, evaluating parameters such as EE %, yield, DC %, mean particle size, ζ , and PDI.

Table 2.5. Encapsulation efficiency (EE %), yield, and drug content (DC %) of NLCs loaded with increasing amounts of compound **1e** (expressed in mg), with or without FITC-CS surface coating. Data are expressed as mean $\pm$ SD.

<i>Samples</i>	<i>EE % $\pm$ SD</i>	<i>Yield % $\pm$ SD</i>	<i>DC % $\pm$ SD</i>
Blank	N.A.	85.3 $\pm$ 2.68	N.A.
Blank (+ 2.3 mL FITC-CS)	N.A.	88.7 $\pm$ 5.36	N.A.
5 mg	100	86.0 $\pm$ 7.26	1.15 $\pm$ 0.06
10 mg	100	87.6 $\pm$ 3.69	2.24 $\pm$ 0.76
15 mg	100	83.9 $\pm$ 4.15	3.47 $\pm$ 1.40
20 mg	100	93.0 $\pm$ 9.18	4.16 $\pm$ 0.80
20 mg (+ 2.3 mL FITC-CS)	100	89.3 $\pm$ 2.2	4.31 $\pm$ 2.20

Notes: N.A. = not applicable; n.r. = not retrievable.

Table 2.6. Hydrodynamic diameter (D_H), polydispersity index (PDI), and zeta potential (ζ) of NLCs loaded with increasing amounts of compound **1e** (expressed in mg), with or without FITC-CS surface coating. Different volumes of FITC-CS solution were tested to evaluate their effect on nanoparticle surface properties. Data are expressed as mean $\pm$ SD.

<i>Samples</i>	<i>Hydrodynamic diameter (D_H) $\pm$ SD (nm)</i>	<i>Polydispersity index (PDI) $\pm$ SD</i>	<i>Zeta potential (ζ) $\pm$ SD (mV)</i>
Blank	40.75 $\pm$ 0.39	0.24 $\pm$ 0.02	-17.61 $\pm$ 4.64
Blank (+ 1 mL FITC-CS)	45.33 $\pm$ 2.46	0.22 $\pm$ 0.03	+5.23 $\pm$ 1.44
Blank (+ 2 mL FITC-CS)	57.53 $\pm$ 1.25	0.18 $\pm$ 0.01	+10.08 $\pm$ 0.26
Blank (+ 2.3 mL FITC-CS)	68.23 $\pm$ 0.44	0.24 $\pm$ 0.02	+19.33 $\pm$ 0.36
Blank (+ 2.5 mL FITC-CS)	n.r.	n.r.	> 50
5 mg	46.79 $\pm$ 1.46	0.15 $\pm$ 0.02	-17.31 $\pm$ 2.97
10 mg	41.55 $\pm$ 1.30	0.23 $\pm$ 0.05	-18.76 $\pm$ 4.27
15 mg	47.89 $\pm$ 0.52	0.27 $\pm$ 0.03	-17.88 $\pm$ 4.36
20 mg	57.15 $\pm$ 1.01	0.28 $\pm$ 0.01	-21.62 $\pm$ 7.58
20 mg (+ 2.3 mL FITC-CS)	83.01 $\pm$ 0.99	0.26 $\pm$ 0.01	+17.33 $\pm$ 2.41

Notes: n.r. = not retrievable.

From a technological standpoint (Table 2.6.), all formulations displayed favorable physicochemical characteristics, with mean particle sizes ranging between approximately 41 and 57 nm, and PDI values consistently below 0.3, indicating good size homogeneity and monodispersity. ζ values remained in the range of -17 to -21 mV across all formulations; these values are in line with those typically

reported in the literature for NLC systems, confirming the expected surface properties of such lipid-based nanocarriers [156–158].

As for encapsulation performance (Table 2.5.), thanks to the highly lipophilic nature of compound **1e**, all formulations achieved 100% EE, with DC % values increasing proportionally with the amount of compound used, reaching up to 4.31% in the 20 mg formulation. However, attempts to encapsulate amounts higher than 20 mg were unsuccessful, as the appearance of a visible precipitate suggested saturation of the system and the presence of non-encapsulated compound **1e** outside the NP matrix.

To develop fluorescent nanocarriers for potential theranostic applications, similarly to what was attempted with the PLGA NPs, blank NLCs were coated with FITC-CS, following the same coating strategy previously applied. A 0.2% w/v solution of FITC-CS in 1% v/v aqueous acetic acid solution was prepared, filtered, and tested at increasing volumes (1, 2, 2.3, and 2.5 mL). As reported in Table 2.6, the results were encouraging: particle size and PDI values remained acceptable, and the surface charge shifted progressively toward positive values, indicating successful coating. The formulation coated with 2.3 mL of solution exhibited an optimal balance, with mean particle sizes of 68.23 ± 0.44 nm and a ζ value of $+19.33 \pm 0.36$ mV. Similarly to what was observed with PLGA NPs, higher coating volumes (e.g., 2.5 mL) resulted in a multimodal size distribution in the Zetasizer analysis, indicative of the presence of two unresolved particle populations, along with irregular ζ values, attributable to excess polymer deposition.

Based on these results, the NLC formulation containing 20 mg of compound **1e** and coated with 2.3 mL of FITC-CS solution was identified as the optimal carrier for compound **1e** and was therefore selected for further investigations.

2.4.6. *In vitro* release of compound **1e** from nanostructured lipid carriers

The release of compound **1e** from the NLCs was evaluated using the dialysis method. Weighted amounts of compound **1e**-loaded NLCs, both coated and uncoated with FITC-CS, were suspended in a PBS/MeOH mixture (80:20, v/v) and compared to a solution of free compound **1e** in the same solvent (Figure 2.10.).

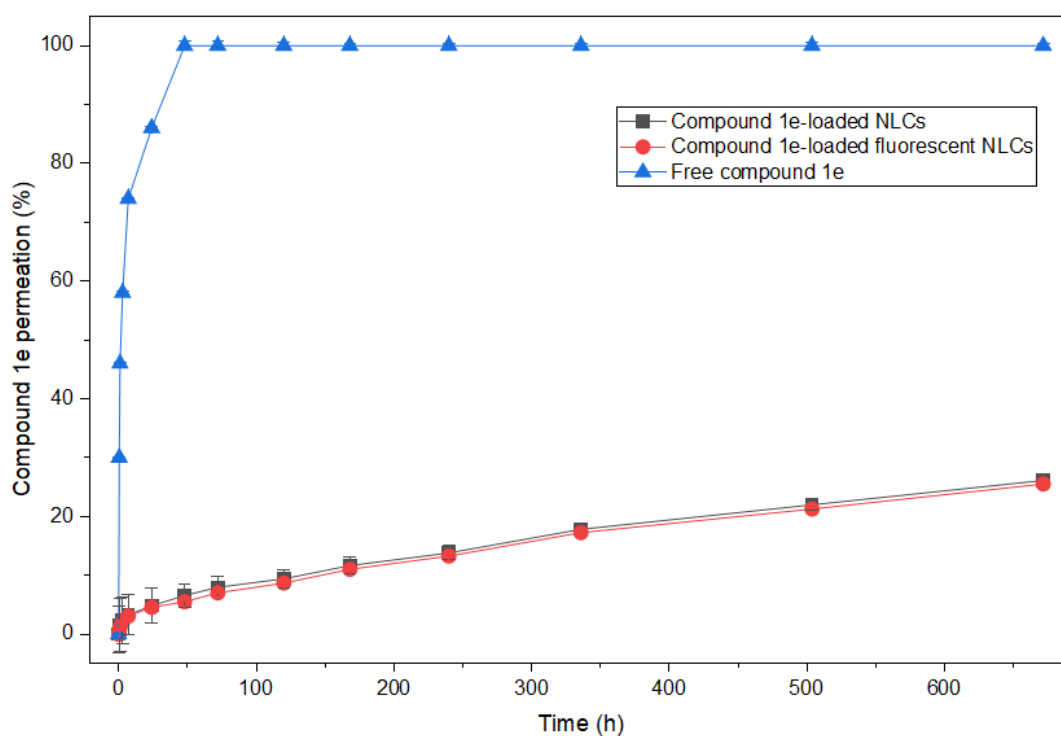


Figure 2.10. *In vitro* release profiles of compound **1e** from uncoated (black squares) and coated (red circles) NLCs, compared to free compound **1e** (blue triangles). Data are presented as the mean $\pm$ SD of three independent experiments. If error bars are not visible, they fall within the data symbols.

The release profile obtained for compound **1e** from the NLCs, both coated and uncoated, shows an extremely limited *in vitro* release, with cumulative percentages not exceeding 25% even after prolonged incubation times (1 month). This behavior reflects the strong capacity of the lipid matrix to retain the chalcone within its structure, significantly limiting its diffusion into the external medium. A relevant

finding from this study is that the coating with FITC-CS, applied to confer fluorescence to the nanosystems and enable their tracking, did not alter the release profile compared to uncoated NLCs. The drug release dynamics are therefore primarily determined by the high affinity of the molecule for the lipid phase rather than by the surface characteristics of the nanosystems.

Our results are fully consistent with findings reported in the literature for other lipophilic drugs encapsulated in similar systems. In particular, Yu *et al.* (2016) reported that the *in vitro* release of the immunosuppressant sirolimus (SRL) from NLCs, prepared by high-pressure homogenization using Precirol® ATO 5 and oleic acid as the lipid phase and Tween 80 as the surfactant, was almost negligible: less than 1% after 2 hours and only 2.15% after 12 hours, compared to an almost complete release from commercial tablets over the same time interval. The authors attributed this behavior to the very strong affinity of the drug for the lipid phase, which ensures nearly 100% EE but limits diffusion into the external medium under acellular conditions [159].

With results that were still incomplete but comparatively more favorable, Marathe *et al.* (2022) developed α -tocopherol succinate-based NLCs for ocular delivery of PTX. These nanosystems were also prepared by high-pressure homogenization, using α -tocopherol succinate as the solid lipid, Capryol™ 90 as the liquid lipid, and stabilized with Tween 80 and Poloxamer 188. Their *in vitro* release was assessed using the dialysis bag method in a release medium composed of 5% methyl β -CD and 2% Tween 80, supplemented with 1 M sodium salicylate in PBS (pH 7.4), added to ensure sink conditions. Once again, release proved to be only partial, with about 40% of PTX released over a prolonged period. The authors attributed this profile to the strong retention of the hydrophobic drug within the lipid core, while

the initial burst was explained by surface-adsorbed molecules. Such a behavior is advantageous for applications requiring prolonged release. However, the authors hypothesized that in a physiological setting, cellular uptake and enzymatic degradation of the lipid matrix would enhance drug liberation [160].

The validity of our experimental setup is strongly supported by the control experiment with the free molecule. Unencapsulated compound **1e** rapidly diffused across the dialysis membrane, reaching plateau levels within two days. This confirms both the suitability and sensitivity of the method and shows that the slow release observed in NLC formulations truly reflects the retention capacity of the lipid matrix rather than a methodological limitation.

Consequently, the limited *in vitro* release observed here should not be interpreted as a predictor of poor *in vivo* behavior. Instead, it stands as a testament to the formulation's stability under the acellular, non-lipolytic conditions of the test. It is reasonable to expect that in a biological environment, where lipases are widely expressed in tissues and often overexpressed in certain tumors [161,162], drug release would be significantly increased, aligning with the observations of Marathe *et al.* [160]. This concept is strongly supported by the work of Porter & Charman (2001) [163] and Shangguan *et al.* (2015) [164], who established that *in vivo* drug liberation from lipid-based systems is primarily governed by lipolysis. Further validating this assumption, Jannin *et al.* (2014) demonstrated that NLCs prepared with Precirol® ATO 5 are highly susceptible to lipase-mediated digestion *in vitro*. Their work, which showed continuous free fatty acid release for over 60 minutes, crucially highlighted that this enzymatic degradation is especially effective for NPs smaller than 200 nm [165].

In our case, therefore, the extremely slow *in vitro* release profile is consistent with the retention mechanisms described for other lipophilic drugs encapsulated in NLCs and should be interpreted as an intrinsic limitation of the acellular experimental model rather than of the formulation itself. It is reasonable to hypothesize that *in vivo*, under physiological conditions, the release of compound **1e** would be considerably higher due to enzymatic lipolysis, and that the low *in vitro* release does not impede actual drug absorption.

2.4.7. Stability evaluation of compound 1e-loaded nanostructured lipid carriers

The extremely limited *in vitro* release of compound **1e** from both coated and uncoated nanosystems reflects its strong affinity for the lipid matrix. While this suggests the compound is well retained, direct thermal analysis was necessary to assess the stability of the carrier itself. To assess the stability of the NLCs over time, lyophilized formulations (with and without FITC-CS coating) were analyzed by TGA immediately after lyophilization and then stored under dry conditions in a desiccator for two months. Portions of the stored samples were withdrawn at monthly intervals and reanalyzed.

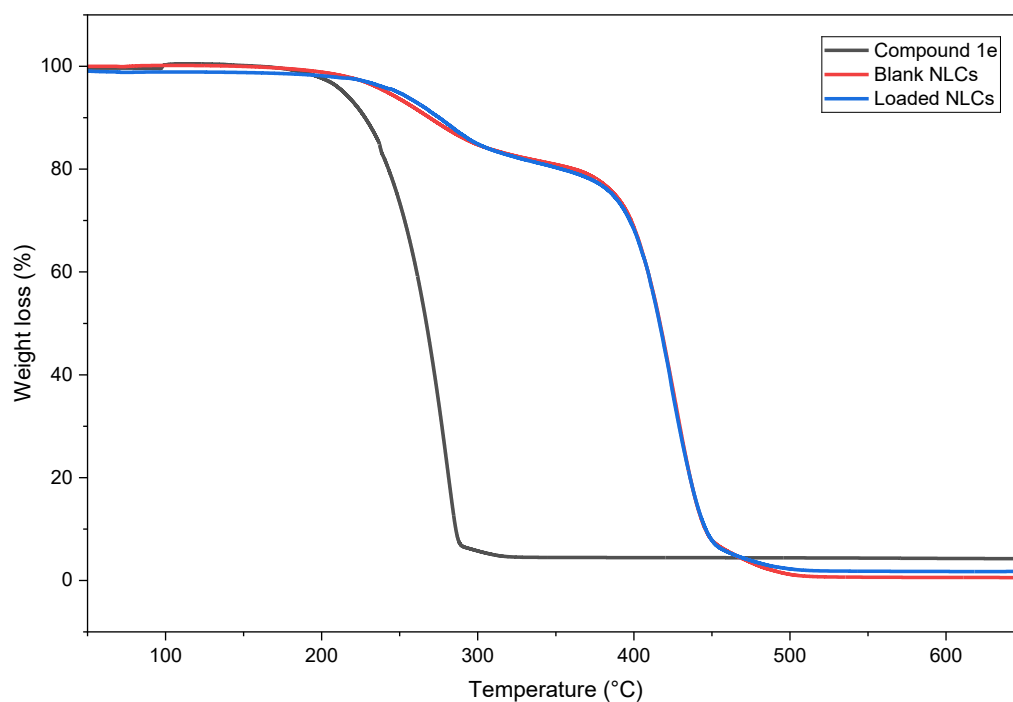


Figure 2.11. TGA profiles of free compound **1e** and uncoated formulations (blank and compound **1e**-loaded NLCs) recorded at time zero over the temperature range 20-800 °C under a nitrogen atmosphere. The **1e**-loaded NLCs exhibit a delayed degradation onset and a more gradual weight loss compared to the free chalcone, indicating improved thermal stability upon encapsulation.

Table 2.7. Thermal parameters of compound **1e** and the uncoated NLC formulations (blank and compound **1e**-loaded) over a period of two months, determined by TGA. The first degradation step (T_{onset1} , T_{max1}) corresponds to a minor initial weight loss, likely due to moisture, volatile components, or partial decomposition. The second step (T_{onset2} , T_{max2}) represents the main thermal decomposition of the material. Residue at 800 °C indicates the remaining mass after complete degradation.

<i>Samples</i>	<i>T_{onset1}</i> (°C)	<i>T_{max1}</i> (°C)	<i>T_{onset2}</i> (°C)	<i>T_{max2}</i> (°C)	<i>Residue</i> <i>at 800</i> <i>°C (%)</i>
Compound 1e	/	/	250.62	281.39	4.09
Blank NLCs	224.27	269.55	390.72	427.30	0.01
Compound 1e-loaded NLCs	238.03	284.16	401.25	423.54	1.62
Blank NLCs (1 month)	223.92	269.26	390.58	427.04	0.05%
Compound 1e-loaded NLCs (1 month)	236.81	282.95	399.97	423.24	1.65%
Blank NLCs (2 months)	222.57	268.86	388.15	426.72	0.08%
Compound 1e-loaded NLCs (2 months)	236.50	282.75	399.66	422.96	1.68%

Figure 2.11. reports the TG curves at time zero for the uncoated formulations, compared with free compound **1e** and blank NLCs, while the corresponding T_{onset} , T_{max} , and residual mass values for all samples during storage are summarized in Table 2.7. The TG curve of free compound **1e** (blue trace) highlights the intrinsic thermal lability of the chalcone: the molecule remains stable up to approximately 250 °C, beyond which it undergoes a sharp decomposition within a narrow

temperature range, leading to almost total weight loss by about 320 °C. This monophasic behavior, characterized by a single degradation step, indicates that the compound breaks down quickly over a narrow temperature range, with negligible mass losses at lower temperatures, consistent with the absence of residual solvents or significant moisture in the pure sample.

In contrast, the blank NLCs (red trace) exhibit a more complex, biphasic degradation profile. An initial, minor weight loss event ($T_{\text{onset}_1} \approx 224$ °C; $T_{\text{max}_1} \approx 270$ °C) is observed, likely attributable to the evaporation of residual water or volatile elements or partial decomposition of less stable fractions. The primary degradation event ($T_{\text{onset}_2} \approx 391$ °C; $T_{\text{max}_2} \approx 427$ °C), which represents the main breakdown of the lipid matrix, occurs at a significantly higher temperature than that of the free chalcone, underscoring the greater thermal stability of the lipid carrier itself [166,167].

Crucially, the TG curve of the compound **1e**-loaded NLCs (green trace) is extremely similar to that of the blank formulation. The characteristic sharp degradation peak of the free chalcone is absent. Instead, its decomposition appears to be integrated within the broader degradation process of the lipid matrix. This thermal behavior provides strong evidence for the successful incorporation of compound **1e** within the NLCs, which effectively delays its decomposition. The data summarized in Table 2.7. further support this interpretation, showing that the key degradation temperatures (T_{onset} and T_{max}) of the loaded NLCs align closely with those of the blank NLCs and are distinctly shifted compared to the free chalcone.

With regard to the topic of stability, the TGA data collected after one and two months of storage provide clear evidence of the long-term stability of the

nanosystems. Both the blank and drug-loaded formulations exhibited only minimal variations in their characteristic degradation temperatures. This remarkable thermal consistency indicates that the structural integrity of the lipid matrix and the successful incorporation of compound **1e** were fully maintained over time. Similarly, the residual mass values remained virtually unchanged. The carbonaceous residue for the loaded formulation, for instance, showed only a minimal increase from 1.62% initially to 1.68% after two months. From these results, we can confidently conclude that no significant degradation of the formulation occurred.

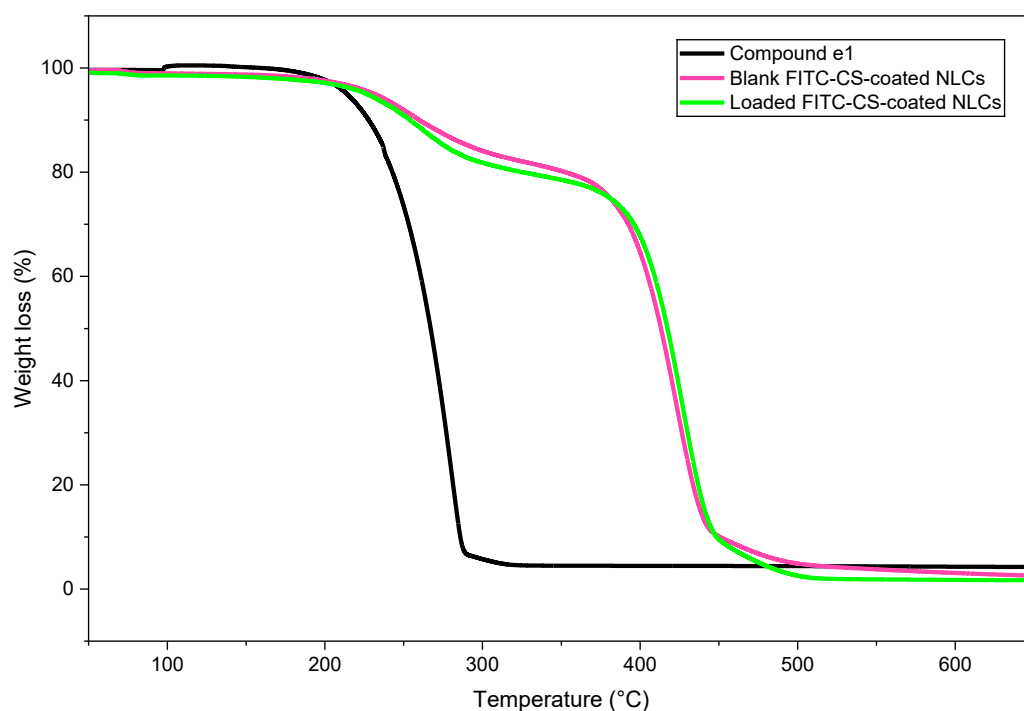


Figure 2.12. TGA profiles of free compound **1e** and coated formulations (blank and compound **1e**-loaded NLCs) recorded at time zero over the temperature range 20-800 °C under a nitrogen atmosphere.

Table 2.8. Thermal parameters of compound **1e** and the coated NLC formulations (blank and compound **1e**-loaded) over a period of two months, determined by TGA.

<i>Samples</i>	<i>T_{onset1}</i> (°C)	<i>T_{max1}</i> (°C)	<i>T_{onset2}</i> (°C)	<i>T_{max2}</i> (°C)	<i>Residue</i> <i>at 800</i> <i>°C (%)</i>
Compound 1e	/	/	250.62	281.39	4.09
Blank FITC-CS-coated NLCs	243.89	259.16	406.35	421.86	1.14
Compound 1e-loaded CF-coated NLCs	250.64	259.75	410.26	425.08	1.17
Blank FITC-CS-coated NLCs (1 month)	242.07	258.22	404.91	420.50	1.22
Compound 1e-loaded FITC-CS-coated NLCs (1 month)	249.03	258.88	408.74	423.14	1.26
Blank FITC-CS-coated NLCs (2 months)	241.05	257.15	403.23	418.99	1.31
Compound 1e-loaded FITC-CS-coated NLCs (2 months)	247.59	257.08	406.98	421.98	1.36

The TG profiles of the FITC-CS-coated NLCs (Figure 2.12.) also show a two-stage decomposition process, but with distinct shifts in temperature compared to their uncoated counterparts. For the blank FITC-CS-coated NLCs, the primary effect of the coating appears to be an increase in the onset temperatures ($T_{\text{onset1}} \approx 244$ °C; $T_{\text{onset2}} \approx 406$ °C) relative to the uncoated blanks ($T_{\text{onset1}} \approx 224$ °C; $T_{\text{onset2}} \approx 391$ °C),

while the $T_{\max}$ values ($T_{\max_1} \approx 259\text{ }^{\circ}\text{C}$; $T_{\max_2} \approx 422\text{ }^{\circ}\text{C}$) are similar or slightly reduced compared to the uncoated blanks ($T_{\max_1} \approx 270\text{ }^{\circ}\text{C}$; $T_{\max_2} \approx 427\text{ }^{\circ}\text{C}$). This suggests that the polymeric shell delays the start of the degradation process without significantly altering its maximum rate.

The thermal behavior of the compound **1e**-loaded FITC-CS-coated NLCs is nearly identical to that of the coated blanks. The characteristic sharp degradation peak of the free chalcone is absent, indicating that the compound's decomposition is incorporated into the broader breakdown of the lipid matrix. Notably, the FITC-CS coating increases the onset temperatures in the loaded formulations ($T_{\text{onset}_1} \approx 251\text{ }^{\circ}\text{C}$; $T_{\text{onset}_2} \approx 410\text{ }^{\circ}\text{C}$) compared to the uncoated NLCs ($T_{\text{onset}_1} \approx 238\text{ }^{\circ}\text{C}$; $T_{\text{onset}_2} \approx 401\text{ }^{\circ}\text{C}$). This highlights the stabilizing role of the FITC-CS shell, which, in addition to being useful for potential applications not only in the therapeutic but also in the diagnostic field due to its fluorescence, effectively delays thermal degradation of compound **1e**.

Concerning stability, similarly to the uncoated systems, the FITC-CS-coated NLCs also displayed excellent preservation during storage, with only marginal variations in T_{onset} and $T_{\max}$ over two months. The residual weights remained practically unchanged as well, increasing only from 1.17% to 1.36% in the loaded systems, thereby confirming the excellent preservation of the formulations.

The results clearly show that compound **1e** is firmly retained within the lipid matrix of both uncoated and FITC-CS-coated NLCs, as reflected by the absence of its sharp degradation peak and the minimal changes in thermal parameters over two months. This strong retention is consistent with the extremely limited in vitro release observed for both formulations. The FITC-CS coating further improves

stability by delaying the start of thermal degradation and, at the same time, offers potential for diagnostic applications through its fluorescence.

These observations confirm that the formulations maintain their structural integrity under non-lipolytic conditions, highlighting their robustness and suitability for sustained therapeutic and diagnostic use.

2.5. Conclusions

In this study, a series of halogenated chalcones (**1a-q**) was successfully synthesized *via* Claisen-Schmidt condensation, affording good to excellent yields across different substitution patterns. Several compounds showed cytotoxic activity with EC₅₀ values in the mid-micromolar range; among them, compound **1e** emerged as the most active derivative, displaying activity against four different cancer cell lines (EC₅₀ = 37-61 μM), with particularly promising results on osteosarcoma cells (EC₅₀ = 4.64 μM), and was therefore selected for further investigations.

Analysis of its physicochemical properties highlighted the typical challenges associated with chalcones: high lipophilicity, poor aqueous solubility, and susceptibility to photodegradation in aqueous media. These features emphasized the need for a suitable nanocarrier system to enhance its therapeutic potential. While initial attempts with PLGA NPs provided encouraging results in terms of colloidal stability and surface functionalization, EE remained unsatisfactory. NLCs, on the other hand, turned out to be far more suitable for such a lipophilic molecule. They allowed complete encapsulation of compound **1e** (up to 20 mg), yielding particles with well-controlled size, narrow distribution, and stable surface charge. The subsequent FITC-CS coating not only endowed the NLCs with a

fluorescent label but also preserved their physicochemical integrity without altering the release profile of the encapsulated drug.

Thermal analysis confirmed that compound **1e** was efficiently protected within the lipid matrix: both coated and uncoated NLCs displayed stable degradation profiles with only minimal variations after two months of storage. In line with this, *in vitro* release experiments revealed a very slow and limited diffusion of compound **1e** from the carriers, a behavior consistent with its strong affinity for the lipid phase and comparable to what has been reported for other lipophilic drugs encapsulated in NLCs.

In conclusion, these findings identified NLCs loaded with 20 mg of compound **1e** and coated with 2.3 mL of FITC-CS solution as the most promising formulation. At present, ongoing studies are focused on the *in vitro* biological evaluation of these systems in comparison with the free drug, with particular emphasis on cellular internalization, taking advantage of the intrinsic fluorescence of the formulations, and on elucidating the mechanisms underlying the observed cytotoxic effects. In parallel, complementary spectroscopic investigations, including FTIR and μ -Raman analyses, together with SEM studies, are being conducted to gain deeper insight into the structural organization and molecular architecture of the developed nanosystems.

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CHAPTER 3. COMPLEXATION OF RUTIN IN A CROSS-LINKED β -CYCLODEXTRIN/PEG POLYMER: EVALUATION OF ANTIANGIOGENIC PROPERTIES

3.1. Rutin

RTN (Figure 3.1.) is a prominent member of the flavonoid family, a class of polyphenolic compounds widely distributed across the plant kingdom responsible for imparting characteristic colors, scents, and flavors. Their roles in nature are manifold: produced by plants as secondary metabolites, flavonoids serve protective functions (e.g., against UV radiation, pathogens, and oxidative stress), enable plants to withstand a wide range of environmental conditions, attract pollinating insects, and regulate cell growth [1]. RTN in particular is found in numerous plant species, including citrus fruits (oranges, lemons, and grapefruits), apples, asparagus, buckwheat, and tea. The compound owes its name to its abundance in *Ruta graveolens*, a plant belonging to the Rutaceae family [2].

RTN has attracted increasing scientific interest due to its wide array of biological activities. Extensive studies have demonstrated its antioxidant [3,4], antiphlogistic [5], anticancer [6], and vascular-protective [7] properties. Particularly relevant in the context of oncology and diseases associated with vascular dysregulation are its antiangiogenic effects [8–10], which position RTN as a promising candidate for the development of combinatorial therapeutic strategies.

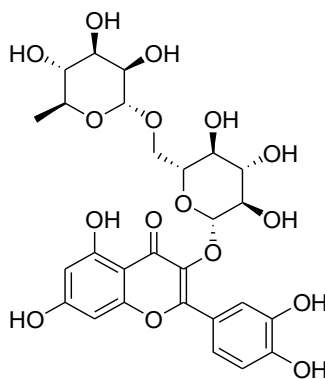


Figure 3.1. Chemical structure of rutin.

This growing attention to flavonoids like RTN reflects a broader trend in contemporary drug discovery, where the search for new synthetic molecules is often hindered by complexity and high costs. In this context, natural products have regained prominence as a valuable source of biologically active and chemically diverse scaffolds [2]. These “privileged structures” can serve as valuable templates for the rational design of new drugs, be used directly as APIs, or form the basis for the development of innovative DDSs, such as nanocarriers, to overcome challenges like poor bioavailability, rapid metabolism, or lack of tissue specificity.

RTN, in particular, exhibits features that make it an ideal candidate for in-depth pharmacological investigation. Its wide availability in both edible and medicinal plant sources, combined with a favorable safety profile and a well-characterized spectrum of biological activities, broadens its potential applications [11]. Beyond traditional therapeutic uses, due to its intrinsic but modest photoluminescence [12], RTN also shows potential in theranostics, an emerging field where a single compound performs both therapeutic and diagnostic functions.

3.1.1. Chemical structure and physicochemical properties of rutin

From a chemical perspective, RTN (also known as quercetin-3-*O*-rutinoside, *rutoside*, sophorin or vitamin P) is a natural flavonoid glycoside [2]. As such, it exhibits the typical structural features of flavonoids: a C6-C3-C6 backbone composed of two aromatic rings (A and B) connected by a three-carbon pyran ring (C), resulting in a total of 15 carbon atoms in its basic scaffold [1] (Figure 3.2.).

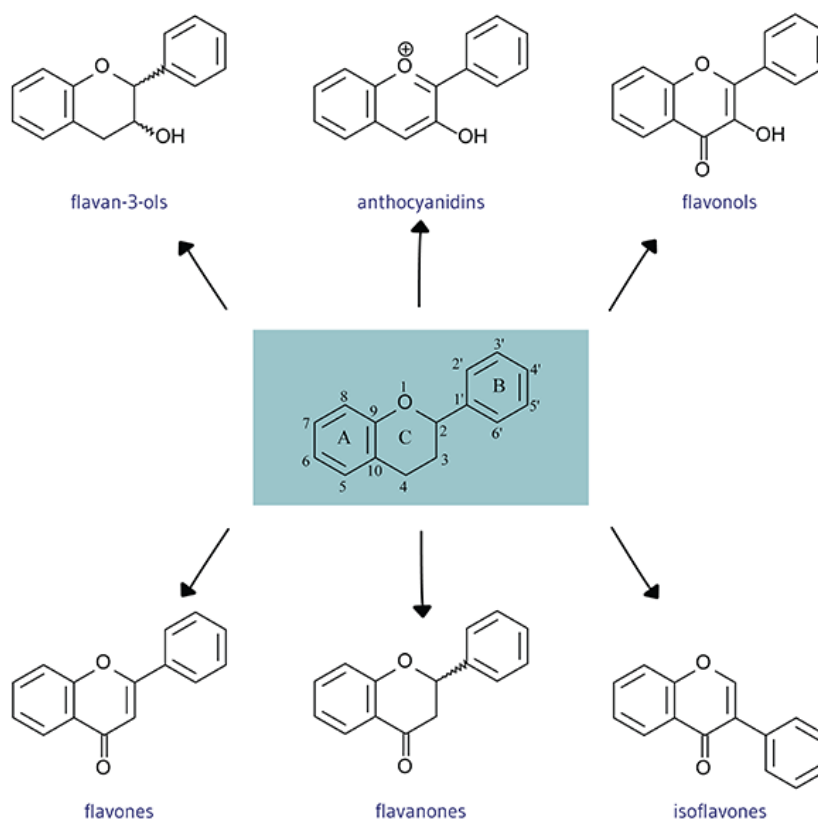


Figure 3.2. Basic flavonoid structure and classification. The central diagram shows the common C6-C3-C6 backbone of flavonoids, consisting of two aromatic rings (A and B) connected by a heterocyclic ring (C). Based on the degree of saturation of the C ring, flavonoids are classified into six major subclasses: flavan-3-ols, anthocyanidins, flavonols, flavones, flavanones, and isoflavones.

Flavonoids themselves are typically classified based on the degree of saturation in their central heterocyclic ring. Those with a C2=C3 double bond, such as

anthocyanidins, flavones, flavonols, and isoflavones, are considered unsaturated, while flavanones and flavan-3-ols are categorized as saturated flavonoids [1]. In addition to this core classification, flavonoid glycosides are distinguished by the type of bond connecting the sugar moiety to the flavonoid nucleus. These can be either *O*-glycosides or *C*-glycosides, depending on whether the sugar is linked via an oxygen or a carbon atom [13]. RTN belongs to the *O*-glycoside subgroup, and more specifically, it is the 3-*O*-rutinoside derivative of quercetin. This means that its flavonol aglycone, quercetin, is glycosylated at the 3-position with a disaccharide known as rutinoside, which consists of one unit of rhamnose and one of glucose [14].

From a physicochemical standpoint, RTN appears as fine, yellow-green crystalline needles. Its water solubility is limited (approximately 0.125 mg/mL) but it dissolves readily in various organic solvents, including pyridine, methanol, glycerol, DMF (dimethylformamide), and ethyl acetate [15]. The molecule has a molar mass of 610.52 g/mol, a melting point of around 125 °C, and a *pK*_a of 6.17, indicating moderate acidity [11].

3.1.2. Pharmacological profile and antiangiogenic properties of rutin

Flavonoids have long garnered significant attention in the pharmacological field due to their broad-spectrum bioactivity, as well as their ubiquitous presence in a wide variety of plant-based foods and traditional medicinal herbs [16]. Numerous studies have underscored their therapeutic potential not only as preventive agents but also as diagnostic and supportive treatments across a wide range of diseases [16–19]. Flavonoids such as quercetin, kaempferol, and apigenin (Figure 3.3.) have demonstrated promising activities in contexts ranging from cardiovascular and

metabolic disorders to neurodegenerative diseases and various types of cancer [20–22].

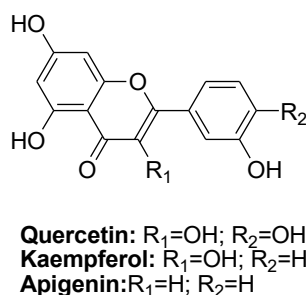


Figure 3.3. Chemical structures of three well-known flavonoids: quercetin, kaempferol, and apigenin. These compounds share the same flavone backbone but differ in the degree of hydroxylation on rings B and C.

RTN is no exception, exhibiting a wide array of pharmacological properties, including antiphlogistic [23], antiallergic [24], antitumor [25–29], antibacterial [30,31], antiviral [32], antiprotozoal [33], hypolipidemic and hypoglycemic [34], cytoprotective [35], gastroprotective [36], and antiplatelet [37] effects. These pharmacological properties are exhibited both as standalone actions and in synergistic combination with established therapeutic agents commonly employed in the management of various pathological conditions. Particularly notable is its ability to strengthen capillary resistance and maintain vascular integrity, largely attributed to its antioxidant and free radical-scavenging activities [38].

Among the diverse biological actions reported for RTN, its antiangiogenic potential has recently attracted growing interest, especially in the context of oncology. It should be noted that scientific interest in the antiangiogenic activity of this flavonoid is not exclusively recent, as evidenced by studies conducted over the years, underscoring its multifaceted and historically explored pharmacological profile.

A significant contribution in this domain stems from the work of Guruvayoorappan *et al.* (2007). Their study comprehensively demonstrated RTN's capacity to inhibit angiogenesis both *in vivo* and *in vitro*. In murine models of B16F-10 melanoma, administration of RTN (200 μ M/kg body weight for 5 consecutive days) resulted in a significant 43.35% reduction in tumor-directed capillary formation, indicating a direct inhibitory effect on tumor vascularization. *In vitro*, using human umbilical vein endothelial cells (HUVECs), RTN showed dose-dependent inhibition of key angiogenic processes: it reduced endothelial cell proliferation by up to 41.02% at 25 μ g/mL, strongly suppressed cell migration (87.5% inhibition at the same concentration), and disrupted capillary-like tube formation in Matrigel assays. Further confirmation of its antiangiogenic activity was provided by the rat aortic ring assay, where RTN (25 μ g/mL) inhibited microvessel sprouting induced by tumor-conditioned medium by 52.8%. Mechanistically, the study revealed that RTN modulates tumor-associated macrophages, exerting a dual effect: it downregulates the expression of pro-angiogenic factors such as VEGF and IL-1 β , while simultaneously promoting the production of TNF- α , a cytokine with known cytotoxic properties at specific concentrations [8].

Further insights were provided by Kumazawa *et al.* (2013), who explored RTN's antiangiogenic potential using *in vivo* models. Through a bio-guided study of subcritical water extracts from *Melia azedarach* leaves, three active flavonoid glycosides were isolated, including RTN. Using the chorioallantoic membrane (CAM) assay in chick embryos, a physiologically relevant *in vivo* model, they demonstrated that pure RTN (50 μ g/egg) significantly inhibited vascular network formation, producing a distinct avascular zone at the site of application. This finding not only confirmed the activity of the plant extract but also clearly

established RTN's direct antiangiogenic role in an integrated biological system. Interestingly, however, when RTN was tested on HUVECs *in vitro*, no antiangiogenic activity was observed. This discrepancy with Guruvayoorappan's earlier results [8] likely reflects methodological differences (e.g., concentrations used and incubation times, which were not specified by Kumazawa), though the authors also speculated that RTN's glycoside form might require metabolic activation to exert its effects [9].

On a molecular level, recent research has clarified how RTN suppresses angiogenesis. A 2021 review identified the STAT signaling cascade (particularly STAT3) as RTN's primary molecular target. By inhibiting STAT3 phosphorylation, RTN prevents its nuclear translocation and the activation of downstream pro-angiogenic genes, including VEGF and matrix metalloproteinases (MMPs). VEGF suppression limits the tumor's ability to promote new blood vessels, while reduced MMP activity hinders extracellular matrix degradation, a key step for endothelial cell migration and vessel sprouting. While other signaling pathways (such as PI3K/Akt/mTOR and MAPK) may also contribute, the STAT3-VEGF-MMP pathway appears to be the dominant mechanism. This multilevel interference not only accounts for RTN's effectiveness in *in vivo* systems but also aligns with its broader antitumor activities, including the inhibition of metastasis and modulation of the tumor microenvironment [10].

3.1.3. Limitations in rutin's bioavailability

RTN is a bioactive flavonoid with a broad pharmacological spectrum. As highlighted in section 3.1.2, it exhibits therapeutic potential across a wide range of pathological conditions and can be effectively employed in therapy, particularly in

combination with conventional drugs. In addition to its multiple pharmacodynamic effects, RTN shows an excellent safety profile, as supported by extensive toxicological evaluations in both acute and chronic settings. Preclinical studies conducted in rodents and other animal models have consistently demonstrated that RTN is well tolerated, even at high doses [11]. For instance, Wilson *et al.* (1947) reported that rats fed a diet containing 1% RTN for 400 days exhibited no signs of organ toxicity, histopathological alterations, or reproductive impairment [39]. More recently, Hamid and Sahib (2021) determined intraperitoneal LD50 values of 1.51 g/kg in male and 1.49 g/kg in female Swiss albino mice, classifying RTN as “practically non-toxic”. Doses below 1.25 g/kg (females) and 0.625 g/kg (both sexes) caused no mortality or morbidity and did not produce significant changes in body weight, hematological parameters, or serum biomarkers of hepatic and renal function (AST, ALT, ALP, bilirubin, creatinine, urea). Histopathological analysis of major organs confirmed the absence of structural abnormalities, further supporting its favorable safety profile [40].

Despite its considerable therapeutic potential, the pharmaceutical application of RTN remains limited. This is primarily due to its poor solubility and permeability, which classify it as a Biopharmaceutics Classification System (BCS) Class IV compound, resulting in very low oral bioavailability [41]. A recent pharmacokinetic study by Domínguez Moré *et al.* (2024) conducted in New Zealand white rabbits demonstrated that the oral administration of pure RTN resulted in extremely low absolute bioavailability ($F = 0.006$; i.e., only 0.6% of the administered dose reached systemic circulation). This limited systemic exposure is attributed to several factors: poor solubility in aqueous media, active efflux from enterocytes, and extensive intestinal metabolism. In the gut, RTN is hydrolyzed to

its aglycone quercetin, then further conjugated to glucuronide and sulfate derivatives. Its relatively high molecular weight, low lipophilicity, and the bulky disaccharide moiety (rutinoside) hinder passive diffusion across biological membranes, reducing absorption across multiple administration routes, not just oral [42]. Further evidence from Carbonaro *et al.* (2005) confirmed minimal absorption in the rat small intestine. Additional barriers include enzymatic degradation by the gut microbiota and significant presystemic metabolism, which together reduce the fraction of bioactive compound reaching systemic circulation [43].

Given these physicochemical and biopharmaceutical limitations, there is a compelling need to develop formulation strategies capable of enhancing RTN solubility, permeability, and overall bioavailability. Over the years, several approaches have been explored. For instance, Mauludin *et al.* (2009) prepared lyophilized RTN nanocrystals, which could be completely redispersed in water. This formulation demonstrated complete dissolution within 30 minutes, significantly exceeding the performance of standard tablets, which typically release only about 55% of the active compound. The enhanced dissolution was attributed to the increased surface area and saturation solubility resulting from particle size reduction [44].

Another widely adopted strategy to address the issue of poor oral bioavailability of poorly soluble compounds like RTN involves the use of self-emulsifying drug delivery systems (SEDDSs). These systems are isotropic mixtures composed of the drug, oil or lipid components, surfactants, and co-surfactants that, upon contact with physiological fluids, spontaneously form emulsions or lipid droplets, typically ranging in size from approximately 50 to 100 nanometers [45]. In a study by Kumar and Bhopal (2012), a SEDDS was developed using edible oil, Tween 80, and a co-

surfactant in a 1:1:8 ratio. The resulting formulation generated nano-sized droplets (~30 nm) and achieved 93% drug release within 30 minutes. The system also showed low turbidity and high dispersibility, highlighting its potential to improve the solubility and bioavailability of RTN [46].

More recently, Babazadeh *et al.* (2016) developed food-grade NLCs as a delivery system for RTN aimed at functional food applications. These carriers, produced using safe and edible components, exhibited high stability during processing and storage and did not negatively affect the appearance of fortified beverages, making them suitable for incorporation into food products [47].

In parallel with these technological strategies, increasing efforts have focused on CD inclusion complexes.

3.1.4. Approaches to enhance rutin bioavailability: cyclodextrin inclusion complexes

Although the formulation strategies discussed above have shown promise in addressing the biopharmaceutical limitations of RTN, their clinical translation may still be hampered by challenges such as large-scale manufacturing, uncertainties regarding long-term physicochemical stability, and the stringent regulatory requirements often associated with innovative DDSs.

In this context, CD complexation has emerged as one of the most effective and reliable alternatives. As discussed in detail in Chapter 1, CDs are cyclic oligosaccharides with a truncated cone-shaped structure that allows them to form inclusion complexes with hydrophobic guest molecules. This encapsulation improves the apparent aqueous solubility of the drug by exposing a hydrophilic exterior to the dissolution medium, while simultaneously protecting the guest

molecule from chemical and enzymatic degradation in biological fluids. Applied to RTN, CD-based complexation offers several key advantages: it is simple, reproducible, and supported by a well-established safety profile already acknowledged by pharmacopeias and regulatory authorities [48]. By enhancing solubility and stability, this strategy directly addresses the main barrier to RTN absorption and results in higher systemic exposure. Consequently, CD inclusion complexes are considered one of the most practical and clinically relevant options for improving the oral bioavailability of RTN [49].

Extensive literature supports this approach, reporting the successful complexation of RTN with both natural and modified CDs, including β -CD, HP- β -CD, HP- γ -CD, and 6-O-maltosyl- β -CD (G2- β -CD). Phase solubility studies have revealed differences in complexation efficiency, with HP- β -CD demonstrating the highest stability constant, followed by β -CD, G2- β -CD, and HP- γ -CD. Spectroscopic analyses have further confirmed that both HP- β -CD and β -CD form particularly stable complexes with RTN, leading to marked improvements in its dissolution rate. Notably, HP- β -CD-based complexes showed the most significant enhancement compared to the pure compound or its physical mixtures. Complexation with HP- β -CD offers several additional benefits beyond solubility enhancement. It provides protection against degradation in the gastrointestinal environment, as demonstrated by reduced hydrolysis in alkaline media and in intestinal homogenates. These stabilizing effects contribute to greater chemical integrity post-ingestion. *In vivo* studies in beagle dogs confirmed that the HP- β -CD/RTN complex significantly increased oral bioavailability, as reflected by elevated plasma concentrations of homovanillic acid, a primary RTN metabolite.

This improved bioavailability is attributed to a combination of enhanced solubility, faster dissolution, and increased stability in gastrointestinal conditions [49].

Supporting these findings, Sri *et al.* developed and characterized inclusion complexes of RTN and its aglycone quercetin with both β -CD and HP- β -CD using kneading and co-evaporation techniques. Characterization in both solid and solution phases confirmed that HP- β -CD complexes provided superior dissolution profiles, outperforming physical mixtures and uncomplexed compounds [50].

Moreover, complexation also influences RTN's pharmacodynamic properties. A study by Nguyen *et al.* evaluated the effect of CD encapsulation on antioxidant activity. Complexes formed with HP- β -CD and HP- γ -CD displayed enhanced radical-scavenging ability in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Specifically, the IC_{50} value for RTN alone was 0.022 mg/mL, while for the RTN/HP- β -CD complex it decreased to 0.014 mg/mL, indicating increased antioxidant efficiency [51].

These findings confirm that CD inclusion complexes provide a promising platform to overcome the biopharmaceutical limitations of RTN, enabling more effective delivery in both pharmaceutical and nutraceutical applications [49]. Within this context, the use of modified CDs, including polymeric derivatives, can be explored as an innovative strategy to further enhance RTN's solubility, stability, and overall bioavailability.

3.2 Cyclodextrin-based polymers (CDPs)

To date, no universally accepted definition of cyclodextrin-based polymers (CDPs) has been established. CDPs are generally described as polymers or polymeric networks that incorporate CDs as key structural or functional components [52].

Based on their architecture, CDPs can be broadly classified into four main categories (Figure 3.4. [52]):

1. **Cyclodextrin-based Polyrotaxanes (CD-PRs):** These are linear polymers in which CD molecules are threaded along the polymer backbone, forming supramolecular assemblies. Bulky end groups are attached to both ends of the polymer chain to prevent the CDs from detaching, thus stabilizing the inclusion structure [53–55].
2. **Grafted Cyclodextrin Polymers (G-CDPs):** In these systems, multiple CD units are covalently grafted onto a polymer chain, allowing a high CD content and providing multiple sites for guest molecule inclusion [52,56].
3. **Cross-linked Cyclodextrin Polymers (C-CDPs):** In this type, CDs are interconnected through cross-linking agents, forming three-dimensional networks. These materials can generate various gel-like systems, among which only the water-soluble variants are suitable for drug delivery applications [52].
4. **Cyclodextrin-based Star Polymers (Star-CDPs):** These structures feature a central CD core from which several polymer arms radiate outward, giving rise to a characteristic star-shaped morphology [52,56].

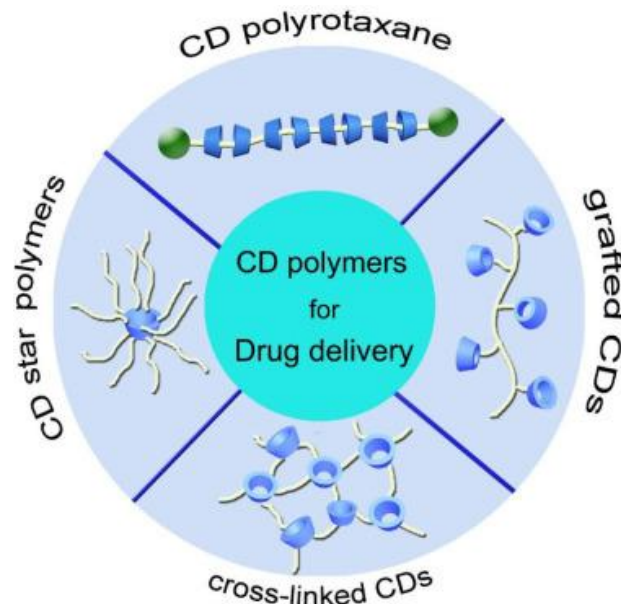


Figure 3.4. Schematic representation of the four main classes of cyclodextrin-based polymers (CDPs): polyrotaxanes (CD-PRs), grafted polymers (G-CDPs), cross-linked polymers (C-CDPs), and star-shaped polymers (star-CDPs).

3.2.1. Advantages of cyclodextrin-based polymers in drug delivery

One of the major challenges in pharmacotherapy is ensuring optimal bioavailability, particularly for APIs with low aqueous solubility. Poor solubility can significantly hinder drug absorption, resulting in subtherapeutic plasma concentrations and reduced clinical efficacy. Moreover, conventional dosage forms often produce a high plasma peak shortly after administration, followed by a rapid decline, necessitating frequent dosing and increasing the risk of side effects or suboptimal therapeutic windows.

To address these limitations, CDPs have emerged as a promising class of materials for advanced drug delivery. These systems combine the molecular inclusion capacity of native CDs with the structural versatility of polymers, leading to enhanced drug solubilization, protection, and controlled release [57].

Compared to native CDs, CDPs offer several advantages. One of the most significant is the tunability of their molecular architecture, which allows modulation of key properties such as particle size, porosity, surface functionality, and degradation rate. This customization facilitates greater drug EE, prolonged drug release, and even covalent conjugation of APIs for more stable formulations [52]. Due to their self-assembling behavior, CDPs can spontaneously form various nanoscale delivery systems (micelles, vesicles, nanosponges, and NPs) capable of enhancing drug solubility and improving bioavailability [58–60]. Additionally, CDPs can be engineered to exhibit biocompatibility, biodegradability, and responsiveness to physiological stimuli (e.g., pH, enzymes, or temperature), making them ideal for smart DDSs. This includes site-specific targeting and triggered release, essential features for maximizing therapeutic efficacy while minimizing systemic toxicity [52].

3.2.2. Therapeutic applications of cross-linked CDPs

Among the various CDP architectures, C-CDPs stand out as the most advantageous for pharmaceutical applications [61]. These polymers are synthesized through chemical cross-linking reactions involving the hydroxyl groups of the CD units, which act as reactive sites for condensation with suitable bifunctional or multifunctional cross-linking agents. Commonly used cross-linkers include epichlorohydrin, dianhydrides, and carbonyldiimidazole, which react with the primary and/or secondary hydroxyl groups of the CDs to form stable three-dimensional polymeric networks [60,62,63]. The degree of cross-linking, the nature of the linker, and the specific reaction conditions can be carefully modulated to adjust the physicochemical properties of the final material, such as porosity,

swelling capacity, mechanical strength, and drug release kinetics, depending on the intended pharmaceutical application [61].

Several advanced formulations exemplify the versatility and efficacy of C-CDPs. In the case of rosuvastatin, incorporation into carboxylate nanosponges synthesized from β -CD and pyromellitic dianhydride resulted in a 5.67-fold increase in oral bioavailability compared to the marketed tablet (Crestor®). This remarkable improvement was attributed to the high specific surface area, enhanced porosity of the nanosponges, and their ability to maintain elevated plasma drug concentrations over an extended period [64].

For sorafenib, a multikinase inhibitor used in the treatment of advanced hepatocellular carcinoma and other solid tumors, inclusion into epichlorohydrin-cross-linked β -CD polymers enabled both enhanced aqueous solubility and reduced systemic toxicity. In particular pulmonary toxicity, a known limitation of sorafenib, was significantly reduced, while its antitumor efficacy remained unchanged [65]. This demonstrates how C-CDPs can not only improve pharmacokinetic properties but also act as protective carriers to limit off-target effects and enhance therapeutic selectivity.

Applications in ophthalmology have also demonstrated the versatility of C-CDPs. Cyclosporine, an immunosuppressive agent commonly used in the treatment of dry eye syndrome and uveitis, was successfully incorporated into transparent ocular inserts composed of HA and HP- β -CD, cross-linked with poly(ethylene glycol) diglycidyl ether (PEGDGE). These inserts, designed for placement in the conjunctival sac, enabled sustained drug release for several hours and enhanced scleral accumulation. By adjusting the HA/HP- β -CD ratio, researchers were able

to modulate the mechanical properties and drug release behavior, achieving good biocompatibility and therapeutic drug levels in ocular tissues [66].

These results highlight the versatility of C-CDPs as advanced DDSs, capable of addressing critical formulation challenges and improving the therapeutic performance of a wide range of APIs.

3.2.3. PEGylated cyclodextrin polymers

Among the various cross-linking agents investigated for the development of CD polymers in pharmaceutical applications, PEG has emerged as one of the most prominent. The incorporation of PEG into CD-based systems gives rise to PEGylated CD polymers, which combine the well-known host-guest inclusion capabilities of CDs with the stealth, hydrophilic, and solubilizing properties of PEG [67]. These materials are especially significant in the context of this study, as they constitute the platform employed for RTN complexation and the investigation of its antiangiogenic potential.

PEG is widely employed in nanomedicine, particularly for the surface modification of nanoparticulate DDSs such as PLGA-based NPs. PEGylation leads to the formation of a hydrophilic corona around the nanocarrier, effectively reducing opsonization and recognition by the MPS. This “stealth” effect extends systemic circulation times and improves the pharmacokinetic profile of the delivery vehicle [68,69]. These features make PEGylated CD copolymers highly suitable for the delivery of poorly water-soluble or biologically labile compounds with short half-lives, including a range of flavonoids, anticancer agents, and peptides [70].

Numerous studies have described the synthesis and characterization of β -CD polymers cross-linked with PEG in diverse architectures. The degree of PEG

substitution, molecular weight, and the type of chemical linkage to the CD scaffold can be precisely tuned, offering a broad design space for developing customized DDSs [67].

One notable example is the work by Salmaso *et al.* (2007), who engineered β -CD/PEG-based hydrogels as versatile platforms for the delivery of both hydrophilic and hydrophobic APIs. These hydrogels were synthesized via cross-linking hexamethylene isocyanate-activated β -CD with diamino-PEG in anhydrous DMSO. Acetic acid was used to modulate gelation kinetics and ensure macroscopic homogeneity. By varying the β -CD/PEG molar ratio (from 0.25:1 to 2:1), the authors were able to tailor the mechanical and swelling properties of the hydrogels. Higher β -CD content significantly increased the rigidity and reduced swelling, while lower ratios enhanced hydrophilicity, resulting in water uptake of up to 800%. These hydrogels demonstrated notable versatility in drug loading. The hydrophilic model protein lysozyme preferentially loaded into low-CD/PEG matrices (0.25:1), due to their higher porosity, with loading efficiency rising from 6% to 20% as PEG content increased. In contrast, the hydrophobic compound β -estradiol achieved exceptional loading in high-CD hydrogels (2:1), primarily through inclusion complexation with CD cavities. Quinine, a moderately hydrophilic molecule with intermediate CD affinity, showed behavior between the two extremes, with loading reaching 290% in CD-rich matrices due to increased interaction with hexamethylene-modified CDs. Drug release studies further underscored the tunability of the CD/PEG hydrogel system. Lysozyme, being hydrophilic and relatively small, was rapidly released from all formulations within 10 hours, consistent with its limited interaction with the CD network. In contrast, both β -estradiol and quinine displayed a biphasic release profile, characterized by

an initial burst phase followed by a slower release. This behavior was closely linked to the affinity of each compound for the β -CD cavities. In particular, hydrogels with a high β -CD content substantially delayed the release of β -estradiol, retaining over 45% of the loaded drug beyond 30 hours. This prolonged retention was attributed to strong inclusion complexation, demonstrating how the β -CD content can be strategically modulated to achieve controlled and sustained drug delivery [71].

Another fascinating example of PEGylated CD polymers was reported by Rojas-Aguirre *et al.* (2019), who synthesized star-shaped β -CD derivatives conjugated with PEG chains of varying molecular weights (5,000, 2,000, and 550 Da) using copper-catalyzed azide-alkyne cycloaddition. The synthesis involved functionalizing the primary face of β -CD with azide groups and modifying the ends of PEG chains with alkyne groups through Steglich esterification. The resulting conjugation yielded β -CD-PEG₅₀₀₀, β -CD-PEG₂₀₀₀, and β -CD-PEG₅₅₀ derivatives in high yields, demonstrating the versatility and efficiency of this synthetic approach. MALDI-TOF spectrometry confirmed predominantly heptasubstituted products for β -CD-PEG₅₀₀₀ and β -CD-PEG₂₀₀₀, although minor hexasubstituted species were detected for the bulkier PEG₅₀₀₀ due to steric hindrance. β -CD-PEG₅₅₀, with a shorter PEG chain, exhibited exclusive heptasubstitution. Biocompatibility studies revealed PEG-length dependent effects. While all derivatives were non-toxic to Vero cells (a renal epithelial model), β -CD-PEG₅₀₀₀ significantly decreased viability in HeLa cells (by 76%) and human peripheral blood monocytes (by 61%) at 500 μ g/mL. In contrast, native β -CD and free PEG₅₀₀₀ showed no toxicity, suggesting that the star-shaped polymer architecture, rather than its individual components, was responsible for cytotoxicity. The dense PEG₅₀₀₀ corona may alter interactions at the cell membrane or activate stress pathways in sensitive cell types.

Conversely, β -CD-PEG₂₀₀₀ and β -CD-PEG₅₅₀ showed no significant toxicity, suggesting that moderate PEG chain lengths mitigate adverse biological effects [72].

More recently, Rincón-López *et al.* (2022) developed a star-shaped polymer named β -CD-PEG5, consisting of seven 5 kDa PEG chains covalently linked to β -CD via triazole groups on the primary face. This architecture enabled amphiphilic self-assembly in aqueous media without the need for additional hydrophobic segments. The critical aggregation concentration (CAC) was remarkably low (0.5 mM), even lower than that of sodium dodecyl sulfate (2.73 mM). Dynamic light scattering (DLS) and transmission electron microscopy (TEM) analyses revealed stable quasi-spherical nanoaggregates (~150 nm) and smaller dimers (~9 nm), the latter acting as precursors in the aggregation process. Molecular simulations confirmed these observations, showing dehydration within the β -CD cavity and the formation of spherical aggregates. Aggregation was shown to be highly responsive to environmental cues. Under acidic conditions (pH 3.8), protonation of triazole linkers promoted electrostatic repulsion, increasing dimer formation (up to 45%). In basic media (pH 8.0), nanoaggregates dominated (96.4%). Increasing ionic strength (0.9-7.5% NaCl) promoted nanoaggregation through PEG dehydration, decreasing particle size by 11-17%. At physiological temperature (37°C), dimers transitioned into nanoaggregates (~152 nm), which remained stable for at least 55 days, with only minimal size reduction (27.5%). The ability of β -CD-PEG5 to aggregate in two distinct ways and respond to stimuli offers enhanced potential for drug delivery applications. Below the CAC, hydrophobic drugs can form stable inclusion complexes within the β -CD cavity. Above the CAC, drugs can be held in two ways: through interaction with β -CD and by being trapped in the PEG corona.

The triazole linkers also function as pH-responsive moieties, while the dense PEG layer confers stealth and colloidal stability. These properties position β -CD-PEG5 as a promising platform for the delivery of a wide range of APIs, with potential for controlled and stimuli-responsive release and enhanced pharmacokinetics [73].

3.3. Aim of the research

The extensive literature reviewed above highlights the significant therapeutic potential of RTN, particularly its antiangiogenic activity, which could be valuable in the context of cancer-related diseases. However, its clinical application is severely limited by poor aqueous solubility, low permeability, and consequently low bioavailability. While CD complexation is a well-established strategy to enhance the solubility of hydrophobic drugs, the use of advanced, functionalized CD-based polymers represents a more sophisticated and tunable approach.

This study was therefore designed to develop and characterize a novel inclusion complex between RTN and a PEG-crosslinked β -CD polymer (β -CPCD). The main goal was to overcome the intrinsic biopharmaceutical limitations of RTN and obtain a supramolecular system capable of enhancing its solubility, stability, and *in vivo* bioavailability, thereby potentiating its antiangiogenic efficacy.

The originality of this work lies in three aspects. First, it employs an innovative carrier, β -CPCD, which synergistically combines the inclusion capacity of CDs with the solubilizing and stealth properties of PEG in a single macromolecular structure. Second, it uses a comprehensive multi-method approach, including in-solution studies, solid-state analyses, computational modeling, and detailed NMR spectroscopy, to provide a deep mechanistic understanding of the host-guest interactions. Finally, the study translates these findings into a relevant *in vivo*

model, evaluating both the efficacy and safety of the complex in zebrafish embryos, providing a preclinical assessment of its antiangiogenic potential and improved toxicological profile compared to free RTN.

By integrating material design, thorough physicochemical characterization, and biological validation, this research establishes a robust foundation for the development of a promising RTN delivery system with potential applications in antiangiogenic therapy.

3.4. Materials and methods

3.4.1. Materials

Rutin (RTN; formula: $C_{27}H_{30}O_{16}$; MW: 610.53 g/mol) was purchased from Sigma-Aldrich Chemie® (St. Louis, MO, USA). PEG-crosslinked β -cyclodextrin polymer (β -CPCD; average MW: 2500 Da) was kindly provided by the Centre for Defence Engineering and Physical Sciences, Cranfield University (Shrivenham, UK). Methanol (MeOH; HPLC grade) was supplied by Merck® (Darmstadt, Germany). The water used in this study was purified by double distillation, followed by deionization and final filtration through 0.22 μ m Millipore® GSWP filters (Bedford, MA, USA). All other chemicals used in this work that are not listed above were of analytical grade.

3.4.2. Synthesis of PEG-crosslinked β -cyclodextrin polymer

The PEG-crosslinked β -cyclodextrin polymer (β -CPCD; average MW: 2500 Da) was synthesized following the procedure described by Luppi *et al.* [74]. The polymer is water-soluble, and its spectral and thermal properties are in agreement

with those reported in the original study, confirming successful crosslinking and the integrity of the β -CD and PEGDGE units.

3.4.3. Preparation of RTN/ β -CPCD inclusion complex and physical mixture

The RTN/ β -CPCD inclusion complex was prepared *via* freeze-drying. Briefly, 6 mg of RTN was dissolved in 1 mL of MeOH to form the organic phase, while β -CPCD was dissolved in 9 mL of ultrapure water to obtain a 0.5% (m/v) solution and stirred until fully solubilized. The RTN solution was added dropwise to the aqueous polymer solution under continuous magnetic stirring at 500 rpm for 5 hours to promote complex formation. The resulting hydroalcoholic mixture was frozen and lyophilized for 72. The obtained powder was stored in a desiccator under anhydrous conditions until further characterization.

For comparative purposes, a physical mixture of RTN and β -CPCD was prepared at the same w/w ratio as that employed for the inclusion complex. The components were accurately weighed and then coarsely blended in a mortar using a pestle until a homogeneous powder blend was obtained.

3.4.4. Determination of aqueous equilibrium solubility of the RTN/ β -CPCD inclusion complex

The aqueous solubility of the lyophilized RTN/ β -CPCD inclusion complex was evaluated by dispersing an excess amount of the lyophilized complex in 10 mL of water. The suspension was maintained under continuous magnetic stirring at 25.0 ± 0.5 °C in the absence of light. At predetermined time points, 1 mL aliquots were collected and filtered through 0.22 μ m nylon syringe filters to remove undissolved solids. The concentration of dissolved RTN was then determined by UV-Vis

spectroscopy at its characteristic absorption wavelength ($\lambda_{\text{max}} = 355 \text{ nm}$). For quantitative analysis, a calibration curve of RTN was prepared in an 80:20 (v/v) MeOH/water mixture over the concentration range of 0.001-0.05 mg/mL, resulting in a correlation coefficient (R^2) of 0.9994. Solubility equilibrium was considered reached when no significant change in concentration was observed between two consecutive measurements (24 hours). After each sampling, the withdrawn volume was immediately replaced with an equal volume of fresh solvent, and all measured concentrations were corrected for cumulative dilution. The assay was carried out in triplicate.

3.4.5. Dissolution profile of the RTN/ β -CPCD inclusion complex

Dissolution studies were conducted following the protocol described in the 44th edition of the United States Pharmacopeia (USP). In brief, free RTN (35 mg), or an equivalent amount provided either as inclusion complex or as physical mixture, was added to 900 mL of water. The dissolution medium was maintained at $37.0 \pm 0.5 \text{ }^\circ\text{C}$ in a thermostatically controlled water bath equipped with a temperature regulation unit, in the absence of light, under constant slow magnetic stirring (100 rpm). At predetermined time points, 1 mL samples were withdrawn, filtered, appropriately diluted, and analyzed by UV-Vis spectroscopy at the characteristic absorption wavelength of RTN to determine the dissolved fraction. After each sampling, the withdrawn volume was immediately replaced with an equal volume of fresh solvent, and all measured concentrations were corrected for cumulative dilution. The assay was carried out in triplicate.

3.4.6. Phase-solubility studies

Solid RTN was added in excess to aqueous solutions of β -CPCD prepared at concentrations ranging from 0 to 10% w/v, in amounts above its solubility limit (see Section 3.1.1). The resulting suspensions were stirred in the dark at 25.0 ± 0.5 °C and then filtered. The filtrates were appropriately diluted with MeOH, and the concentration of solubilized RTN was determined by UV-Vis spectrophotometry. To establish the equilibrium time, a series of experiments were conducted and terminated at different intervals. Phase-solubility diagrams were then constructed, and the slopes were evaluated. Consistent slope values indicated that equilibrium was reached after 24 hours. All experiments were performed in triplicate.

3.4.7. UV-Vis titration studies

A stock solution of free RTN (0.125 mg, 0.04 mM) was prepared by dissolving the compound in MeOH. Separately, increasing concentrations of β -CPCD (0-1% m/v) were prepared in water. Each β -CPCD solution was then combined with aliquots of the RTN stock solution to obtain samples with a final water/MeOH ratio of 80:20 (v/v) and a total volume of 5 mL. All mixtures were stirred magnetically at 500 rpm in the dark for 5 hours to ensure interaction between RTN and the carrier. After the incubation period, the samples were analyzed by UV-Vis spectroscopy to monitor changes in the absorption spectra of RTN as a function of β -CPCD concentration.

3.4.8. Dynamic light scattering (DLS) analysis

The aggregation behavior of free β -CPCD and the RTN/ β -CPCD inclusion complex in aqueous solution was investigated by DLS. Samples were prepared in

ultrapure water and equilibrated under magnetic stirring (500 rpm) prior to analysis. D_H , PDI, and ζ were measured according to the same experimental protocol described in Section 2.3.10, at 25 ± 0.5 °C.

3.4.9. Thermogravimetric analysis (TGA)

Thermal decomposition profiles of free RTN, β -CPCD, the RTN/ β -CPCD inclusion complex, and the corresponding physical mixture were assessed by TGA following the procedure described in Section 2.3.13. All measurements were performed in triplicate.

3.4.10. Proton nuclear magnetic resonance (^1H NMR) characterization

Solutions of RTN and the RTN/ β -CPCD inclusion complex, both prepared at a final concentration of 15 mM, were dissolved in a D_2O/CD_3OD mixture (80:20, v/v) and pipetted into 5-mm NMR tubes. Deuterated solvents were purchased from Merck. Proton nuclear magnetic resonance (^1H NMR) spectra were acquired at 300 K on a Varian 500 MHz spectrometer equipped with a ONE_NMR probe and operating at 499.74 MHz, using 64 scans. The residual D_2O signal at 4.79 ppm served as an internal reference.

3.4.11. μ -Raman spectroscopic characterization

μ -Raman spectra of RTN, β -CPCD, their physical mixture, and the solid-state RTN/ β -CPCD inclusion complex were recorded using a portable spectrometer (BTR 111 Mini-Ram™, B&W Tek, Newark, NJ, USA) equipped with a 785 nm diode laser and a thermoelectrically cooled CCD detector, delivering up to 280 mW of laser power at the excitation port. Spectra were acquired in the 500-1800 cm^{-1}

range (resolution: 8 cm^{-1} , $40\text{ s} \times 16\text{ scans}$). Instrument calibration was performed with the silicon band at 520.6 cm^{-1} . A BAC151B Raman microscope with an $80\times$ objective (spot size $\sim 25\text{ }\mu\text{m}$, working distance $\sim 1.25\text{ mm}$) was used, with an effective laser power of $\sim 40\text{ mW}$ at the sample.

3.4.12. Molecular docking studies

Docking studies examining the interaction of RTN with the monomeric unit of β -CPCD (β -CPCDm) were executed using the *Maestro* software package (Schrödinger Inc.). The three-dimensional structure of RTN (PubChem CID: 5280805) was retrieved and prepared for docking using the *LigPrep* module. Preparation included the application of the OPLS4 force field and the use of *Epik* to generate possible ionization states at a physiological pH of 7.4. The receptor model for β -CPCDm was constructed based on the native structure of β -CD (PubChem CID: 444041). Using the *Maestro Builder* tool, a PEG chain was introduced *via* an ether linkage to one of the primary hydroxyl groups of a glucose unit. The resulting β -CPCDm structure was subsequently energy-minimized using the Polak-Ribiere conjugate gradient (PRCG) algorithm (5000 steps) within the *MacroModel's Macrocycle Conformational Sampling* (MCS) tool. For the docking calculations, a cubic grid with side dimensions of 120 Å was centered on the β -CPCDm molecule to encompass its entire volume as the potential binding site. Docking was executed using the *Glide XP* (extra precision) flexible docking protocol at 300 K and a pH of 7.0 ± 2.0 . The generated poses were ranked according to their Glide XP interaction energy score. The top-ranking poses for the RTN/ β -CPCDm complex were further evaluated using the *Molecular Mechanics*

Generalized Born Surface Area (MM-GBSA) method to calculate the binding free energy (ΔG_{Bind}).

3.4.13. Biological activity

3.4.13.1. In vivo assessment of antiangiogenic activity in zebrafish embryos: culture and treatment protocol

Adult zebrafish of both sexes were maintained in a continuous flow-through aquarium system under standard laboratory conditions [75]. The environment was regulated at 28.5 ± 0.5 °C with a 14/10-hour light/dark photoperiod. Fish were fed a diet of live brine shrimp (*Artemia salina*) three times daily. Embryos were obtained via natural spawning, collected, and examined under a stereomicroscope. Only fertilized eggs with normal morphology were selected for experimentation. Chorions were carefully removed using fine forceps, and dechorionated embryos were individually transferred into 96-well microplates containing 0.2 mL of embryo medium per well. Treatments were applied from 24 to 72 hours post-fertilization (hpf), with embryos exposed to varying concentrations of free RTN, β -CPCD, or the RTN/ β -CPCD complex. Free RTN was solubilized in 0.2% (v/v) DMSO. 2-Methoxyestradiol (2 μ M), a compound with well-documented antiangiogenic properties, was employed as a positive control. All experimental procedures were rigorously conducted in accordance with the ethical principles of the European Directive 2010/63/EU and the guidelines set forth in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Results are presented as the mean $\pm$ SD, and statistical significance was determined using Student's *t*-test.

3.4.13.2. Assessment of toxicity: zebrafish embryo viability

The safety profile of β -CPCD was assessed by monitoring zebrafish embryo viability over a 96 hpf, a timeframe widely recognized for early toxicity studies that do not require further ethical authorization according to EU guidelines. Embryos were exposed to β -CPCD at concentrations ranging from 1 to 10 mg/mL. Survival was recorded daily via microscopic examination. Each experimental condition was performed in triplicate, using at least 20 embryos per concentration. The primary endpoint was the survival rate (%), calculated at 96 hpf as:

$$\% \text{ Survival} = \frac{\text{Number of live embryos}}{\text{Total number of embryos}} \times 100$$

A negative control group exposed to 0.2% DMSO alone was included to account for any solvent effects. Results are expressed as mean $\pm$ SD.

3.4.13.3. Assessment of anti-angiogenic effects via endogenous alkaline phosphatase quantification

The quantitative assessment of endogenous alkaline phosphatase (EAP) activity, a well-established marker for vascular development, was conducted in accordance with the protocol established by Iannuzzi *et al.* [76]. Briefly, at 72 hpf, embryos were dehydrated through a graded MeOH series and rinsed three times with 1 M diethanolamine buffer (pH 9.8). Embryos were then incubated with 0.5 mg/mL *p*-nitrophenyl phosphate disodium salt for 30 minutes at room temperature. The enzymatic reaction was stopped by adding 2 M NaOH, and the OD of the resulting yellow product, proportional to EAP activity, was measured at 405 nm using a Multiskan GO microplate reader (Thermo Scientific). Vascular growth was

quantified relative to the control, and the percentage of inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{OD_{\text{control}} - OD_{\text{treated}}}{OD_{\text{control}}} \times 100$$

Where OD_{control} represents the mean OD of untreated embryos (normal vascular development), and OD_{treated} represents the OD of embryos exposed to the test compounds (free RTN or RTN/ β -CPCD complex).

3.4.14. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data from triplicate experiments are expressed as mean $\pm$ SD. Group differences were evaluated by one- or two-way ANOVA, followed by Bonferroni or Dunnett post-hoc tests. A p-value < 0.05 was considered statistically significant.

3.5. Results and Discussion

RTN is a naturally occurring flavonoid with promising therapeutic properties. However, its clinical application is significantly limited by its poor water solubility and low membrane permeability, which result in poor oral bioavailability. To overcome these drawbacks, RTN was complexed with a PEG-crosslinked β -CD polymer, aiming to enhance its solubility and promote its biological activity. The RTN/ β -CPCD inclusion complex was prepared by freeze-drying a hydroalcoholic solution containing the two components. A small amount of MeOH (10% v/v) was necessary to increase RTN solubility and facilitate its encapsulation within the polymeric CD network.

The lyophilized product showed a striking increase in aqueous solubility, which can be attributed to the formation of a stable inclusion complex, improved wettability, and a probable reduction in crystallinity caused by freeze-drying. To gain a deeper mechanistic insight into the host-guest interactions, the complex was extensively characterized in both solution and solid states.

3.5.1. In-solution studies of RTN/ β -CPCD inclusion complex

Inclusion of RTN into β -CPCD led to a dramatic increase in the bioflavonoid's water solubility, from 0.125 to 6.6 mg/mL, representing an approximately 50-fold enhancement compared to its intrinsic solubility. This enhanced solubility resulted in a significantly accelerated dissolution rate. As evidenced in Figure 3.5., dissolution profiling demonstrated that 45.6% (w/w) of the RTN/ β -CPCD complex dissolved within the initial 5 minutes, reaching complete dissolution within 60 minutes. The rapid dissolution behavior of the RTN/ β -CPCD system can be attributed to the combined contribution of multiple physicochemical factors. In addition to the intrinsic increase in aqueous solubility provided by inclusion complexation, the lyophilization process yielded a solid characterized by low crystallinity and a highly porous, fluffy morphology, which favored rapid interaction with the dissolution medium. Moreover, the pronounced hydrophilic character of the polymeric CD enhanced water penetration and wetting of the solid surface, further promoting dissolution. In contrast, dissolution of both uncomplexed RTN and the physical RTN/ β -CPCD mixture proceeded significantly slower. After 60 minutes, dissolution efficiencies reached only 10.0% (w/w) for uncomplexed RTN and 23.7% (w/w) for the physical mixture. These results

demonstrate that physical blending with β -CPCD is insufficient to confer the solubility enhancement achieved through true molecular inclusion complexation.

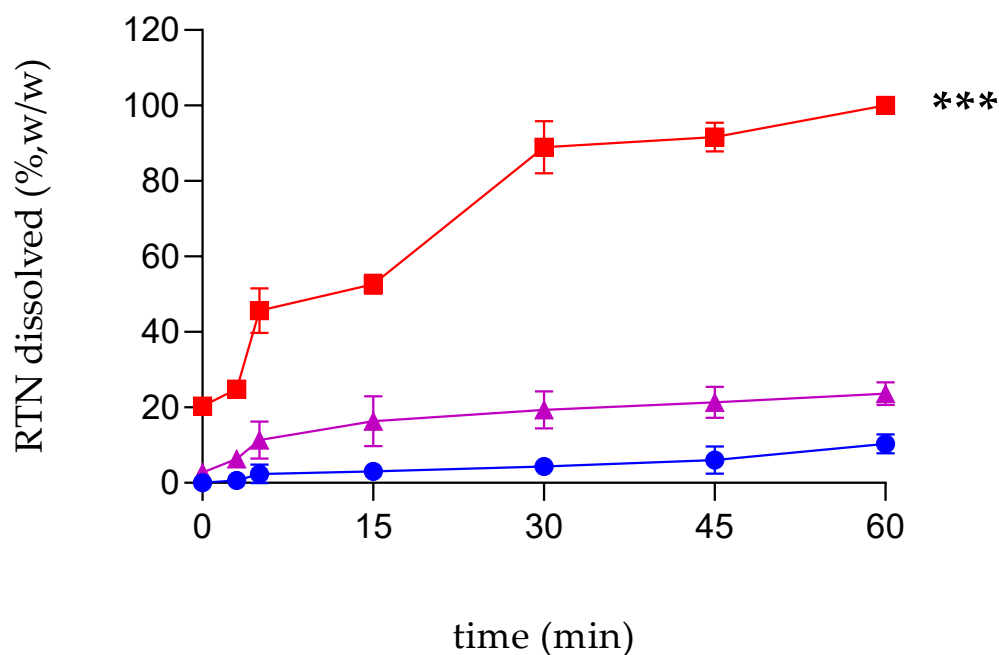


Figure 3.5. Dissolution profiles of free RTN (blue circles), physical mixture (purple triangles), and RTN/ β -CPCD inclusion complex (red squares) in water at 25 ± 0.5 °C. Data are presented as the mean $\pm$ SD of three independent experiments. If error bars are not visible, they fall within the data symbols. All dissolution data for the inclusion complex were statistically significant compared to free RTN (** $p < 0.001$).

The improvement in dissolution performance observed for the RTN/ β -CPCD complex is consistent with previously reported studies on RTN inclusion complexes with native CDs. For instance, Sri *et al.* (2007) observed that the kneaded inclusion complex of RTN with HP- β -CD in a 1:2 molar ratio exhibited a dissolution efficiency at 30 minutes (DE_{30}) of $86.80\% \pm 0.260\%$. DE_{30} is a quantitative parameter that evaluates both the extent and the rate of drug dissolution

within a specified timeframe (in this case, 30 minutes). It is determined by calculating the area under the dissolution curve from time zero to 30 minutes, then expressing that area as a percentage of the maximum possible value (i.e., the area of a rectangle representing 100% dissolution over the same interval). In the same study, more than 90% of the bioflavonoid was released within the first 10 minutes, compared to $15.43\% \pm 0.30\%$ for pure RTN. By comparison, the RTN/ β -CD inclusion complex showed a lower DE₃₀ value of $69.43\% \pm 0.28\%$. It should be noted that these dissolution studies were conducted using a USP paddle apparatus in 900 mL of distilled water containing 0.75% sodium lauryl sulfate at 37°C and 50 rpm, conditions that favor dissolution due to the presence of the surfactant [50]. In contrast, the present study utilized pure water at a controlled temperature of 25 ± 0.5 °C, entirely without surfactants or other additives that could artificially boost solubility. Due to these substantial differences in methodology, the dissolution profiles and DE₃₀ values obtained are not directly comparable in a strict quantitative sense. Nevertheless, the fact that the RTN/ β -CPCD inclusion complex achieved a DE₃₀ of approximately 70% under these more challenging, surfactant-free conditions represents a particularly impressive outcome and highlights the exceptional solubilizing efficacy of the polymeric CD.

Similarly, Sharma *et al.* (2013) in their review emphasized that complexation with CDs, particularly HP- β -CD, significantly enhances the dissolution rates of RTN over the pure compound and physical mixtures, attributing this behavior to improved solubility, faster dissolution, and greater gastrointestinal stability, thereby positioning RTN as a promising candidate for treating chronic diseases [49].

To gain deeper insight into the host-guest interaction mechanism, a phase-solubility study was conducted (Figure 3.6.). The resulting diagram, plotting the concentration of dissolved RTN against the concentration of β -CPCD (0-10%, m/v), the solubility of RTN increased linearly with the concentration of β -CPCD, culminating in a solubility of 2.3 mg/mL at the maximum polymer concentration. This represents a dramatic increase over the intrinsic solubility of RTN in water. This pronounced solubilizing effect is likely due to the polymer's high hydrophilicity and its three-dimensional network. This structure facilitates the formation of a supramolecular system in which RTN is partially encapsulated within the β -CD cavities and, more predominantly, engages in physical interactions with the hydrated PEG chains, resulting in a non-inclusion complex [77].

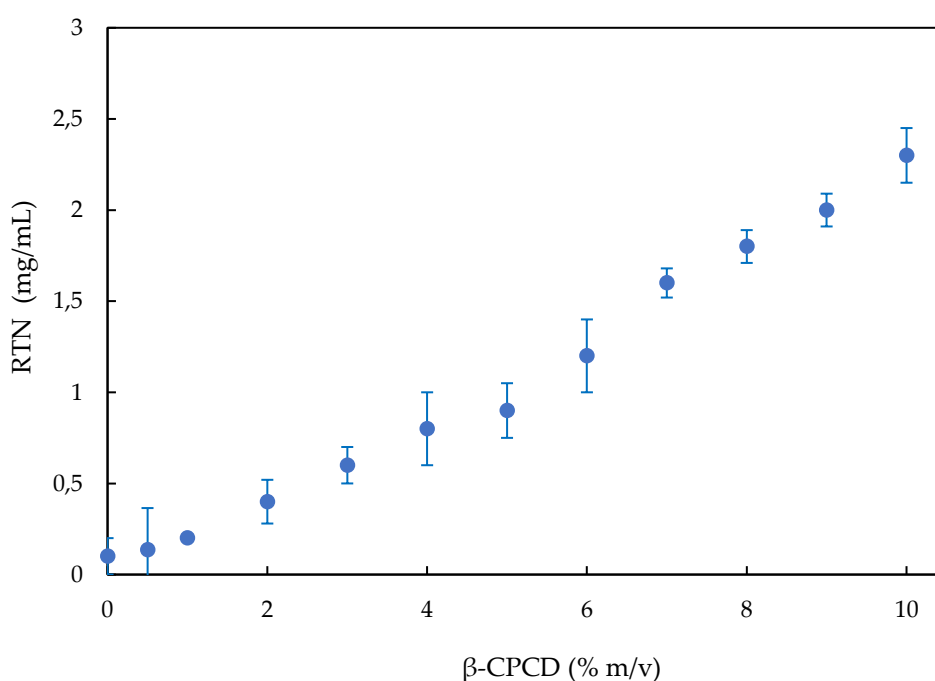


Figure 3.6. Phase-solubility diagram of RTN as a function of increasing β -CPCD concentration in water at 25.0 ± 0.5 °C. Experiments were performed in triplicate $\pm$ SD. If error bars are not visible, they fall within the data symbols.

To unambiguously confirm the formation of the inclusion complex, a UV-Vis titration study was performed in a hydroalcoholic solution (water/MeOH, 80:20 v/v). The concentration of RTN was maintained at 0.04 mM, while the concentration of β -CD polymer was gradually increased.

Figure 3.7. shows the spectra of RTN alone and in the presence of the CD polymer. RTN exhibits two intense absorption bands: one at 256 nm and another at 351 nm. Specifically, the band at 256 nm corresponds to the $\pi \rightarrow \pi^*$ electronic rearrangement of the phenyl group, while the band at 351 nm is associated with the benzene ring of the pyrocatechol unit [78]. A weaker band around 300 nm, attributed to the heterocyclic C-ring, is also present [79]. Upon incremental addition of β -CPCD, which does not exhibit intrinsic absorption in this spectral region, a pronounced increase in the intensity of all absorption bands (hyperchromic effect) was observed. These spectral changes indicate a modification of the microenvironment surrounding RTN's chromophores.

The observed effects are consistent with partial inclusion of the drug molecule into the apolar cavities of the β -CD units, as supported by NMR data. This inclusion complex, stabilized by hydrophobic and van der Waals interactions, alters both the electron density and the solvation shell of the chromophoric groups [78,80,81]. Simultaneously, the overall increase in signal intensity suggests additional physical interactions between RTN and the hydrated PEG chains of the polymer network [82,83].

Collectively, the UV-Vis titration data confirm a multifaceted host-guest interaction, involving both encapsulation of RTN within β -CD cavities and its physical entrapment within the hydrophilic polymer matrix. This strong affinity provides a clear explanation for the substantial improvement in RTN's solubility.

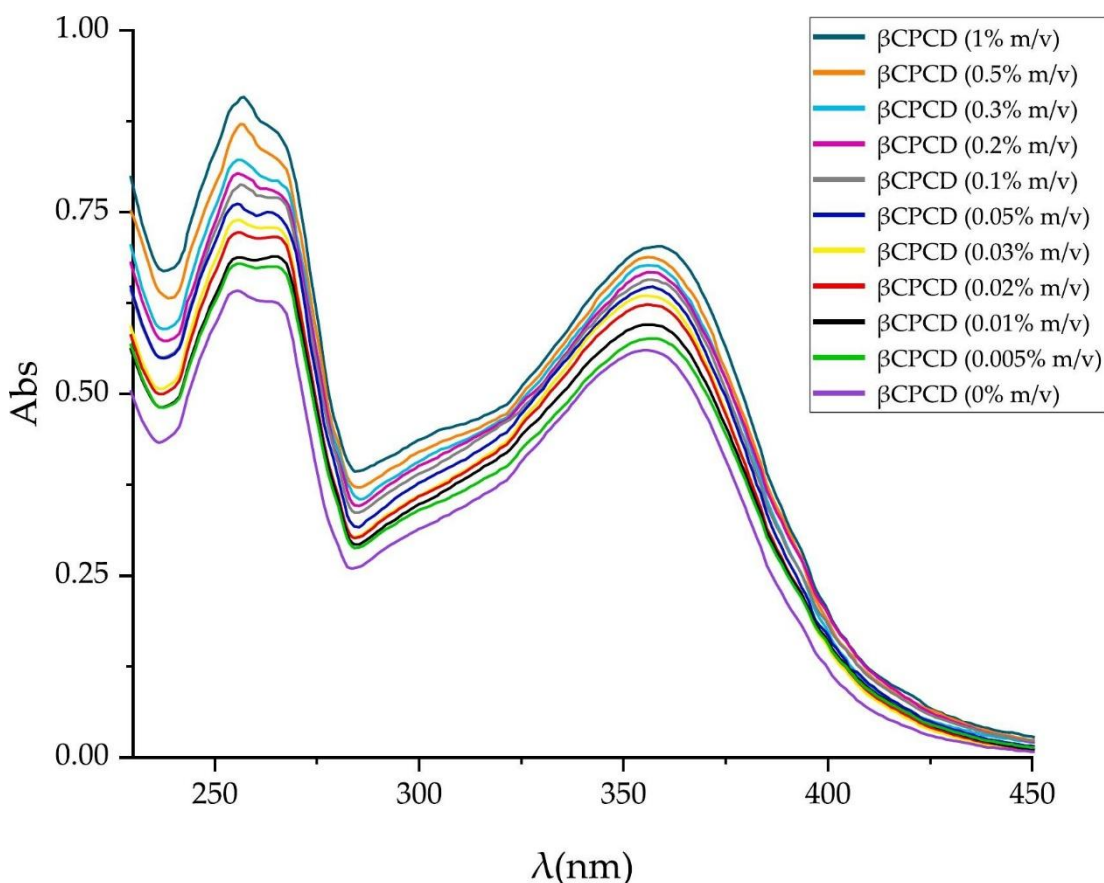


Figure 3.7. UV-Vis absorption spectra of RTN in the absence and presence of increasing concentrations of β -CPCD in a 80:20 (v/v) water/MeOH mixture at 25.0 ± 0.5 °C. All measurements were performed in triplicate.

3.5.2. Self-assembly behavior and aggregation properties of β -CPCD and RTN/ β -CPCD complexes in aqueous solution

As established in the literature, CDPs are capable of undergoing spontaneous self-assembly in aqueous environments, forming nanoscale aggregates through non-covalent interactions. This phenomenon holds significant technological relevance, particularly in drug delivery and pharmaceutical formulation. Nanoaggregates formed from CDPs not only improve the apparent solubility and stability of poorly water-soluble drugs but also enhance their transport across biological membranes. Compared to their monomeric CD counterparts, these polymeric aggregates are

more likely to interact with and transiently modulate tight junctions, facilitate passive diffusion, or exploit endocytic pathways, thereby promoting higher cellular uptake and bioavailability of the encapsulated compounds [84].

Therefore, elucidating the self-assembly behavior of β -CPCD and assessing the impact of RTN complexation on its aggregation dynamics is of critical importance.

To investigate these aspects, DLS and ζ measurements were carried out on aqueous solutions containing increasing concentrations of either free β -CPCD or its inclusion complex with RTN.

Table 3.1. Hydrodynamic size (D_H), polydispersity index (PDI), and zeta potential (ζ) of free β -CPCD nanoaggregates. Data are expressed as mean $\pm$ SD. Measurements below 1 mg/mL were not feasible due to the instrument's detection limits.

β-CPCD (mg/mL)	Peak 1 Size (nm) $\pm$ SD	Peak 2 Size (nm) $\pm$ SD	PDI $\pm$ SD	$\zeta \pm$ SD
1 mg/mL	15.72 nm $\pm$ 3.50	75.87 nm $\pm$ 7.13	0.29 $\pm$ 0.09	-12.27 $\pm$ 8.26
2 mg/mL	15.73 nm $\pm$ 2.00	76.42 nm $\pm$ 6.85	0.31 $\pm$ 0.05	-12.19 $\pm$ 9.94
3 mg/mL	12.21 nm $\pm$ 0.93	81.39 nm $\pm$ 9.05	0.33 $\pm$ 0.04	-11.72 $\pm$ 6.95
4 mg/mL	10.78 nm $\pm$ 6.15	83.67 nm $\pm$ 3.17	0.33 $\pm$ 0.04	-11.25 $\pm$ 6.62
5 mg/mL	11.39 nm $\pm$ 0.54	85.17 nm $\pm$ 8.78	0.34 $\pm$ 0.02	-11.17 $\pm$ 4.83
10 mg/mL	11.61 nm $\pm$ 0.76	85.71 nm $\pm$ 7.03	0.34 $\pm$ 0.06	-9.63 $\pm$ 4.96
15 mg/mL	15.04 nm $\pm$ 2.83	87.43 nm $\pm$ 2.99	0.44 $\pm$ 0.02	-9.56 $\pm$ 3.98
20 mg/mL	14.58 nm $\pm$ 3.60	94.46 nm $\pm$ 5.12	0.46 $\pm$ 0.01	-7.05 $\pm$ 4.93
25 mg/mL	13.80 nm $\pm$ 5.23	114.10 nm $\pm$ 22.23	0.47 $\pm$ 0.10	-6.87 $\pm$ 4.89

Table 3.2. Hydrodynamic size (D_H), polydispersity index (PDI), and zeta potential (ζ) of RTN/ β -CPCD inclusion complex nanoaggregates (molar ratio 1:2; concentration refers to β -CPCD). Data are expressed as mean $\pm$ SD. Measurements below 1 mg/mL were not feasible due to the instrument's detection limits.

RTN/ β-CPCD (mg/mL)	Peak 1 Size (nm) $\pm$ SD	Peak 2 Size (nm) $\pm$ SD	PDI $\pm$ SD	$\zeta \pm$ SD
1 mg/mL	12.51 nm $\pm$ 0.61	81.48 nm $\pm$ 4.42	0.31 $\pm$ 0.10	-10.56 $\pm$ 5.66
2 mg/mL	14.46 nm $\pm$ 2.71	81.97 nm $\pm$ 10.63	0.34 $\pm$ 0.05	-10.05 $\pm$ 5.52
3 mg/mL	14.73 nm $\pm$ 3.76	82.51 nm $\pm$ 7.45	0.37 $\pm$ 0.02	-9.83 $\pm$ 6.94
4 mg/mL	13.88 nm $\pm$ 3.54	82.68 nm $\pm$ 3.32	0.37 $\pm$ 0.02	-7.13 $\pm$ 6.34
5 mg/mL	11.35 nm $\pm$ 1.94	93.95 nm $\pm$ 4.58	0.39 $\pm$ 0.03	-5.84 $\pm$ 5.58
10 mg/mL	17.54 nm $\pm$ 3.79	99.93 nm $\pm$ 3.08	0.43 $\pm$ 0.004	-5.43 $\pm$ 4.66
15 mg/mL	18.12 nm $\pm$ 4.57	114.41 nm $\pm$ 5.81	0.46 $\pm$ 0.02	-5.21 $\pm$ 3.69
20 mg/mL	N.D.	N.D.	N.D.	-5.09 $\pm$ 3.38
25 mg/mL	N.D.	N.D.	N.D.	-4.13 $\pm$ 3.57

Notes: N.D. = not detectable.

The results summarized in Table 3.1. and 3.2. show a clear bimodal size distribution for free β -CPCD, with two populations consistently detected at all tested concentrations. The smaller population, stable between 10 and 16 nm irrespective of polymer concentration, most likely corresponds to unaggregated polymer chains. In contrast, the larger fraction, which grows from 75 nm to 114 nm as the polymer concentration increases, points to the formation of nanoaggregates that expand progressively with higher β -CPCD content. This behavior suggests that additional polymer chains progressively incorporate into existing aggregates, while the proportion of free polymer remains essentially constant. ζ values for the free β -CPCD solutions ranged from -12.27 mV to -6.87 mV, displaying a gradual decrease with increasing concentration. These relatively low absolute values, far from the

± 30 mV range typical of moderate colloidal stability, suggest limited electrostatic repulsion among particles, thereby favoring aggregation. A consistently high PDI was recorded for all β -CPCD solutions, reflecting the presence of the two distinct size populations observed in the bimodal distribution.

The inclusion of RTN within the β -CPCD cavity clearly affected the aggregation behavior. Compared to the free polymer at equivalent concentrations, the inclusion complex exhibited larger D_H (81-114 nm) and higher PDI, particularly at concentrations above 10 mg/mL, where reliable size determination was no longer obtainable. This suggests that guest encapsulation causes the aggregates to become less uniform and more structurally heterogeneous. Also, the ζ decreased from -10.56 mV to -4.13 mV, showing that RTN reduces the surface charge, although it remained negative at all concentrations.

These results demonstrate that both the concentration of β -CPCD and and RTN encapsulation significantly influence aggregate size and surface charge, underscoring the dynamic self-assembly of this system.

3.5.3. ^1H NMR characterization of RTN/ β -CPCD inclusion complex

The formation of the RTN/ β -CPCD inclusion complex was characterized by ^1H NMR spectroscopy. The investigation focused particularly on the aromatic protons of the RTN molecule, which constitute highly sensitive probes of changes in their local electronic environment upon complex formation. When the spectra of free RTN were compared with those of the RTN/ β -CPCD complex (Figure 3.8.), all signals ascribable to the flavonol moiety exhibited downfield shifts.

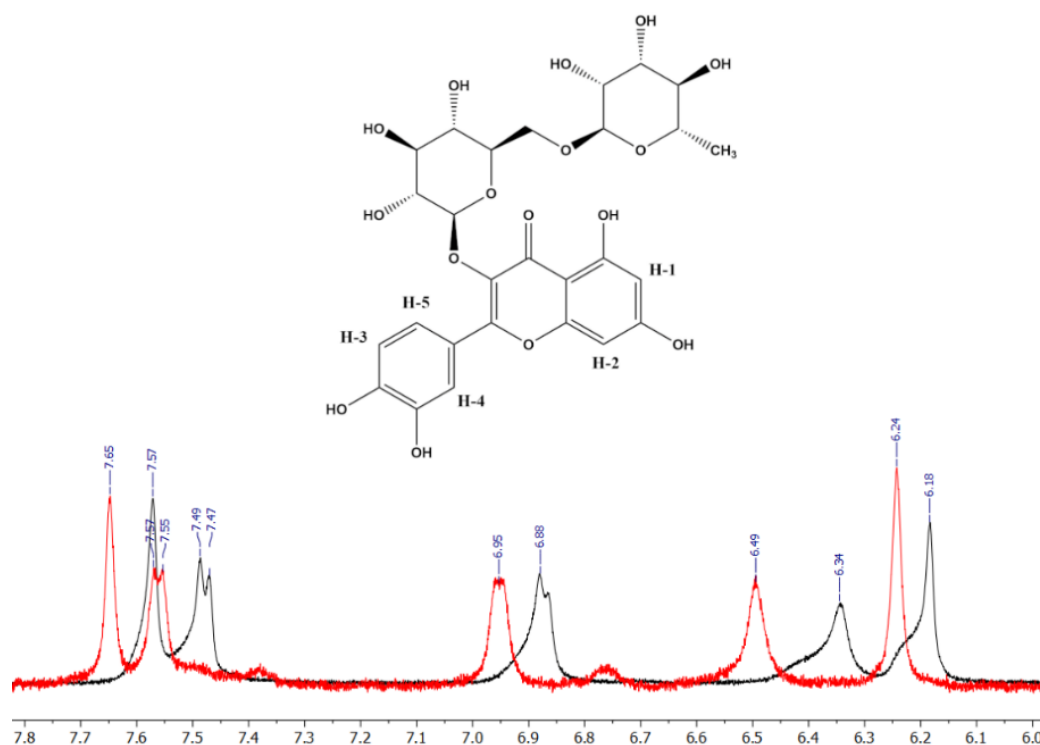


Figure 3.8. Overlay of ^1H NMR spectra of free RTN (black) and the RTN/ β -CPCD inclusion complex (red) in the aromatic region (δ 7.8-6.0 ppm).

Table 3.3. Comparison of the aromatic proton resonances (δ , ppm) of RTN in its free form and after complexation with β -CPCD.

<i>Proton assignment</i>	Free RTN (δ , ppm)	RTN/ β -CPCD inclusion complex (δ , ppm)	$\Delta\delta$ (ppm)
<i>H-1</i>	6.18	6.24	+ 0.06
<i>H-2</i>	6.34	6.49	+ 0.15
<i>H-3</i>	6.84	6.95	+ 0.11
<i>H-4</i>	7.48	7.56	+ 0.08
<i>H-5</i>	7.57	7.65	+ 0.08

All proton chemical shifts for free RTN and for the inclusion complex are reported in Table 3.3. The $\Delta\delta$ values, ranging from +0.06 to +0.15 ppm, indicate a consistent deshielding of the aromatic protons, reflecting their involvement in interactions with the CD cavity. Closer inspection of the spectra reveals faint upfield shoulders near H-3, H-4, and H-5, suggesting the presence of differently bound RTN populations. This observation supports a scenario in which a fraction of the RTN molecules is fully encapsulated within the β -CPCD cavity, while another fraction interacts more superficially with the polymeric framework. By contrast, no additional features are observed for H-1 and H-2, indicating that these protons are likely positioned outside the host cavity and experience only limited interaction.

3.5.4. μ -Raman characterization of RTN/ β -CPCD inclusion complex

To further elucidate the capacity of β -CPCD to interact with RTN, a μ -Raman analysis was conducted. This spectroscopic technique is particularly valuable for the study of CD inclusion complexes, as it enables the characterization of intermolecular interactions involved in the complexation pathway, thereby distinguishing between a mere physical mixture and genuine complex formation. This approach, previously validated for similar systems [85–87], relies on monitoring key spectral changes in the vibrational fingerprint of RTN upon its incorporation into the β -CPCD matrix. Therefore, the investigation involved a comparative assessment of the spectral features of the RTN/ β -CPCD inclusion complex against those of the physical mixture and the individual components. The corresponding μ -Raman spectra, recorded at 300 K in the 650-1800 cm^{-1} region, are shown in Figure 3.9.

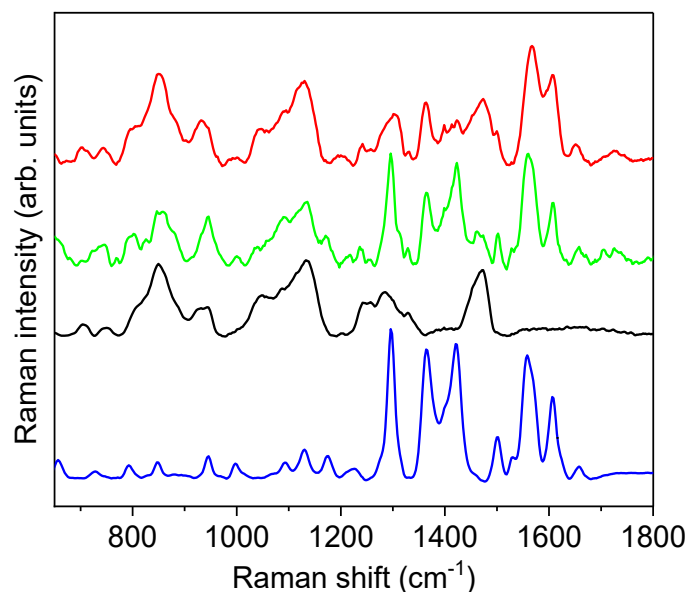


Figure 3.9. Comparison of μ -Raman spectra of RTN (blue), β -CPCD (black), their physical mixture (green), and the RTN/ β CPCD inclusion complex (red) in the 650-1800 cm^{-1} range at 300 K.

For RNT (Figure 3.9., blue trace), across the 650-1230 cm^{-1} spectral window, a sequence of low-intensity bands is evident at at $\sim 796 \text{ cm}^{-1}$, $\sim 849 \text{ cm}^{-1}$, $\sim 946 \text{ cm}^{-1}$, $\sim 1000 \text{ cm}^{-1}$, $\sim 1092 \text{ cm}^{-1}$, $\sim 1108 \text{ cm}^{-1}$, $\sim 1131 \text{ cm}^{-1}$. These spectral features are diagnostically associated with deformation modes involving C-C, C-H, and C-O bonds localized within the constituent benzene, heterocyclic, mannopyranosyl, and glucopyranosyl rings of the molecule [88]. A more prominent band centered at $\sim 1296 \text{ cm}^{-1}$ is attributable to bending vibrations of C-C-H and C-O-H groups situated on the benzene, heterocyclic, and mannopyranosyl rings. Furthermore, distinct peaks are observable at $\sim 1367 \text{ cm}^{-1}$ and $\sim 1422 \text{ cm}^{-1}$. The former is assigned to a C-O stretching mode specific to the dihydroxyphenyl ring, while the latter originates from a coupled C-O stretching vibration that engages both the benzene and the heterocyclic ring systems. Progressing to higher wavenumbers, the spectrum reveals a set of bands at at $\sim 1502 \text{ cm}^{-1}$, $\sim 1556 \text{ cm}^{-1}$ and $\sim 1608 \text{ cm}^{-1}$.

These are assigned to C=C aromatic stretching vibrations. The signal at 1502 cm^{-1} is characteristic of the benzene ring alone, the one at 1556 cm^{-1} arises from coupled vibrations of the benzene and dihydroxyphenyl rings, and the band at 1608 cm^{-1} is interpreted as a collective stretching mode involving the benzene, heterocyclic, and dihydroxyphenyl rings. The most intense feature in this high-frequency region, located at $\sim 1657\text{ cm}^{-1}$, is unequivocally linked to the stretching vibration of the carbonyl group (C=O) of the heterocyclic ring.

Shifting focus to the host molecule, the μ -Raman spectrum of β -CPCD (Figure 3.9., black trace) presents the characteristic vibrational signature expected for a β -CD derivative. Key resonances are identified at $\sim 851\text{ cm}^{-1}$ (attributed to breathing vibrations of the glucopyranose rings), $\sim 944\text{ cm}^{-1}$ (symmetric C-O-C stretching), $\sim 1032\text{ cm}^{-1}$ (C-O stretching), alongside bending modes of CH and CH₂ groups at 1250 cm^{-1} and 1466 cm^{-1} , respectively [89]. Superimposed upon this CD framework are additional vibrational modes stemming from the PEG component, manifesting as a shoulder at $\sim 806\text{ cm}^{-1}$, and distinct bands at $\sim 887\text{ cm}^{-1}$ (C-O-C stretch), $\sim 1040\text{ cm}^{-1}$ (C-C stretch), $\sim 1283\text{ cm}^{-1}$, and $\sim 1473\text{ cm}^{-1}$ (CH₂ stretching) [90].

The spectral profile of the physical mixture (Figure 3.9., green trace) was found to be essentially a linear combination of the individual spectra of RTN and β -CPCD. The absence of any significant band broadening, wavenumber displacement, or the emergence of new features indicates that no substantive intermolecular interactions occur between the components in this blended state, which is consistent with a mere physical mixing rather than complexation.

In stark contrast, the μ -Raman spectrum of the RTN/ β -CPCD inclusion complex (Figure 3.9., red trace) displays pronounced spectral modifications that

unequivocally demonstrate the successful encapsulation of RTN within the CD cavity. To gain deeper insight into these alterations, particular attention must be given to the 1500-1700 cm^{-1} spectral window (Figure 3.10.), which is especially informative as it lacks major contributions from the β -CPCD scaffold and therefore isolates the vibrational signatures of the guest molecule.

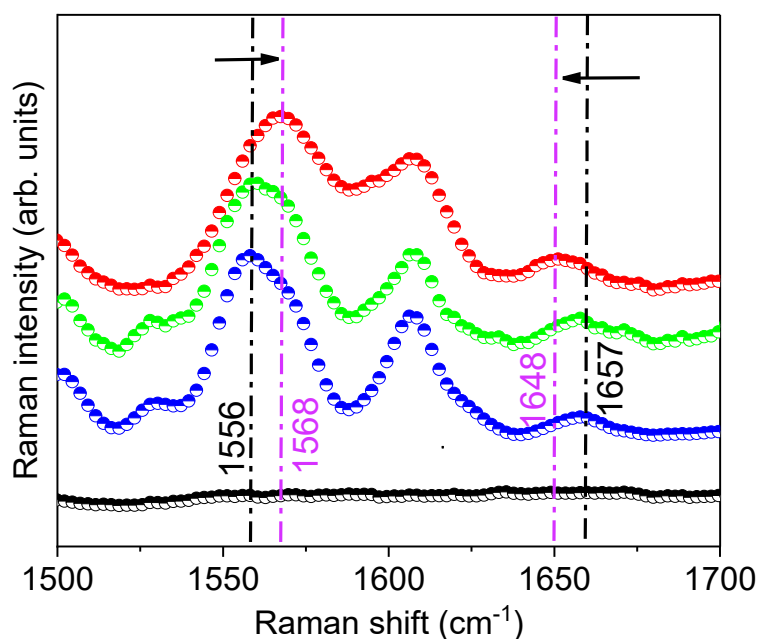


Figure 3.10. Comparison of μ -Raman spectra of RTN (blue circular markers), β -CPCD (black circular markers), their physical mixture (green circular markers), and the RTN/ β -CPCD inclusion complex (red circular markers) highlighting the 1500-1700 cm^{-1} spectral window at 300 K.

A detailed examination of 1500-1700 cm^{-1} range reveals a notable shift of the C=C stretching vibration to higher wavenumbers for the benzene and dihydroxyphenyl rings of RTN upon complexation. Specifically, the band centre shifts from approximately $\sim 1556 \text{ cm}^{-1}$ in the physical mixture to $\sim 1568 \text{ cm}^{-1}$ in the inclusion complex spectrum. This blueshift provides direct experimental confirmation of

specific intermolecular interactions between the bioflavonoid and the β -CPCD host, strongly suggesting the involvement of these aromatic rings in the complexation process. The observed shift indicates a significant alteration in the local electrostatic environment surrounding these C=C bonds, leading to a reduction in the associated electron density. This phenomenon is consistent with the partial or complete insertion of the benzene and/or dihydroxyphenyl rings into the relatively non-polar cavity of the CD, resulting in a shielded electronic environment for these molecular oscillators. Furthermore, analysis of this spectral region shows a complementary redshift of the carbonyl stretching band, which moves from its original position at $\sim 1657\text{ cm}^{-1}$. Given the well-established sensitivity of the C=O stretching frequency to environmental polarity and conjugation effects, this shift is particularly revealing [85]. In RTN, the carbonyl group is integrated into an extended π -conjugated system encompassing the heterocyclic and adjacent phenolic rings. The observed redshift strongly suggests the formation of intermolecular hydrogen bonds, potentially involving the carbonyl oxygen at the C6 position or other proximal oxygen atoms. Such interactions would facilitate a redistribution of electron density across the flavonoid framework, thereby promoting stabilization of the host-guest complex.

3.5.5. Computational insights into the RTN/ β -CPCD inclusion complex

To elucidate the specific interactions governing the inclusion of RTN within β -CPCD, and to assess the most favorable conformations, binding affinities, and thermodynamic stability of the host-guest complex, molecular docking studies were conducted. Through XP flexible docking, five binding docking poses were predicted, with docking scores ranging from -2.87 to -3.66 kcal/mol. The most

stable geometry, corresponding to the highest-scoring pose, is illustrated in Figure 3.11.

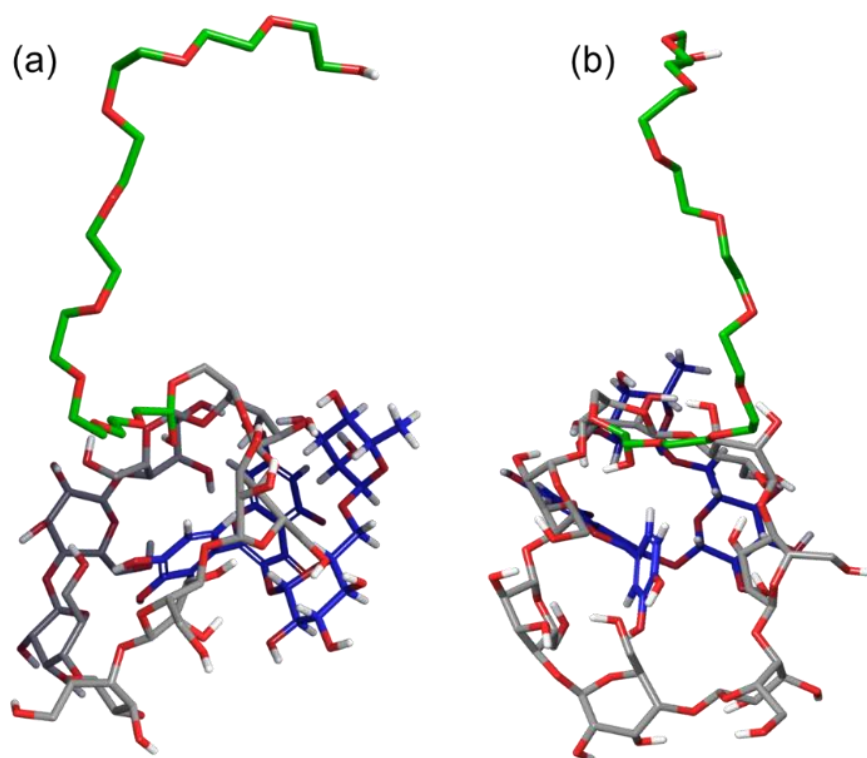


Figure 3.11. (a) Frontal and (b) lateral perspectives of the RTN/β-CPD inclusion complex in its top-scoring docking pose. RTN is represented in blue, β-CD in grey, and the PEG chain in green.

Consistent with its bulky and relatively rigid branched structure, RTN was found to partially penetrate the β-CD cavity. The primary site of inclusion was the hydrophobic hydroxyphenyl ring of the quercetin moiety, which fits into the apolar core of the macrocycle. In contrast, the highly hydrophilic sugar moiety remained outside the cavity, fully exposed to the solvent [78]. The PEG chain was oriented away from the host, retaining flexibility and solvation while preventing steric hindrance at the cavity entrance.

The stability of the inclusion complex arises from a combination of intermolecular hydrogen bonding, hydrophobic interactions, and electrostatic effects. A key stabilizing contribution was a strong hydrogen bond (2.070 Å, 135.030°) between the H73 atom of RTN's hydroxyphenyl ring and the O30 atom of a β -CD glucopyranose unit (Figure 3.12a). Additional stability was provided by a network of four further hydrogen bonds involving the O11 atom of RTN's heterocyclic ring and three hydroxyl groups from its sugar moiety, engaging with β -CD in a “chelating” effect that reinforces the binding, particularly in the solid state (Figure 3.12b).

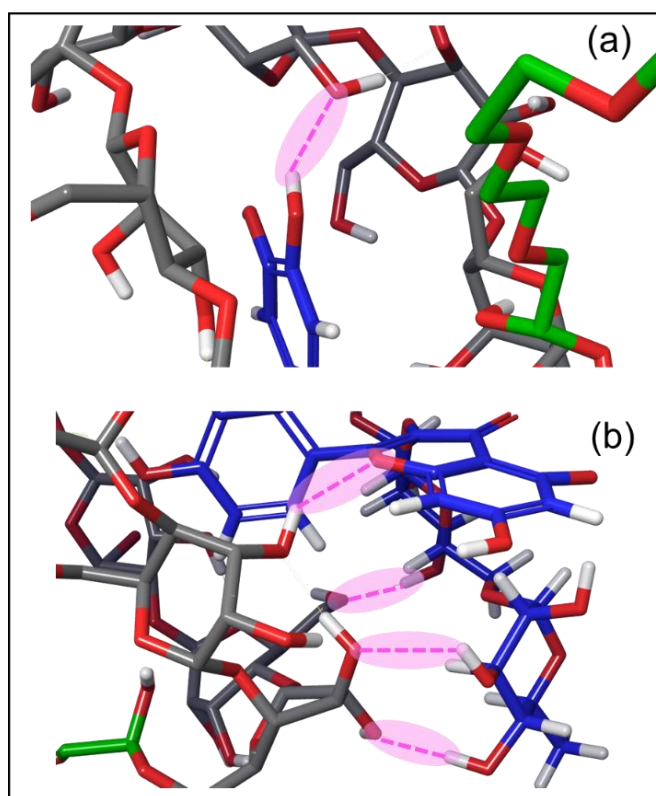


Figure 3.12. Close-up of the hydrogen bonding interactions (purple dashed lines) in the RTN/ β -CPCD inclusion complex. **(a)** Hydrogen bond formed between the H73 atom of RTN and the O30 atom of β -CPCD. **(b)** Additional hydrogen bonds involving the O11 atom and three hydroxyl hydrogen atoms of the RTN sugar moiety with β -CPCD.

Notably, the predicted hydrogen bond involving the heterocyclic ring is consistent with a redistribution of electron density in this region, offering a plausible explanation for the C=O stretching frequency shift observed in μ -Raman spectroscopy.

Finally, the partial inclusion of the nonpolar hydroxyphenyl ring is further stabilized by short-range van der Waals interactions, which shield this portion of the molecule from the aqueous medium. Conversely, the polar and water-soluble PEG chain remains fully solvated and outward-oriented, enhancing the overall solubility and biocompatibility of the final complex.

To assess the stability of the complex from a thermodynamic perspective, a MM-GBSA analysis was carried out. This method allowed the calculation of the binding free energy (ΔG Bind), together with the energetic contributions arising from different types of molecular interactions, for the docked RTN/ β -CPCD complex. The results are summarized in Table 3.4.

Table 3.4. Energetic contributions from the MM-GBSA analysis of the RTN/ β -CPCD docked inclusion complex (values in kcal·mol⁻¹).

ΔG Bind	ΔG Coul	ΔG Coval	ΔG HB	ΔG HP	ΔG Solv	ΔG VdW
- 30.719	- 9.111	+ 4.417	- 3.013	- 10.303	+ 3.024	- 15.773

Notes: ΔG Bind = total binding free energy; ΔG Coul = electrostatic contribution; ΔG Coval = covalent contribution; ΔG HB = hydrogen bonding contribution; ΔG HP = hydrophobic contribution; ΔG Solv = polar solvation contribution; ΔG VdW = van der Waals contribution.

The MM-GBSA calculation yielded a markedly favorable binding free energy ΔG_{Bind} of $-30.719 \text{ kcal}\cdot\text{mol}^{-1}$, indicating the formation of a robust and thermodynamically stable complex. The breakdown of the total binding energy reveals that the host-guest association is mainly driven by van der Waals forces ($-15.773 \text{ kcal}\cdot\text{mol}^{-1}$) and hydrophobic interactions ($-10.30 \text{ kcal}\cdot\text{mol}^{-1}$). This trend agrees with the typical non-covalent encapsulation mechanism observed in β -CD inclusion complexes. The hydrogen-bonding contribution ($-3.013 \text{ kcal}\cdot\text{mol}^{-1}$), although not the major driving force, confirms the existence of specific directional interactions between the hydroxyl groups of RTN and the polar rim of the CD cavity, in line with the docking results. Conversely, covalent energy ($+4.417 \text{ kcal}\cdot\text{mol}^{-1}$) and polar solvation energy ($+3.024 \text{ kcal}\cdot\text{mol}^{-1}$) contribute unfavorably to the binding. The positive ΔG_{Cov} value suggests the presence of torsional strain, most likely within the RTN backbone, together with minor structural rearrangements of the CD cone upon complexation. The unfavorable solvation contribution reflects an energetic penalty associated with dehydrating the polar surfaces of RTN prior to complex formation. Notably, the magnitude of this penalty is nearly compensated by the stabilizing effect of newly established intermolecular hydrogen bonds, highlighting the balance between desolvation and complexation forces.

As established in the literature [91], MM-GBSA data can also be employed to estimate the enthalpic (ΔH) and entropic (ΔS) contributions to binding, under the assumption of a single dominant conformational pathway. The calculated values ($\Delta H = -21.819 \text{ kcal}\cdot\text{mol}^{-1}$; $\Delta S = +0.029 \text{ kcal}\cdot\text{mol}^{-1}$) indicate that the formation of the RTN/ β -CPCD complex is an exothermic and predominantly enthalpy-driven process.

3.5.6. At solid state characterization of RTN/ β -CPCD inclusion complex

The freeze-dried RTN/ β -CPCD inclusion complex was analyzed in the solid state by TGA, in comparison with free RTN, the β -CD polymer, and a physical mixture of the two components prepared as described in Section 3.4.3. Figure 3.13. shows the TG profiles recorded between 20 and 800 °C.

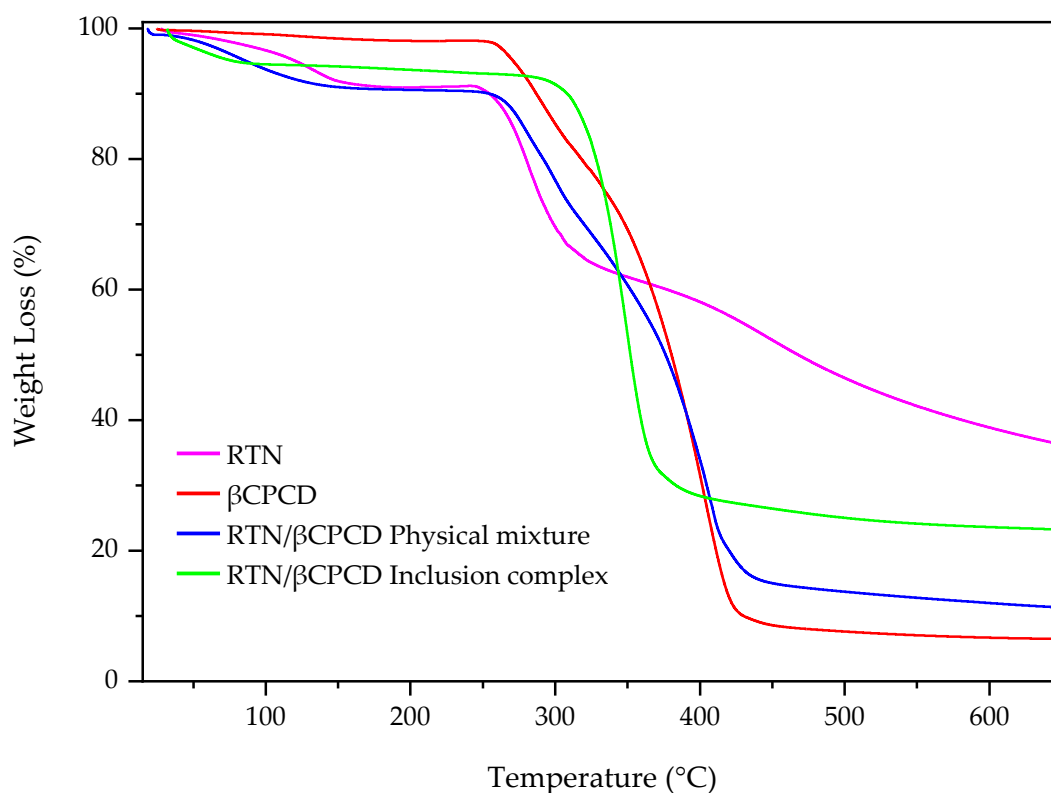


Figure 3.13. TGA profiles of free RTN, β -CPCD polymer, their physical mixture, and the freeze-dried RTN/ β -CPCD inclusion complex, recorded in the temperature range of 20-800 °C under nitrogen atmosphere. The inclusion complex shows a delayed onset of thermal degradation and a more gradual decomposition profile compared to the individual components and the physical mixture, indicating enhanced thermal stability as a result of complexation.

Free RTN displays a multistep degradation profile. An initial mass loss of about 8% is observed between 30 and 120 °C, corresponding to the release of adsorbed water. Thermal degradation of the flavonoid begins around 260 °C and proceeds

through distinct stages, leaving a final residue of approximately 38%. Such a pattern is in line with the reported instability of RTN under heat, known to undergo oxidative and structural decomposition when exposed to elevated temperatures.

The β -CD polymer exhibits minimal weight loss at low temperatures, indicating low moisture content. Its thermal decomposition begins around 280 °C and proceeds in two main stages: the first is likely associated with the degradation of the PEG segments, while the second corresponds to the breakdown of the more rigid CD units. The process ultimately leaves a final residue of approximately 6%, consistent with the material's composition.

The thermal profile of the physical mixture essentially reflects the additive contribution of its individual components: degradation initiates at the characteristic temperatures of the free species, with RTN decomposing first followed by the β -CD polymer, without appreciable shifts or alterations in the degradation pattern. This outcome confirms the absence of significant solid-state interactions, indicating that the two components coexist physically rather than forming an inclusion complex. However, it is worth noting that the physical mixture exhibited pronounced hygroscopicity, as shown by weight loss below 140 °C.

In contrast, the RTN/ β -CPCD inclusion complex exhibits a markedly altered thermal behavior compared to the free components and their physical mixture. While a minor initial mass loss, attributable to residual moisture, is still observed, the onset of thermal degradation shifts to a significantly higher temperature of 300 °C, representing an increase of approximately 50 °C for RTN and 20 °C for the β -CPCD polymer relative to the respective free species. Furthermore, decomposition progresses in a more gradual and continuous manner, without the sharp transitions observed in the free components or the physical mixture. This enhanced thermal

stability can be attributed to the confinement of RTN within the β -CPCD cavity, which restricts molecular mobility and reduces reactivity, thereby delaying degradation.

These findings highlight the role of β -CPCD as an effective stabilizing carrier, confirming its potential for incorporation into formulations where improved thermal resistance of RTN is desired.

3.5.7. In vivo anti-angiogenic studies using zebrafish embryos

The zebrafish embryo is widely recognized as a simple, cost-effective, and translationally relevant *in vivo* model for the early-stage screening of bioactive compounds. Its natural transparency allows real-time, non invasive visualization of vascular development, making it particularly suitable for studying angiogenesis. In this work, angiogenic progression was quantitatively assessed by measuring the activity of EAP, an enzyme physiologically released by endothelial cells during neovascularization. Because EAP activity correlates with the extent of blood vessel formation, it serves as a functional and reliable biomarker of angiogenesis.

To evaluate the anti-angiogenic potential of RTN, both the free compound and its inclusion complex with β -CPCD were tested and compared to 2-methoxyestradiol, an endogenous metabolite of 17 β -estradiol with well-documented anti-angiogenic activity, used as a positive control corresponding to 50% EAP inhibition. Prior to efficacy evaluation, a tolerability assay was performed by exposing zebrafish embryos to increasing concentrations of free RTN (0.1-1 mg/mL). Free RTN exhibited a marked dose-dependent toxicity: only the lowest concentration (0.1 mg/mL) was non-toxic, whereas higher doses caused over 90% mortality and morphological abnormalities. Conversely, the RTN/ β -CPCD inclusion complex

showed no significant toxicity, even at the highest tested concentration (1 mg/mL), suggesting that the β -CD polymer mitigates the intrinsic cytotoxicity of free RTN. To ensure completeness in the *in vivo* evaluation, the tolerability and potential anti-angiogenic activity of β -CPCD alone were also assessed (Figure 3.14). Embryos exposed to β -CPCD at concentrations ranging from 1 to 10 mg/mL exhibited high survival rates (close to 100%), indicating that the polymer itself is biocompatible and non-toxic at these concentrations. Moreover, the β -CPCD polymer showed no anti-angiogenic activity, not even at weak levels, confirming the absence of any interference with vascular development. These data attribute the anti-angiogenic effect observed in the complex exclusively to RTN, whose efficacy is enhanced by the formulation.

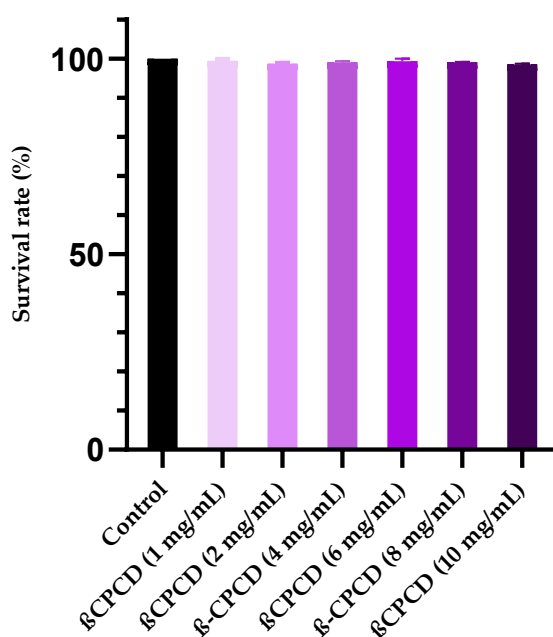


Figure 3.14. Percentage survival of zebrafish embryos at 96 hours post-fertilization after exposure to escalating concentrations of β -CPCD (1-10 mg/mL). Data are expressed as mean $\pm$ SD from six independent assays performed on six distinct zebrafish embryo batches.

Based on these findings, anti-angiogenic activity was assessed at 0.1 mg/mL for free RTN and across the 0.1-1 mg/mL range for the RTN/ β -CPCD complex. As illustrated in Figure 3.15., free RTN produced only a modest inhibition of EAP (~10%), probably due to its poor aqueous solubility and consequently limited bioavailability. In stark contrast, the inclusion complex exhibited a clear dose-dependent enhancement of anti-angiogenic efficacy, achieving EAP inhibition levels comparable to those of 2-methoxyestradiol at the highest tested dose.

It is reasonable to assert that the RTN/ β -CPCD complex improves the therapeutic index of RTN by modulating both its pharmacokinetic and pharmacodynamic properties. Encapsulation within the hydrophilic β -CPCD polymer increases RTN solubility and stabilizes the molecule, thereby reducing direct cellular exposure to free RTN and minimizing toxic effects. The improved solubility and stabilization facilitate RTN diffusion through cell layers and enhance its accumulation in vascular tissues. Moreover, the β -CD units exposed on the polymer surface can interact with membrane components, such as cholesterol and phospholipids, partially destabilizing the lipid bilayer and promoting cellular uptake [92,93]. Collectively, these mechanisms support more effective delivery to endothelial targets, highlighting the potential of the β -CPCD nanoformulation to overcome the limitations of free RTN and serve as a promising strategy for targeted anti-angiogenic therapy.

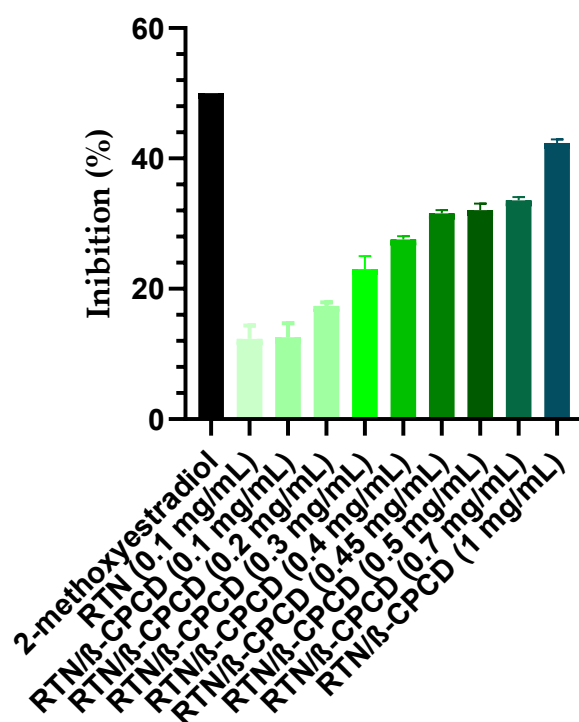


Figure 3.15. Comparative analysis of the anti-angiogenic activity of free RTN and its inclusion complex at varying concentrations, using 2-methoxyestradiol as a positive control. Anti-angiogenic efficacy was quantified as the percentage reduction in EAP activity. Data are expressed as mean $\pm$ SD from six independent assays performed on six distinct zebrafish embryo batches.

3.6. Conclusions

In this work, we successfully designed, developed, and validated a supramolecular carrier based on a novel, functionalized, highly hydrophilic β -CD polymer (β -CPCD) to improve the pharmacological profile of RTN. The inclusion complexation strategy effectively addressed the intrinsic limitations of this bioflavonoid, namely its poor aqueous solubility and consequently limited bioavailability, while enhancing its biological activity.

μ Raman spectroscopy and molecular docking revealed specific shifts in the characteristic vibrational bands of RTN upon binding to monomeric β -CPCD,

providing strong evidence of the stable supramolecular assembly between the flavonoid and the polymer. *In vitro* characterization demonstrated a marked improvement in dissolution in aqueous medium, approximately 50-fold higher than free RTN, with rapid release kinetics, as well as enhanced thermostability. Computational modeling, consistent with NMR and μ -Raman data, suggested a preferential binding mode in which the hydrophobic flavonoid core of RTN is partially inserted into the β -CD cavity, while the sugar moiety remains solvent-exposed together with the polymeric PEG chain.

A central aspect of this study was the toxicological evaluation of the β -CPCD carrier, performed *in vivo* for the first time using zebrafish (*Danio rerio*) embryos. The polymer exhibited a favorable safety profile across concentrations of 1 to 10 mg/mL, with no observed lethality or malformations after 96 hours. In contrast, free RTN induced marked toxicity at concentrations as low as 0.25 mg/mL. Importantly, the RTN/ β -CPCD inclusion complex not only mitigated RTN's toxicity but also enhanced its pharmacological efficacy, producing dose-dependent inhibition of angiogenesis comparable to that of the reference antiangiogenic drug, 2-methoxyestradiol.

Collectively, these findings underline the potential of β -CPCD as a versatile and biocompatible delivery platform capable of enhancing the solubility, permeability, stability, and therapeutic window of poorly soluble bioactive molecules such as RTN. Beyond the specific case of RTN, this work contributes to the broader field of CD-based DDSs by demonstrating how rational supramolecular design, supported by a combination of computational modeling, spectroscopic validation, and early-phase toxicological screening, can provide a strong foundation for translational research. Looking forward, further studies will be necessary to extend

the evaluation of this innovative delivery system into more advanced preclinical models, including pharmacokinetic and biodistribution analyses, as well as long-term toxicity assessments.

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CHAPTER 4. CHITOSAN NANOPARTICLES INCORPORATING A BICALUTAMIDE/HP- β -CYCLODEXTRIN INCLUSION COMPLEX AS A NOVEL DRUG DELIVERY SYSTEM FOR TRIPLE-NEGATIVE BREAST CANCER

4.1. Triple-negative breast cancer

Triple-negative breast cancer (TNBC) represents, within the heterogeneous landscape of breast malignancies, the most aggressive subtype and the one associated with the poorest prognosis [1]. According to the guidelines provided by the American Society of Clinical Oncology (ASCO), TNBC is defined by the absence or very low expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2). More specifically, tumors are classified as triple-negative when ER and PR expression is $\leq 1\%$, and HER2 status is scored as 0 or 1+, or 2+ with a negative in situ hybridization (ISH) test, as determined by immunohistochemistry [2,3].

For this reason, TNBC, which accounts for approximately 15-20% of all breast cancers, represents the only subtype that cannot benefit from either hormonal or HER2-targeted therapies. This therapeutic limitation has established systemic chemotherapy as the cornerstone of TNBC clinical management for decades [4,5]. However, subsequent studies of molecular profiling have unveiled a more complex scenario, demonstrating that TNBC is not a single, uniform neoplasm but rather a profoundly heterogeneous condition with distinct subtypes, each defined by a

distinct expression pattern and clinical behavior [1,6]. This molecular stratification has laid the groundwork for more tailored therapeutic approaches [6].

4.1.1. Lehmann classification and subtypes

The pivotal classification proposed in 2011 by Lehmann *et al.* identified six distinct subtypes (Figure 4.1. [5]): basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) [7]. BL1 and BL2, which together account for the majority of TNBC cases (approximately 75%), are both driven by proliferative programs but through distinct mechanisms: BL1 is enriched in genes involved in cell cycle regulation and DNA repair, whereas BL2 is characterized by activation of growth factor signaling pathways and glucose metabolism [8]. The M and MSL subtypes share a phenotype driven by genes that enhance cell motility and epithelial-mesenchymal transition, processes closely linked to tumor invasiveness. The MSL subtype, however, is further distinguished by lower expression of proliferation markers and by the presence of stemness-associated genes, reflecting features commonly associated with stem cell biology [8]. The IM subtype is characterized by the overexpression of genes involved in immune activation and signaling, and shares some similarities with the BL subtypes. Interestingly, despite this molecular profile suggestive of a strong immune response, IM tumors often develop mechanisms that allow them to evade immune surveillance. These include the recruitment of immunosuppressive cells within the tumor microenvironment and the activation of immune checkpoint pathways, which effectively inhibit T-cell activity. Clinically, this subtype usually presents with a high histological grade but is paradoxically associated with a relatively favorable prognosis [1,8]. Finally, the

LAR subtype is predominantly characterized by the expression of hormonal pathways, including those mediating steroid and androgen/estrogen metabolism, as well as peroxisome proliferator-activated receptor (PPAR) signaling [9,10]. It exhibits a luminal-like gene profile characterized by the co-expression of transcription factors like FOXA1 and GATA3, enrichment of luminal cytokeratins (CK7/8, CK18, CK19), and a lack of basal markers, all of which are linked to a relatively more favorable prognosis compared to other TNBC subtypes. From a clinical perspective, LAR tumors are less proliferative, occur more frequently in older patients, and typically have an indolent disease course, even though they generally carry a higher overall mutational burden [11].

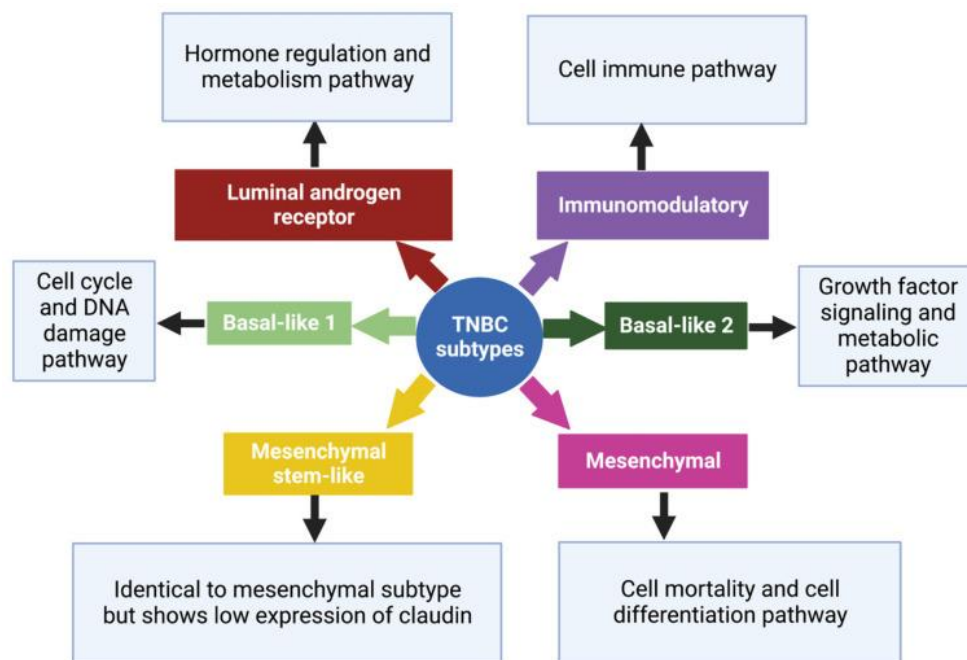


Figure 4.1. Schematic representation of the six molecular subtypes of triple-negative breast cancer as defined by Lehmann *et al.* (2011), each characterized by distinct gene expression profiles and biological pathways.

4.1.1. Clinical and diagnostic features

It is well-established that TNBC is the most aggressive subtype of breast cancer from a clinical viewpoint. Its clinical behavior is characterized by rapid tumor growth, early metastatic dissemination, and a high propensity for both local and distant recurrence [5,12]. These hallmarks translate into a markedly poorer prognosis compared to other BC subtypes, with patients often experiencing shorter disease-free survival and facing an increased risk of mortality. Most metastatic events occur within the first three years after diagnosis, and the likelihood of distant recurrence within five years is nearly three times greater than in non-TNBC cases. In contrast, late relapses beyond this period are rare (<3%) [12–14]. Metastatic spread in TNBC shows a strong tropism for visceral organs, most commonly the lungs, distant lymph nodes, and liver, along with a particularly high incidence of CNS involvement, which poses a major therapeutic challenge. As a consequence, the five-year overall survival rate for TNBC patients remains around 60%, markedly lower than for other subtypes, underscoring the urgent need for more effective therapeutic options [12,15].

The high mortality rate associated with TNBC seems to be partly attributable to delayed diagnosis. TNBC is frequently detected at an advanced stage, when eradication is no longer feasible [16,17]. Current diagnostic approaches largely rely on histopathological assessment and immunohistochemistry, which are operator-dependent, time-consuming, and may suffer from inter-laboratory variability [18]. To improve diagnostic efficiency and allow earlier detection, advanced and rapid technologies are being investigated. These include immuno-positron emission tomography (immuno-PET), nanobiosensors, analysis of circulating tumor nucleic acids, circulating tumor cells, and blood-based liquid biopsies [19].

4.1.2. Epidemiological features

Epidemiologically, TNBC shows marked variation across populations. It is more frequently diagnosed in younger women, particularly those under 40 and in premenopausal age, underscoring the need for age-specific prevention and early detection strategies [5,20]. Racial and ethnic disparities are also evident: women of African or Hispanic origin are nearly twice as likely to develop TNBC compared to White women and often present with more advanced disease and worse survival outcomes [20,21]. Genetic factors partly contribute to this trend, as BRCA1/2 mutations are more common in Black women. These alterations have been observed to correlate strongly with the onset of TNBC, particularly in BRCA1 carriers, where the majority of breast cancers present a triple-negative phenotype. Such mutations not only increase susceptibility to the disease but also shape its biological behavior, conferring defects in DNA repair mechanisms that favor tumorigenesis [22]. Additionally, recent studies have identified distinct DNA methylation patterns in Black women diagnosed before age 50, suggesting the presence of unique epigenetic profiles in this group [23].

However, biological differences alone cannot fully explain the disparities in outcome. The elevated mortality observed in Black women is not solely a function of tumor biology but is profoundly exacerbated by systemic barriers. These include inequitable access to screening programs, delays in diagnosis, and financial obstacles to obtaining high-quality and/or innovative treatments. As a result, survival rates remain consistently lower among Black women across all stages of disease compared with White women. Consequently, reducing the burden of TNBC demands progress in both research and therapy, complemented by strategies that

ensure prevention, equitable access to care, and attention to the wider social determinants of health [20].

4.1.3. Main therapeutic approaches

Given the issues outlined in the previous sections, TNBC represents one of the most complex challenges in contemporary breast oncology. In contrast to hormone receptor-positive subtypes, which are amenable to endocrine therapy (e.g., tamoxifen, aromatase inhibitors), and HER2+ tumors, for which targeted agents such as trastuzumab have revolutionized clinical management, TNBC still lacks established and specific therapeutic options [24]. Its pronounced biological and clinical heterogeneity precludes the adoption of a universal treatment strategy, requiring instead personalized therapeutic strategies based on patient and tumor specific parameters, including age, stage (TNM), histological grade and type, and, most importantly, molecular subtype [5,11,24]. At present, the management of TNBC, as with many other solid tumors, still relies primarily on the therapeutic triad of surgery, radiotherapy, and systemic chemotherapy [11].

4.1.3.1. Surgical management

When technically feasible, surgical resection represents the cornerstone of initial management. Depending on disease extent, procedures may include lumpectomy or mastectomy with axillary lymph node dissection [25]. In addition to eradicating the primary tumor, surgery can prevent severe complications such as spinal cord compression or pathological fractures, allow resection of selected metastases, and alleviate local symptoms including chest wall infiltration, recurrence, or bone pain [26].

4.1.3.2. Role of radiotherapy

Radiotherapy is frequently administered in the adjuvant setting following mastectomy, aiming to eliminate residual malignant cells and reduce the risk of locoregional recurrence [26]. It also plays an important role in the management of locally advanced or metastatic disease, where it can provide meaningful palliation. Nevertheless, recurrence rates of 7-12.6% within five years and the emergence of radio resistance limit its long-term efficacy [27,28].

4.1.3.3. Systemic chemotherapy

In the absence of conventional receptor-targeted therapies, systemic chemotherapy represents the standard of care in TNBC management. Its administration can be neoadjuvant, adjuvant, or palliative, depending on the clinical setting and disease stage [5]. Among the most widely employed agents, anthracyclines and taxanes represent the standard backbone of therapy [29]. Anthracyclines (e.g., DOX, epirubicin) intercalate into DNA, impair repair pathways, and exert direct cytotoxicity, while also stimulating antitumor immune responses, particularly through CD8⁺ T cells [30,31]. Despite high initial response rates, recurrences are common, overall survival remains limited, and cumulative cardiotoxicity restricts repeated use [5]. Taxanes (e.g., PTX, DTX, cabazitaxel) disrupt microtubule function, inhibit mitosis, and impair angiogenesis [32]. They can be administered alone or in combination with anthracyclines, achieving pathological complete response (pCR) rates of at least 40% in TNBC [5,11]. PTX is often preferred due to its reversible binding to microtubules, and its albumin-bound formulation (nab-PTX) offers improved tolerability and safety. Nonetheless, resistance and off-target toxicities remain significant challenges, reinforcing the rationale for combination

strategies [5]. More recently, platinum-based agents (e.g., carboplatin, cisplatin) have gained attention, particularly in tumors with BRCA1/2 mutations or homologous recombination deficiency. By inducing DNA crosslinks and apoptosis, they enhance pCR rates when incorporated into neoadjuvant regimens, highlighting their value as additions to conventional chemotherapy [11].

In parallel with this traditional multimodal approach, a number of emerging strategies are currently being explored (Figure 4.2. [11]). Although these strategies often do not constitute standalone treatments, they have the potential to complement and enhance the efficacy of conventional therapies, thereby providing additional avenues to improve outcomes in TNBC patients.

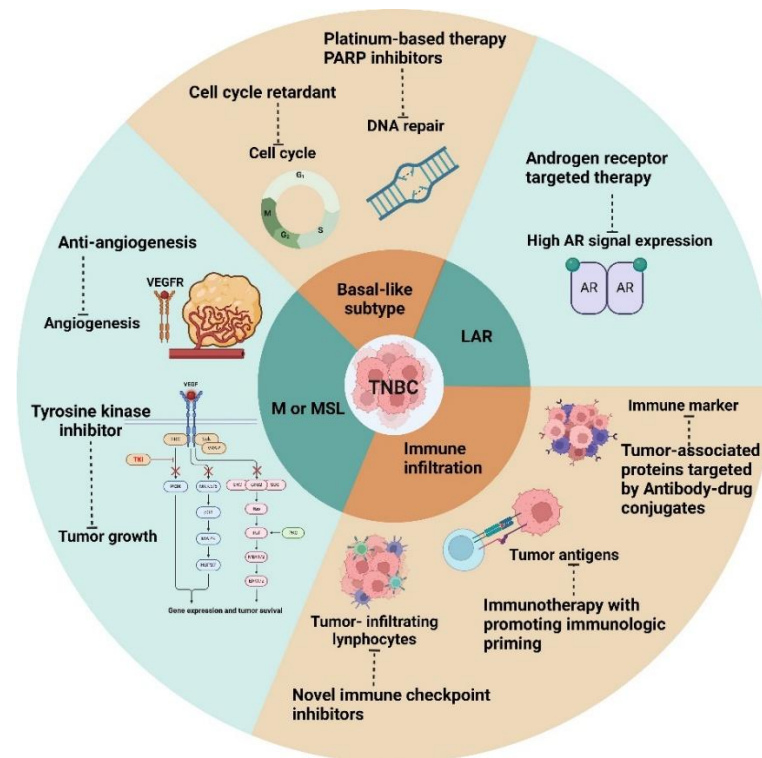


Figure 4.2. Overview of therapeutic strategies for triple-negative breast cancer across its main subtypes, ranging from conventional treatments (chemotherapy, surgery, and radiotherapy) to emerging targeted therapies and immunotherapeutic approaches.

4.1.4. Emerging targeted and biological therapies

The therapeutic landscape of TNBC has progressively evolved beyond conventional chemotherapy, with a growing number of targeted and biological agents now under investigation or already integrated into clinical practice [11].

4.1.4.1. DNA damage response–targeted therapies: PARP inhibitors

Among the targeted strategies investigated in TNBC, Poly(ADP-ribose) polymerase (PARP) inhibitors represent a consolidated therapeutic option. Recently approved for the treatment of metastatic BC in patients harboring BRCA mutations, these drugs show efficacy in about 20% of cases. This rate essentially mirrors the prevalence of BRCA alterations within the general population [5,33].

PARP inhibitors function as "smart drugs" by exploiting the inherent vulnerability of tumors with BRCA deficiencies. The BRCA1 and BRCA2 genes are essential for maintaining genomic stability, primarily through their role in repairing DNA double-strand breaks. While BRCA2 directly recruits the RAD51 protein to initiate homologous recombination, BRCA1 acts as a broader orchestrator of the repair process. In patients with mutations in these genes, this crucial mechanism is compromised, leading to a greater susceptibility to genomic instability [34].

This specific vulnerability forms the therapeutic basis for PARP inhibitors. Like BRCA genes, PARP family enzymes are involved in DNA repair, but they specialize in resolving single-strand breaks [35]. Consequently, inhibiting PARP in a cell already deficient in BRCA function blocks this residual repair pathway. The resulting accumulation of unresolved DNA damage becomes catastrophic for the cancer cell. This phenomenon, known as synthetic lethality, selectively triggers cell

death in tumors that lack a functional repair system, explaining the marked sensitivity of BRCA-mutated cancers, including TNBC, to this approach [5,33].

In the context of BRCA-mutated TNBC, olaparib and talazoparib are currently approved by the FDA, whereas veliparib and niraparib are still being tested in clinical trials [5].

4.1.4.2. Tyrosine kinase inhibitors: targeting EGFR and VEGFR pathways

Another area of interest is represented by tyrosine kinase inhibitors (TKIs). Tyrosine kinases are enzymes involved in the transmission of signals that control key cellular processes such as proliferation, survival, and angiogenesis [36,37]. In TNBC these kinases, often functioning as growth factor receptors, are aberrantly activated or overexpressed, thereby sustaining uncontrolled tumor progression. Therefore, pharmacological inhibition of these pathways can suppress proliferative and survival signaling, ultimately limiting tumor growth while reducing the non-specific cytotoxicity typically associated with conventional chemotherapy [38].

Within this group, particular attention has been directed toward inhibitors of the EGFR. EGFR is a transmembrane tyrosine kinase receptor involved in the regulation of proliferation, invasion, and apoptosis [39,40]. Its overexpression has been consistently observed in TNBC, often at levels markedly higher than in normal tissue, thus making it a promising therapeutic target [41]. Several approaches have been tested in this regard, including small-molecule inhibitors such as gefitinib and erlotinib, as well as monoclonal antibodies like cetuximab. Combinatorial regimens that integrate EGFR inhibitors with standard chemotherapeutics, for instance gefitinib with carboplatin and DTX, are currently under clinical evaluation [42]. Moreover, strategies based on RNA interference

directed against EGFR mRNA are being explored as a potential therapeutic alternative [5].

Another strategy focuses on targeting the VEGFR, a key mediator of angiogenesis and vascular permeability, both of which are critical for tumor growth and metastatic dissemination. Inhibition of this pathway impairs tumor vascularization and consequently hampers disease progression [29]. Bevacizumab, a monoclonal antibody directed against VEGF, has been tested in TNBC and received FDA approval in this setting. The addition of bevacizumab to neoadjuvant chemotherapy has been associated with improved pCR rates, highlighting its potential clinical benefit [43].

4.1.4.3. Immunotherapy

Within the expanding landscape of novel therapeutic strategies, immunotherapy has emerged as a prominent strategy within the breast cancer treatment landscape. While most breast cancers respond poorly to such approaches, TNBC is a notable exception due to its more immunogenic features. These include a high tumor mutational burden and significant infiltration of CD8⁺ T lymphocytes, characteristics shared with immunotherapy-responsive cancers like melanoma and lung cancer that enhance tumor visibility to the immune system. These properties have paved the way for the clinical implementation of ICIs in TNBC [11,44].

Under physiological conditions, T lymphocytes are able to recognize and destroy abnormal cells. However, tumor cells can evade immune surveillance by exploiting inhibitory pathways. One of the most relevant mechanisms involves the overexpression of programmed cell death protein 1 (PD-L1) on tumor cells, which binds to programmed death-ligand 1 (PD-L1) receptors on T cells and delivers

inhibitory signals that induce functional exhaustion, ultimately suppressing antitumor activity. A similar mechanism is mediated by cytotoxic T-lymphocyte antigen 4 (CTLA-4), which downregulates T cell function through its interaction with the surface receptors CD80 and CD86 [5].

Given that TNBC frequently exhibits elevated PD-L1 expression alongside dense lymphocytic infiltration, blocking the PD-1/PD-L1 signaling pathway has become a promising therapeutic strategy [31]. In this context, the FDA has approved pembrolizumab and nivolumab for several solid tumors, while ongoing studies are investigating their efficacy in TNBC [5]. Similarly, CTLA-4 inhibitors, including tremelimumab, which is among the most advanced in clinical trials for TNBC, are being evaluated following their success in other malignancies [31].

4.1.4.4. Antibody-drug conjugates

Among monoclonal antibody-based strategies, antibody-drug conjugates (ADCs) represent a particularly promising class of therapeutics. These agents exploit the high specificity of antibodies to selectively deliver cytotoxic drugs directly to tumor cells, thereby enhancing antitumor efficacy while minimizing systemic toxicity typically associated with conventional chemotherapy [45]. In the case of TNBC, the only ADC currently approved is sacituzumab govitecan (SG), which received FDA authorization in 2016 for patients with metastatic TNBC who had already undergone at least two prior lines of therapy [46]. SG is composed of a humanized anti-Trop-2 monoclonal antibody (HuRS7) conjugated with SN-38, the active metabolite of the chemotherapeutic irinotecan. Following administration, HuRS7 binds Trop-2, a surface antigen frequently overexpressed in TNBC, and directs the targeted delivery of SN-38. SN-38 is able to exert its antineoplastic effect both after

intracellular release and within the tumor microenvironment. Before SG, topoisomerase I inhibitors had not been used in metastatic TNBC, making SG a novel cytotoxic option for this disease [47].

4.1.4.5. PI3K/AKT/mTOR pathway inhibition

Another promising class of agents for TNBC, although not yet part of routine clinical practice due to lack of FDA approval, includes PI3K/AKT/mTOR inhibitors. This pathway is frequently hyperactivated in TNBC, where it plays a central role in regulating cellular proliferation, survival, metabolism, and angiogenesis. Consequently, targeting key components of this pathway could disrupt these processes, thereby slowing tumor growth and potentially sensitizing cancers to other therapies [24,48].

Promising compounds under investigation include mTOR inhibitors (e.g., everolimus, temsirolimus), AKT inhibitors (such as ipatasertib and capivasertib), and PI3K inhibitors (including buparlisib and taselisib). These agents are primarily being evaluated in combination with chemotherapy or other targeted therapies, as they have shown limited efficacy when used as monotherapy in TNBC [5,48].

4.1.4.6. Androgen receptor-targeted therapies

Last but not least, an additional promising approach for TNBC treatment focuses on targeting the androgen receptor (AR). Although TNBC is defined by the absence of classic hormone receptors, molecular profiling has identified the LAR subtype, accounting for approximately 22-35% of TNBC cases, which is characterized by functional AR expression. In this context, AR signaling appears to drive tumor

initiation, progression, and metastasis, providing a strong rationale for anti-androgen therapy [49–51].

The clinical potential of these agents has been deeply investigated in phase II studies, offering meaningful evidence of efficacy. For example, the UCBG 12-1 trial evaluated abiraterone acetate, a CYP17 inhibitor, in combination with prednisone in AR+ TNBC ($\geq 10\%$ nuclear expression), reporting a six-month clinical benefit rate of 20% and a median progression-free survival of 2.8 months [52]. A separate trial with enzalutamide, a second-generation AR inhibitor, showed more promising outcomes, with a 16-week clinical benefit rate of 33%, a median progression-free survival of 3.3 months, and a median overall survival of 17.6 months [53]. Despite these encouraging findings, no anti-androgen has yet received FDA approval for TNBC, and their use remains investigational [5]. However, these agents are occasionally prescribed off-label by certain oncology specialists [51].

This body of evidence has paved the way for further exploration of AR blockade in TNBC management, highlighting the AR as a clinically relevant target whose inhibition could further interfere with tumor growth and progression. Among the agents investigated, BCL, a first-generation nonsteroidal anti-androgen, was the first drug tested in this context [7,54].

4.2. Bicalutamide

Patented in 1982 and approved for medical use in 1995, BCL is an orally administered nonsteroidal anti-androgen (NSAA) that has become widely established in clinical practice [55]. Its primary pharmacological activity consists of a highly selective and potent antagonism of the AR, functioning as a pure antagonist with reported IC_{50} values ranging from 159 to 243 nM [56,57]. By

competitively inhibiting the binding of endogenous ligands such as testosterone and dihydrotestosterone, BCL blocks AR-mediated signaling pathways, exerting an anti-androgenic effect without altering systemic hormone levels, as it functions exclusively via receptor blockade rather than suppressing androgen synthesis [58].

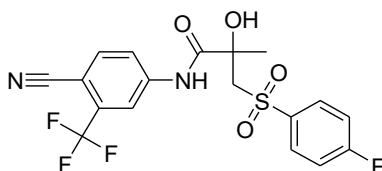


Figure 4.3. Chemical structure of bicalutamide.

Originally marketed under the trade name Casodex® by AstraZeneca, and now available in numerous generic formulations, BCL's primary clinical application is in the management of prostate cancer (PC) [59]. Its standard use in this setting involves a 50 mg/day dose, administered in combination with a luteinising hormone-releasing hormone (LHRH) analogue or following orchiectomy for advanced disease. This dosage is also employed in asymptomatic or mildly symptomatic patients with metastatic castration-resistant PC after the failure of initial androgen deprivation therapy. Furthermore, it is an option for men with metastatic castration-resistant PC whose disease has progressed during or after DTX chemotherapy. A higher monotherapy dose of 150 mg/day is approved in Europe for selected patients with locally advanced, non-metastatic PC [60].

Beyond its established role in prostate oncology, BCL is also used in several clinical contexts. In men, it mitigates the testosterone “flare-up” observed at the initiation of GnRH agonist therapy [61]. In women, it has been employed to treat androgen-dependent dermatological and hair disorders, as well as hyperandrogenism associated with polycystic ovary syndrome (PCOS), typically

in combination with an oral contraceptive [62–64]. Moreover, BCL is a component of feminizing hormone therapy in transgender women, generally administered at 50 mg/day alongside estrogen supplementation [65,66]. In pediatric endocrinology, it is used to manage peripheral precocious puberty in boys, particularly in cases of familial male-limited precocious puberty (testotoxicosis), in combination with aromatase inhibitors (e.g., anastrozole) [67–69]. Additionally, it has been applied in the treatment of priapism in men, with regimens ranging from 50 mg weekly to 50 mg on alternate days [70,71].

The clinical relevance of BCL has also been re-evaluated in oncology beyond PC, particularly within the subgroup of TNBC characterized by androgen receptor expression (AR+ TNBC) [7]. This therapeutic repositioning, from PC to TNBC, reflects an emerging trend in oncology, where established agents with well-defined safety profiles are increasingly explored in new tumor contexts sharing common molecular drivers.

Owing to its broad clinical utility and well-documented safety profile, BCL is included in the World Health Organization's List of Essential Medicines and is marketed in over 80 countries, primarily as 50 mg and 150 mg oral tablets, with an additional 80 mg formulation available in Japan [72–74]. To date, no alternative routes of administration or formulations are commercially available [75].

4.2.1. Chemical structure and physicochemical properties

From a chemical standpoint, BCL (Figure 4.3., *N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropan amide) is a chiral small molecule built around a substituted propanamide core. Its structure features a hydroxyl group, a sulfonyl-linked 4-fluorophenyl moiety, and

a 4-cyano-3-(trifluoromethyl)phenyl substituent [76]. This molecular architecture integrates lipophilic domains with polar functionalities, giving rise to an amphiphilic profile. However, BCL is predominantly hydrophobic, with an experimentally determined $\log P$ of 2.92, which accounts for its extremely poor aqueous solubility ($<4 \mu\text{g/mL}$) [77]. Consequently, it is classified under the BCS as a Class II compound, defined by high permeability but low solubility/dissolution rate [78].

Commercial formulations are supplied as a racemic mixture containing equimolar proportions of the (*R*)- and (*S*)-enantiomers, although the pharmacological activity resides almost entirely in the (*R*)-enantiomer ((*R*)-BCL). In fact, (*R*)-BCL exhibits approximately 30-fold higher affinity for ARs compared with its (*S*)-counterpart, and is responsible for the clinically relevant anti-androgenic effects [79,80].

In terms of physicochemical properties, BCL is described as a fine white to off-white crystalline powder, with a molecular weight of 430.37 g/mol [81]. Notably, its solid-state behavior is governed by conformational polymorphism, which plays a pivotal role in ensuring consistent pharmaceutical performance. The compound exists in at least two crystalline forms (I and II), differing in molecular conformation: Form I adopts an open arrangement and represents the thermodynamically stable polymorph, whereas Form II displays a closed arrangement and is metastable. These structural differences translate into distinct physicochemical parameters: Form I exhibits a melting point of approximately 192 °C and limited aqueous solubility, while Form II melts at about 189 °C and demonstrates approximately 2.4-fold higher solubility. An amorphous phase of BCL can also be generated, but it is highly unstable and rapidly recrystallizes to

Form II at room temperature within about a week, underscoring the importance of solid-state control for this drug [82].

Irrespective of its solid form, BCL displays intrinsically low aqueous solubility, whereas its solubility is comparatively higher in a variety of organic solvents. The solubility, expressed as mole fraction, decreases according to the following order: *n*-propyl acetate > acetonitrile > ethyl acetate > methanol > ethanol > 1-butanol > *n*-propanol > isopropanol > isobutanol > toluene > cyclohexane > *n*-hexane. [83]. These findings indicate that BCL preferentially partitions into lipophilic environments, suggesting that lipid-based DDSs may represent a suitable strategy for its formulation [72]. Furthermore, BCL exhibits a *pK*_a of approximately 12, consistent with the presence of a weakly acidic hydroxyl group, which remains largely unionized under physiological conditions [84].

4.2.2. Therapeutic repositioning of bicalutamide in triple-negative breast cancer

As previously mentioned, BCL was the very first anti-androgenic agent to be evaluated in the challenging setting of TNBC, thereby demonstrating that ARs blockade could exert clinically meaningful antitumor activity in this specific patient population.

4.2.2.1. Preclinical evidence

The first preclinical study to systematically highlight the potential role of BCL in TNBC was the landmark work by Lehmann and colleagues, published in the *Journal of Clinical Investigation* in 2011 [7]. As extensively discussed in the previous sections, this study reshaped the understanding of TNBC by dismantling the notion of a single, homogeneous disease entity and classifying it into six distinct

molecular subtypes based on gene expression profiling of 587 TNBC tumors. Among these, the luminal LAR subgroup emerged as particularly noteworthy. It is characterized by a unique transcriptional signature highly enriched in hormone-regulated signaling pathways, especially those governed by the AR. These tumors displayed ARs mRNA levels approximately nine-fold higher than those of other TNBC subtypes, along with robust expression of downstream AR target genes. The functional significance of AR dependence was further corroborated in cell line models. The authors identified a series of TNBC cell lines representative of each molecular subtype. LAR cell lines (MDA-MB-453, SUM185PE, CAL-148, MFM-223) exhibited markedly elevated AR expression, both at mRNA and protein levels. When exposed to BCL, a panel of 30 TNBC cell lines revealed a strikingly differential sensitivity: while most BC cell lines showed no response, even at high concentrations ($IC_{50} > 500 \mu M$), all LAR lines demonstrated a substantial reduction in cell viability, with mean IC_{50} values around $227 \mu M$. These findings were validated *in vivo* through xenograft models. Tumors derived from LAR cell lines (SUM185PE and CAL-148) implanted into nude mice displayed a significant growth inhibition upon BCL treatment, showing that the antitumor efficacy of BCL extended beyond cell culture models into living systems [7].

Thus, the study by Lehmann *et al.* not only elucidated the molecular heterogeneity of TNBC but also provided the first therapeutic rationale for introducing anti-androgen therapy in this patient subgroup.

4.2.2.2. *Clinical evidence and ongoing trials*

This rationale was first tested clinically in the multicenter TBCRC-011 trial conducted by Gucalp *et al.*, which evaluated the efficacy and safety of BCL in the management of TNBC [54]. This study specifically enrolled patients with AR+, ER/PR- TNBC. Out of 452 patients initially screened, 424 underwent central immunohistochemical testing for AR. AR expression was detected in 12% (51/424) of ER/PR- tumors. Not all eligible patients, however, received BCL: 8 patients were excluded, 15 continued on systemic therapies that were still providing clinical benefit, and 28 were ultimately enrolled to receive oral BCL at a daily dose of 150 mg. The treated cohort had a median age of 66 years (range 41-83) and generally favorable performance status (median ECOG 0). Most patients presented with visceral metastases and had already received, on average, one prior chemotherapy regimen for metastatic disease. The primary endpoint, the clinical benefit rate at 6 months (defined as the proportion of evaluable patients achieving complete response, partial response, or stable disease lasting ≥ 6 months), was achieved. Among 26 evaluable patients, 5 (19%; 95% CI: 7-39%) achieved durable disease stabilization, although no confirmed complete or partial responses were observed. Nevertheless, this result was considered clinically relevant: in metastatic BC, where chemotherapy is often highly toxic, achieving prolonged disease stabilization with a well-tolerated oral agent is a meaningful therapeutic outcome. Median progression-free survival was 12 weeks (95% CI: 11-22). Two patients experienced particularly durable disease control, remaining on treatment for 57+ and 11+ cycles, respectively. A notable case involved a patient with unresectable locally advanced disease who, after 6 months of stable disease under BCL, was able to undergo mastectomy; however, postoperative analysis revealed HER2 positivity,

raising the possibility of cross-talk between AR and HER2 pathways. The safety profile of BCL was favorable, with no severe toxicities reported. Serum levels of testosterone (total and free), estradiol, and sex hormone-binding globulin showed no significant changes or discernible patterns during treatment, indicating that BCL's pharmacological activity is mediated exclusively through AR antagonism rather than systemic modulation of androgen levels [54].

The results of the TBCRC-011 trial provided the clinical rationale to further explore more effective therapeutic strategies based on the combination of BCL with other targeted agents, with the aim of improving clinical outcomes in AR-dependent BC. Several clinical trials are currently underway to test this hypothesis. Among these, the ongoing study NCT03090165 is investigating the therapeutic potential of combining ribociclib, a selective CDK4/6 inhibitor, with BCL in patients with AR+ TNBC. This approach aims to exploit the functional interplay between AR signaling and cell-cycle regulation, with the ultimate goal of improving clinical outcomes in this particularly aggressive tumor subtype [85]. Along the same line, a Belgian trial is evaluating the combination of BCL with abemaciclib, another CDK4/6 inhibitor [86].

These efforts highlight the potential of drug repurposing, especially when combined with rational drug combinations, in the challenging context of cancer therapy. This is particularly relevant for TNBC, where effective treatment options are still considerably limited.

4.2.3. Limitations in bicalutamide's pharmaceutical and pharmacokinetic profile

BCL is generally considered as a safe drug, with a favorable tolerability profile in both sexes. Since TNBC predominantly affects women, it is particularly relevant to consider the safety data in female patients. In this population, the drug remains well tolerated: given the limited physiological role of androgens in women, the adverse effects associated with pure anti-androgens are generally mild. The most common events include gastrointestinal discomfort and skin reactions. Even when administered at higher doses than the conventional 50 mg/day, up to 600 mg/day, BCL has maintained good tolerability, with few additional adverse events [73]. The main safety concern remains hepatotoxicity: abnormal liver function tests, particularly elevated liver enzymes, may occur, typically within the first 3 to 6 months of treatment. For this reason, regular monitoring of liver function is recommended during the early phase of therapy [87]. Another clinically relevant limitation is its teratogenic potential, as antiandrogen exposure during pregnancy carries a risk of birth defects (e.g., ambiguous genitalia in male fetuses). Therefore, adequate contraception is mandatory in women of childbearing potential receiving BCL [88].

Despite this reassuring safety record, the pharmaceutical performance of BCL is constrained by important pharmacokinetic limitations. The drug is efficiently absorbed after oral administration owing to its lipophilicity; nonetheless, as a BCS Class II compound, its low aqueous solubility makes dissolution in the gastrointestinal tract the rate-limiting step for absorption [72]. This poor solubility results in nonlinear, saturable absorption kinetics: while oral bioavailability is relatively high at low doses, it progressively decreases as the dose increases. Specifically, steady-state plasma concentrations (C_{ss}) increase proportionally only up to approximately 50 mg/day; between 50 and 300 mg/day, the increase becomes

less than proportional, and at doses above 300 mg/day a complete plateau is observed, with no further rise in exposure. As a result, BCL shows variable and sometimes incomplete bioavailability. Combined with the high interpatient variability in systemic exposure (up to 15-fold differences in C_{ss}), this poses a significant risk of subtherapeutic drug levels at the tumor site [80].

Furthermore, the hydrophobic nature of BCL precludes its development into parenteral liquid formulations, restricting its administration to the oral route and necessitating relatively high doses to achieve therapeutic efficacy. Such requirements can negatively impact patient compliance [81].

From a pharmacokinetic standpoint, additional constraints further limit its clinical performance. BCL is administered as a racemate, although only the (*R*)-enantiomer is pharmacologically active and reaches steady-state levels about 100-fold higher than the inactive (*S*)-form. Its elimination is markedly slow, with a half-life of roughly one week, leading to a 10-fold accumulation in plasma during daily dosing. The drug is also highly bound to plasma proteins (>96%), a factor that reduces the fraction available for tissue penetration. Metabolism occurs almost exclusively in the liver, mainly through CYP3A4-mediated oxidation and glucuronidation, with excretion balanced between urine and feces. Although food does not significantly alter overall bioavailability, high-fat meals can slightly increase peak concentrations, suggesting solubilization effects in the upper gastrointestinal tract. Furthermore, systemic exposure is subject to wide interindividual variability and tends to be higher in Japanese compared with Caucasian patients [80]. This profile, characterized by slow elimination, extensive protein binding, enantioselective activity, and variable systemic exposure, provides a compelling rationale for the development of advanced DDSs for BCL. Novel formulations designed to enhance

dissolution and stabilize pharmacokinetics could not only improve therapeutic efficacy in established indications like PC but also unlock its full potential in emerging applications like TNBC.

4.2.4. Enhancing bicalutamide's bioavailability: current state of art

To overcome the biopharmaceutical challenges associated with BCL, various formulation strategies have been explored and reported in the literature. A notable example is the work of Li *et al.* (2011), who developed a BCL nanodispersion through a combination of anti-solvent precipitation and spray-drying. In their study, lactose was employed as a hydrophilic excipient to prevent particle growth and aggregation. This process yielded NPs with a mean diameter of approximately 330 nm and a narrow size distribution, a dramatic reduction compared to the raw drug's irregular particles, which instead measured several hundred micrometers. Remarkably, the lactose-based nanodispersion released 94% of the drug within 10 minutes *in vitro*, compared to 60% and 38% released by Casodex® and standard BCL tablets, respectively. This enhanced dissolution was attributed to both the vastly increased surface area and the improved wettability conferred by the lactose matrix [89].

Following the anti-solvent precipitation approach, Meer *et al.* (2011) applied a similar strategy to produce microcrystals stabilized by a polymer-surfactant system. They used hydroxypropyl methylcellulose and Poloxamer 407 to prevent crystal growth and aggregation. This approach reduced the mean particle size of BCL to 24 µm and increased the specific surface area. Although the particles were larger than the nanodispersion reported by Li *et al.* and remained in the micrometer range, solid-state analysis revealed an additional benefit: a partial reduction in

crystallinity, shown by a lower melting enthalpy in differential scanning calorimetry (DSC) measurements. This feature, together with the modest reduction in particle size, contributed to an improved dissolution rate compared to the raw drug. This example highlights that anti-solvent precipitation can be effectively modulated using different stabilizers to control particle size and solid-state properties, with both mechanisms contributing to enhanced dissolution [90].

Advancing this technology further into the nanoscale, Pokharkar *et al.* (2012) successfully produced BCL nanocrystals with a mean particle size of 168 nm using the novel amphiphilic polymer Soluplus® as a singular stabilizer. This study is particularly significant as it provided the first *in vivo* pharmacokinetic data for a nanocrystalline BCL formulation. The nanocrystals exhibited a fivefold increase in kinetic solubility and an 85% dissolution within 60 minutes. Most importantly, oral administration to rats demonstrated a 3.5-fold increase in both the maximum plasma concentration ($C_{\max}$) and the area under the curve (AUC) compared to the pure drug. Importantly, from a technological perspective, the nanocrystals produced by Pokharkar *et al.* preserved the metastable Form II of BCL employed in the preparation of this nanoformulation. As outlined in Section 4.2.1, this polymorph, although less thermodynamically stable than Form I, is characterized by higher free energy and a slightly higher aqueous solubility. In conventional bulk material, Form II tends to recrystallize into the more stable Form I, thereby limiting its practical utility. In this case, however, stabilization at the nanoscale through Soluplus® prevented such a transition, allowing the formulation to retain the dissolution advantages of Form II while maintaining excellent physical stability over a six-month period [91].

Moving beyond merely improving dissolution to enhance systemic bioavailability, a more refined strategy involves actively targeting the drug to its site of action. Lee *et al.* (2012) pioneered this approach for BCL by developing a magnetic NP-based DDS. They synthesized superparamagnetic $\gamma\text{-Fe}_2\text{O}_3$ NPs with a diameter of approximately 23 nm and loaded them with BCL using an emulsion technique with ethylene glycol as a surfactant [92]. The system was designed not merely to enhance dissolution but for magnetic drug targeting, an innovative nanotechnology approach that uses external magnetic fields to guide and accumulate NPs directly at the target site. This strategy is distinct from ligand-mediated (“active”) targeting, which relies on surface-conjugated ligands (e.g., monoclonal antibodies) to bind specific receptors [93]; magnetic targeting instead uses applied magnetic fields to physically direct magnetic particles to the desired location. Magnetic targeting has been extensively investigated in preclinical models as a means to increase local drug concentration while minimizing systemic exposure [94]. The BCL-loaded magnetic NPs exhibited significant cytotoxicity against a panel of cancer cell lines, including a 56% inhibition of human prostate cancer cells (DU145), while the unloaded magnetic NPs proved non-toxic [92].

The most advanced evolution of these strategies is represented by the work of Ray *et al.* (2016), who developed a PLGA NP system for BCL. This approach moves beyond immediate release to achieve sustained and controlled drug delivery. Using a single emulsion-solvent evaporation method, they encapsulated BCL within the biodegradable PLGA polymer matrix, producing spherical NPs of approximately 270 nm. A key feature of this system is its biphasic release profile: an initial burst release of 58% within 24 hours, attributed to drug molecules on or near the particle surface, followed by a slow, sustained release of up to 98% over 120 hours (5 days)

as the polymer matrix gradually degrades. This release kinetics, which best fit the Higuchi model, suggests a diffusion-controlled mechanism. Most importantly, the system demonstrated superior biological performance. *In vitro* studies on DU145 prostate cancer cells showed that the PLGA NPs enhanced cellular uptake via energy-dependent endocytosis and significantly increased cytotoxic potency. The IC_{50} was achieved within 48 hours for the nanoformulation, compared to 72 hours for the free drug, confirming a more rapid and efficient therapeutic effect [95].

A significant contribution to the advancement of BCL formulation was also provided by a study carried out by the research group with which I collaborated, published by De Gaetano *et al.* (2022). In this work, the authors systematically investigated the use of highly soluble CDs, namely HP- β -CD and SBE- β -CD, to overcome the poor aqueous solubility of BCL. By exploiting the peculiar supramolecular architecture of CDs, characterized by a hydrophobic cavity capable of hosting lipophilic molecules and a hydrophilic outer surface promoting water solubility, the team successfully formed stable inclusion complexes with this drug. The complexes were prepared using the freeze-drying method and extensively characterized both in the solid state and in solution. DSC and X-ray diffraction analyses confirmed the amorphous nature of the complexes, as indicated by the disappearance of crystalline BCL peaks and shifts in thermal events, suggesting strong interactions between the drug and the CDs. Phase-solubility studies yielded AL-type diagrams, consistent with a 1:1 stoichiometry, and revealed stability constants (K_c) of 9018 M^{-1} for BCL/HP- β -CD and 4530 M^{-1} for BCL/SBE- β -CD, indicating a higher affinity of BCL for HP- β -CD. Additionally, UV-vis spectroscopy showed a hyperchromic effect on BCL's absorption band at 272 nm, more pronounced with HP- β -CD, while ^1H -NMR spectroscopy and molecular

dynamics simulations confirmed the inclusion of the 4-cyano-3-(trifluoromethyl)phenyl ring within the CD cavities. The amorphous complexes obtained by freeze-drying showed a 10-fold and 7-fold increase in water solubility for HP- β -CD and SBE- β -CD, respectively, and a complete dissolution achieved within 10 minutes. Beyond the physicochemical benefits, this strategy also translated into a marked biological advantage. *In vitro* studies against androgen-insensitive prostate cancer cell lines (DU145 and PC3) demonstrated that the BCL/CD inclusion complexes significantly enhanced the anticancer activity of the drug compared to unformulated BCL [81].

4.3. Aim of the research

Building upon this solid background, the primary objective of the research presented in this chapter was to develop a novel DDS for the therapeutic repositioning of BCL in AR+ TNBC. To this end, CS NPs incorporating a BCL/HP- β -CD inclusion complex were developed. This formulation strategy was conceived to provide multiple advantages: on one hand, to ensure a sustained and controlled release of BCL; on the other, to exploit receptor-mediated mechanisms for selective tumor targeting and, at the same time, to protect the drug against premature physical and chemical degradation.

To the best of our knowledge, the encapsulation of BCL into CS-based NPs has not previously been reported. This gap reflects the inherent properties of unmodified CS, which is well-suited for encapsulating hydrophilic compounds but is typically incompatible with hydrophobic molecules like BCL [96]. To overcome this limitation, we adopted a strategic two-step approach: first, BCL was pre-complexed with HP- β -CD to improve its apparent solubility and mask its hydrophobicity,

following the procedure described by De Gaetano *et al.* [81]; second, the resulting inclusion complex was successfully incorporated into a CS matrix. Among the available options, HA was selected as the gelling polyanion because of its strong affinity for CD44 receptors, which are frequently overexpressed in several tumors and correlate with higher invasiveness and poorer prognosis [97,98]. This receptor-mediated interaction was expected to promote the selective uptake of the NPs by cancer cells via receptor-mediated endocytosis, thereby maximizing therapeutic efficacy while minimizing off-target effects.

In summary, the present work proposes a synergistic nanoplatform that combines the solubility-enhancing capacity of CDs with the biocompatibility, mucoadhesive properties, and tumor-targeting potential of CS/HA NPs. This approach paves the way for repositioning BCL as a more effective and targeted therapy for AR+ TNBC.

4.4. Materials and methods

4.4.1. Materials

Bicalutamide (BCL; formula: $C_{18}H_{14}F_4N_2O_4S$; MW: 430.37 g/mol), chitosan low-molecular-weight (CS; formula: $(C_{12}H_{24}N_2O_9)_n$; average MW 50,000-190,000 Da), 2-hydroxypropyl- β -cyclodextrin (HP- β -CD; average MW: 1380 g/mol), hyaluronic acid (HA; formula: $(C_{14}H_{21}NO_{11})_n$; average MW 25,000 Da), and D-(+)-trehalose dihydrate were purchased from Sigma-Aldrich Chemie® (St. Louis, MO, USA). Methanol (MeOH; HPLC grade) was supplied by Merck® (Darmstadt, Germany). The water used in this study was purified by double distillation, followed by deionization and final filtration through 0.22 μ m Millipore® GSWP

filters (Bedford, MA, USA). All other chemicals used in this work that are not listed above were of analytical grade.

4.4.2. Preparation of BCL/HP- β -CD inclusion complex

The BCL/HP- β -CD inclusion complex, previously characterized in detail by De Gaetano *et al.* [81], was prepared in a 1:1 molar ratio using the freeze-drying method. Briefly, BCL (1 mg, 2.32×10^{-6} mol) was dissolved in MeOH (1 mL), while HP- β -CD (3.2 mg, 2.32×10^{-6} mol) was dissolved in ultrapure water (4 mL) under gentle magnetic stirring at room temperature until complete dissolution. The methanolic BCL solution was then added dropwise to the aqueous HP- β -CD solution under continuous stirring at 500 rpm. The resulting mixture was transferred into lyophilization flasks, frozen, and subsequently lyophilized for 72 yielding the BCL/HP- β -CD inclusion complex as a solid powder.

4.4.3. Preparation of blank chitosan nanoparticles

Blank CS NPs were prepared using the ionotropic gelation method, employing HA as the crosslinking agent. Specifically, 10 mg of CS were dissolved in 5 mL of an aqueous acetic acid solution (1%, v/v), and the so obtained dispersion was magnetically stirred overnight to achieve complete polymer solubilization. The following day, the pH of the CS solution was adjusted to 5 by the dropwise addition of 2 M NaOH, after which the solution was filtered through 0.22 μ m nylon syringe filters to eliminate impurities or undissolved residues that could compromise NP formation. Subsequently, 3 mg of HA were dissolved in 3 mL of pH 5 buffer, and this solution was added dropwise to the CS solution under constant magnetic stirring (500 rpm). The resulting opalescent suspension was further stirred for 30

minutes to promote complete NP formation. NPs were isolated through a two-step centrifugation process: initially, larger aggregates were removed by centrifugation at 5000 rpm for 15 minutes; subsequently, a series of centrifugations at 13,000 rpm for 30 minutes each was performed. After each cycle, the supernatant was discarded, while the pellet containing CS NPs was carefully recovered and redispersed in 0.5 mL of ultrapure water. All pellets were finally pooled, supplemented with trehalose (2.5% w/v) as cryoprotectant, and lyophilized for 72 h. The resulting dry powders were stored in a desiccator under anhydrous conditions until further characterization.

4.4.4. Preparation of BCL/HP- β -CD-loaded chitosan nanoparticles

To prepare BCL-loaded CS NPs, the lyophilized BCL/HP- β -CD inclusion complex, obtained as described in Section 4.4.2, was incorporated into the previously filtered aqueous CS solution. Subsequently, the crosslinking agent solution (3 mg of HA dissolved in 3 mL of pH 5 buffer) was added dropwise to the polymeric solution containing the inclusion complex, under continuous magnetic stirring (500 rpm), to promote NP formation. The resulting suspension was then processed and purified following the same protocol applied for blank CS NPs. The supernatants collected during centrifugation were used for the indirect quantification of BCL and the determination of HP- β -CD content.

4.4.5. Technological characterization of blank and BCL/HP- β -CD-loaded chitosan nanoparticles

To assess the efficiency of NP formation and the extent of BCL encapsulation, blank and BCL/HP- β -CD-loaded CS NPs were subjected to a comprehensive

technological characterization. While the detailed methodology for particle size, PDI, and ζ measurements has been described in Section 2.3.11, the production yield, EE %, and DC % were specifically calculated for the systems studied here using the following equations:

$$\text{Yield \%} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \cdot 100$$

$$\text{EE \%} = \frac{\text{Initial BCL content} - \text{Free BCL}}{\text{Initial BCL content}} \cdot 100$$

$$\text{DC \%} = \frac{\text{Initial BCL content} - \text{Free BCL}}{\text{Weight of recovered NPs}} \cdot 100$$

The amount of BCL encapsulated within the CS NPs was determined indirectly by calculating the difference between the initial amount of BCL used in the preparation (1 mg per inclusion complex) and the residual BCL detected in the supernatant after NP isolation. The supernatants collected from the centrifugation cycles were pooled, concentrated using a rotary evaporator, and the resulting residues were reconstituted in 3 mL of MeOH, filtered through a 0.22 μm syringe filter, and analyzed by UV-Vis spectroscopy at the characteristic absorption maximum of BCL ($\lambda_{\text{max}} = 272 \text{ nm}$). The concentration of BCL in the samples was determined using a calibration curve prepared from standard BCL solutions over the relevant concentration range, which exhibited excellent linearity ($R^2 = 0.998$). These data were then used to calculate EE % and DC %, providing a quantitative assessment of the EE and the overall performance of the formulated NPs.

4.4.6. Determination of encapsulated HP- β -CD in BCL/HP- β -CD-loaded chitosan nanoparticles

The amount of HP- β -CD encapsulated within the CS NPs was quantified by indirect UV-vis spectrophotometric assay, adapting with minor modifications the method described by Cannavà *et al.* (2013) [99]. The analytical principle of this technique is based on the ability of CDs to form inclusion complexes with phenolphthalein; when such a complex is formed, the absorbance of the indicator's colored form decreases proportionally to the CD concentration [100].

To determine the fraction of HP- β -CD not incorporated into the NPs, the supernatants obtained after centrifugation of the BCL/HP- β -CD-loaded NP suspension were collected and evaporated to dryness under vacuum using a rotary evaporator. The dried residues were reconstituted in 1.5 mL of PBS (pH 7.4) and filtered through a 0.22 μ m syringe filter. To each sample, 1.5 mL of a mixture consisting of alkaline borate buffer (pH 10.0) and an ethanolic phenolphthalein solution (0.02% w/v) in an 80/20 (v/v) ratio was then added. Upon complex formation between HP- β -CD and phenolphthalein, the characteristic pink coloration of the indicator progressively fades. The decrease in color intensity, measured spectrophotometrically at 554 nm, is directly proportional to the concentration of CD present in the solution.

A thermodynamic rationale supports the reliability of this assay in this specific context, where the presence of BCL could potentially interfere with the accuracy of the results. Literature data indicate that the HP- β -CD/phenolphthalein inclusion complex exhibits a higher stability constant ($K_{PP} \approx 2.0 \times 10^4 \text{ M}^{-1}$, for HP- β -CD with an average MW of 1380 g/mol [100]) than the HP- β -CD/BCL inclusion complex ($K_{BCL} = 9.018 \times 10^3 \text{ M}^{-1}$ [81]). Given its higher association constant,

phenolphthalein is thermodynamically favoured to occupy the HP- β -CD cavity over BCL, ensuring that the spectrophotometric response reflects the effective HP- β -CD concentration rather than artefacts arising from residual drug-CD interactions.

For quantitative analysis, a calibration curve was constructed over the concentration range 0.013-0.1% (w/v) of HP- β -CD. Standard solutions of known concentration were treated under identical conditions to those applied to the samples. The calibration curve exhibited linearity with $R^2 = 0.9842$.

The amount of HP- β -CD effectively encapsulated within the NPs was calculated by subtracting the quantity of non-entrapped HP- β -CD (determined in the supernatants) from the total amount originally used in the formulation process:

$$\text{Encapsulated HP-}\beta\text{-CD \%} = \frac{\text{Initial HP-}\beta\text{-CD content} - \text{Free HP-}\beta\text{-CD}}{\text{Initial HP-}\beta\text{-CD content}} \cdot 100$$

4.4.7. In vitro release of BCL from BCL/HP- β -CD-loaded chitosan nanoparticles

The *in vitro* release profile of BCL from CS NPs was evaluated using the dialysis bag method. Briefly, lyophilized BCL/HP- β -CD-loaded formulations, corresponding to a theoretical BCL content of 1 mg, were reconstituted in 1 mL of PBS (pH 7.4) and transferred into dialysis membranes (MWCO 3.5 kDa) pre-hydrated in the same buffer for 12 h. The sealed membranes were immersed in 100 mL of PBS and the system was maintained under magnetic stirring (150 rpm) at 37.0 ± 0.5 °C in a thermostatically controlled water bath. At predetermined time intervals, the entire external medium was withdrawn and immediately replaced with an equal volume of fresh medium to maintain sink conditions. Collected samples were evaporated to dryness under reduced pressure using a rotary

evaporator, reconstituted in 3 mL of MeOH, filtered through a 0.22 μm syringe filter, and analyzed spectrophotometrically (see Section 4.4.5. for analytical details). For comparison, the release profile of the free BCL/HP- β -CD complex (equivalent to 1 mg of BCL) was evaluated under the same conditions. Drug release was expressed as the cumulative percentage of BCL released over time. All experiments were performed in triplicate and results are reported as mean $\pm$ SD.

The data obtained from the *in vitro* release studies were analyzed using different mathematical models in order to elucidate the release mechanism. The release profiles were fitted to the zero-order model (cumulative percentage of drug released vs. time), the first-order model (log cumulative percentage of drug remaining vs. time), the Higuchi model (cumulative percentage of drug released vs. square root of time), and the Hixson-Crowell cube root law (cube root of the initial percentage minus cube root of the percentage remaining vs. time). In addition, the Korsmeyer-Peppas model was used to analyze the mechanism of drug release from the polymeric system [100]. The model that best described the release kinetics was selected based on the highest correlation coefficient (R^2) obtained from the linear regression analysis of each plot [101,102].

4.4.8. In vitro permeation of HP- β -CD from BCL/HP- β -CD-loaded chitosan nanoparticles

The *in vitro* permeation profile of HP- β -CD from CS NPs was evaluated using the same dialysis bag method as described for BCL release. In brief, freshly prepared BCL/HP- β -CD-loaded CS NPs, redispersed in a total volume of 2 mL of ultrapure water, were transferred into dialysis membranes (MWCO 3.5 kDa) that had been pre-hydrated in PBS for 12 hours prior to use. Once filled, the membranes were

carefully sealed and immersed in 100 mL of fresh PBS. The experiments were conducted under constant magnetic stirring (150 rpm) at 37.0 ± 0.5 °C. At predetermined time intervals, the entire volume of the release medium was withdrawn and immediately replaced with an equal volume of fresh PBS to maintain constant experimental conditions. The collected samples were evaporated to dryness under reduced pressure using a rotary evaporator, reconstituted in 1.5 mL of PBS and filtered through a 0.22 μ m syringe filter. Quantification of the permeated HP- β -CD was then performed spectrophotometrically using the phenolphthalein complexation method, following the same analytical procedure adopted for the determination of non-encapsulated HP- β -CD (Section 4.4.6.). CD permeation was expressed as the cumulative percentage of HP- β -CD diffused through the dialysis membrane over time. All experiments were performed in triplicate, and results are reported as mean $\pm$ SD.

4.4.9. Thermogravimetric analysis (TGA)

Thermal decomposition profiles of free BCL, HP- β -CD, blank CS NPs, BCL/HP- β -CD-loaded CS NPs, and the corresponding physical mixture were assessed by TGA following the procedure described in Section 2.3.13. All measurements were performed in triplicate.

4.4.10. Biological activity

4.4.10.1. In vitro studies: cell cultures and treatments

The TNBC cell line MDA-MB-231 was kindly provided by the IRCCS Ospedale Policlinico San Martino (ICLC, Genoa, Italy). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-

glutamine, and 1% non-essential amino acids. Cultures were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂.

In the biological assays, three different forms of BCL were tested: the free drug, the inclusion complex with HP- β -CD (BCL/HP- β -CD), and CS/HA NPs containing BCL in its complexed form with HP- β -CD (BCL/HP- β -CD-loaded NPs), prepared as described in Sections 4.4.2. and 4.4.4., respectively. Free BCL was freshly dissolved in DMSO immediately before use, whereas both the inclusion complex and the NP formulations were readily dispersible in aqueous medium. The final concentration of DMSO in the culture medium was maintained at 0.1% (v/v) in all treatment groups to ensure consistent experimental conditions.

Subconfluent cells were exposed for 72 hours to free BCL (25-100 μ M), HP- β -CD, BCL/HP- β -CD inclusion complex, blank CS/HA NPs, or BCL/HP- β -CD-loaded NPs, with concentrations expressed as equivalent BCL molar concentrations. Cultures treated with vehicle only (0.1% DMSO) served as controls (CTR).

4.4.10.2. Cell viability assay (SRB assay)

To evaluate cell viability and proliferation, the sulforhodamine B (SRB) assay was conducted. This colorimetric method relies on the ability of SRB, an anionic dye, to electrostatically bind to basic amino acid residues of cellular proteins under mildly acidic conditions. After fixation and staining, the amount of bound dye, quantified spectrophotometrically, is directly proportional to the total cellular protein content and, consequently, to the number of viable cells. MDA-MB-231 cells were seeded in 24-well plates (Greiner Bio-One, Italy) at a density of 45,000 cells/well and, after 24 h of growth, treated with free BCL, HP- β -CD, BCL/HP- β -CD inclusion complex, blank CS/HA NPs, or BCL/HP- β -CD-loaded NPs at

concentrations ranging from 25 to 100 μ M of BCL equivalent. Control cells were treated with the vehicle alone (DMSO 0.1%). At the end of the incubation period (72 h), the cells were first fixed in the wells with a 10% (v/v) trichloroacetic acid (TCA) solution for 1 hour at 4°C. Subsequently, after washing the cells with distilled water to remove acid residues, they were incubated for 30 minutes in the dark at room temperature with a 0.4% (w/v) SRB solution in 1% acetic acid. Four washes with 1% acetic acid were then performed to remove unbound SRB. The dye was finally solubilized in 300 μ L of 10 mM Tris-base under agitation for 5 minutes. Absorbance was measured using a spectrophotometer reader (GloMax® Discover System, Promega, TM397) at a wavelength of 565 nm. Results were reported as the percentage of viable cells relative to untreated controls. Additionally, the LC₅₀ and fiducial limits (95% confidence interval) were calculated using the Litchfield and Wilcoxon method.

4.4.11. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data from triplicate experiments are expressed as mean $\pm$ SD. Group differences were evaluated by one- or two-way ANOVA, followed by Bonferroni or Dunnett post-hoc tests. A p-value < 0.05 was considered statistically significant.

4.5. Results and Discussion

BCL is an orally administered NSAA that exerts its therapeutic effect through competitive inhibition of AR⁺. This specific mechanism has made it a reference drug in several androgen-dependent disorders and, most notably, in oncology for

the treatment of PC, with its use now being extended to other AR+ malignancies. Despite this broad pharmacological relevance, the clinical impact of BCL is critically limited by its hydrophobic nature. To address these issues, a novel delivery system was engineered in which BCL was first complexed with HP- β -CD (1:1 molar ratio), a water-soluble CD, in order to improve its solubility. The resulting BCL/HP- β -CD complex was then encapsulated in CS NPs cross-linked with HA. This dual strategy was designed to obtain a nanosystem capable of providing a water-dispersible form of BCL with controlled and sustained release, while also enabling active targeting to the tumor site and thereby enhancing the drug's pharmacological potential. The resulting formulations underwent a comprehensive technological characterization in order to assess their pharmaceutical suitability, with TGA providing additional confirmation of thermal stability and drug incorporation.

4.5.1. Technological profiling of BCL/HP- β -CD-loaded chitosan nanoparticles and their blank counterparts

The hybrid nanosystem containing the BCL/HP- β -CD inclusion complex was prepared using the ionotropic gelation method, selected for its operational simplicity and mild reaction conditions. Conducted in a weakly acidic aqueous environment, this technique relies on the electrostatic interaction between the cationic polymer CS, positively charged through protonation of its amino groups under these conditions, and an anionic agent, here represented by HA. Such interactions drive the spontaneous self-assembly of colloidal NPs in water, typically ranging in size from 100 to 2000 nm. This approach is not only straightforward but also inherently safe, as it is conducted entirely in water and

avoids the use of organic solvents. Consequently, all components, including the active drug, must be water-compatible [103,104]. Since BCL is poorly soluble in water, its solubility was enhanced through prior complexation with HP- β -CD, an FDA-approved CD for parenteral, oral, rectal, dermal, and ocular administration, which is highly water-soluble compared to native β -CD [105]. The inclusion complex was previously investigated by De Gaetano *et al.* (2022) [81], who reported an approximately tenfold increase in the aqueous solubility of BCL following complexation. The nanosystems were then prepared by incorporating the lyophilized BCL/HP- β -CD complex into the CS solution, followed by the dropwise addition of an aqueous solution of the cross-linking agent.

Table 4.1. Encapsulation efficiency (EE %), yield, and drug content (DC %) of CS/HA NPs obtained in the absence (A) and presence (B) of the BCL/HP- β -CD inclusion complex. For BCL/HP- β -CD-loaded NPs, the percentage of HP- β -CD encapsulated is also reported. Data are expressed as mean $\pm$ SD.

<i>Samples</i>	EE % $\pm$ SD	Encapsulated HP-β-CD % $\pm$ SD	Yield % $\pm$ SD	DC % $\pm$ SD
(A) Blank NPs	N.A.	N.A.	72.9% $\pm$ 3.3	N.A.
(B) BCL/HP-β-CD-loaded NPs	90.4% $\pm$ 2.1	90.3% $\pm$ 5.0	67.8% $\pm$ 2.5	6.4% $\pm$ 1.6

Notes: N.A. = not applicable.

Table 4.1 reports the EE %, production yield, and DC % of the nanosystems, as well as the EE of HP- β -CD for the BCL/HP- β -CD-loaded NPs. A series of samples were prepared either without (A) or with (B) the BCL/HP- β -CD inclusion complex.

Production yield of the NPs remained relatively consistent across all samples, ranging from $67.8\% \pm 2.5$ for the BCL/HP- β -CD-loaded NPs to $72.9\% \pm 3.3$ for the blank NPs. The observed consistency indicates that ionotropic gelation is a solid approach for producing NPs with reproducible features.

With regard to the drug-loaded NPs specifically, EE % was particularly high, reaching $90.4\% \pm 2.1$. This high EE % reflects the effectiveness of the prior complexation of BCL with HP- β -CD, which enhanced the drug's water compatibility and facilitated its stable incorporation within the CS/HA matrix during NP formation. In parallel, the amount of HP- β -CD actually encapsulated was also high, around $90.3\% \pm 5.0$. This elevated CD encapsulation can be attributed to the high water solubility of this macrocycle, which makes it an ideal candidate for the preparation of CS-based hybrid NPs *via* the ionotropic gelation method, which, as noted above, is carried out entirely in aqueous solution. The encapsulation was determined using a colorimetric method based on the interaction between HP- β -CD and the colored indicator phenolphthalein, allowing indirect quantification of the fraction of CD not incorporated into the NPs. The close correspondence between the EE % of BCL and the EE % of HP- β -CD indicates that the inclusion complex is efficiently incorporated into the NPs. From a technological perspective, these findings are particularly significant, as they demonstrate that the combination of ionotropic gelation and CD complexation constitutes a reliable strategy for encapsulating lipophilic drugs in water-dispersible nanoparticulate systems.

The DC % also displayed high reproducibility, with a value of $6.4\% \pm 1.6$, indicating that the NPs maintain a consistent and predictable loading of BCL relative to their mass. It is interesting to note that, despite the relatively large size

of the BCL/HP- β -CD complex compared to the free drug, the formulation efficiently accommodates it without compromising NP formation or production yield.

Table 4.2. Hydrodynamic diameter (D_H), polydispersity index (PDI), and zeta potential (ζ) of CS/HA NPs obtained in the absence (A) and presence (B) of the BCL/HP- β -CD inclusion complex. Data are expressed as mean $\pm$ SD.

<i>Samples</i>	<i>Hydrodynamic diameter (D_H) $\pm$ SD (nm)</i>	<i>Polydispersity index (PDI) $\pm$ SD</i>	<i>Zeta potential (ζ) $\pm$ SD (mV)</i>
<i>(A) Blank NPs</i>	111.73 $\pm$ 8.63	0.20 $\pm$ 0.002	-31.6 $\pm$ 1.39
<i>(B) BCL/HP-β-CD-loaded NPs</i>	167.19 $\pm$ 8.31	0.19 $\pm$ 0.008	-30.7 $\pm$ 1.14

Table 4.2. reports the key technological parameters related to the particle size and surface charge of the nanosystems, both blank and loaded with the BCL/HP- β -CD inclusion complex. These parameters are essential for characterizing the physicochemical properties of the NPs and, consequently, for predicting their behavior in biological environments, including maintenance of particle size in suspension, consistent cellular uptake, and efficient biodistribution, as well as other relevant aspects of *in vivo* performance [106].

The D_H of the NPs shows a clear increase upon loading with the BCL/HP- β -CD inclusion complex. Blank CS/HA NPs exhibit a relatively small and uniform size, with a mean D_H of 111.73 $\pm$ 8.63 nm. Upon encapsulation of the BCL inclusion complex, the mean D_H increases to 167.19 $\pm$ 8.31 nm. This increase is consistent with the expected behavior for drug-loaded NPs, where the internal incorporation

of a substance, particularly a bulky supramolecular complex such as BCL/HP- β -CD, leads to expansion of the polymeric matrix.

The PDI provides information on the uniformity of particle size distribution within a sample. Both blank and BCL/HP- β -CD-loaded NPs exhibit low PDI values (<0.3), indicative of a narrow and homogeneous size distribution. Such homogeneity is essential in pharmaceutical applications, as it ensures uniform cellular interaction, predictable pharmacokinetics, and, importantly, reduced variability in drug release profiles. The slight reduction in PDI upon BCL loading may reflect a stabilizing effect of the inclusion complex on NP self-assembly during the ionotropic gelation process.

ζ measurements reveal that both blank and BCL/HP- β -CD-loaded NPs possess consistently negative surface charges, approximately -31.6 ± 1.39 mV and -30.73 ± 1.14 mV, respectively. This behavior is in line with other systems reported in the literature in which HA is employed as the cross-linking agent [107–109]. Unlike NPs reticulated with smaller agents such as TPP, which often display positive surface charges, HA-based NPs retain a strong negative ζ due to the intrinsic anionic character of HA, arising from its deprotonated carboxyl groups in aqueous solution [110]. It is important to emphasize that the net surface charge is strongly influenced by the ratio between CS and the cross-linker [111]. In CS/HA NPs, when the CS/HA ratio is not excessively high (as in the present formulation using 10 mg of CS and 3 mg of HA, which ensures a significant HA content) the bulkier HA chains tend to dominate the particle surface, exposing their negatively charged groups and thereby contributing to a net negative ζ [108]. Conversely, in CS/TPP systems, the amount of TPP typically employed does not fully neutralize the cationic amino groups of CS, leaving residual positive charges exposed on the

surface and often resulting in a positive ζ [112]. Thus, the negative potential observed in CS/HA NPs reflects both the inherent anionic character of HA and its preferential surface localization relative to CS, particularly under balanced compositional conditions.

ζ is a primary indicator of colloidal stability, reflecting the electrostatic repulsion between particles. The high negative potential of around ± 30 mV indicates that these NPs are highly stable in aqueous solution and resist aggregation. Importantly, the encapsulation of the BCL/HP- β -CD inclusion complex does not alter this behavior, demonstrating that the nanosystems maintain their electrostatic stability even after drug loading.

In view of the fact that the results obtained with this formulation strategy were consistently positive across all the technological parameters evaluated, the nanosystems were judged to be suitable for pharmaceutical application. Consequently, no further optimization attempts were undertaken, as the system already exhibited satisfactory performance for the objectives of this study.

4.5.2. Thermogravimetric characterization

To assess the successful incorporation of the BCL/HP- β -CD inclusion complex within the NPs and to evaluate potential improvements in thermal stability, TGA was performed. The comparison included all individual components in their free form (BCL and HP- β -CD), the lyophilized inclusion complex, the unloaded (blank) and loaded NPs, and a physical mixture obtained by blending blank NPs with the lyophilized complex. For the purpose of solid-state characterization, the samples were lyophilized in the absence of cryoprotectants (specifically trehalose) to avoid alterations in the thermal profiles.

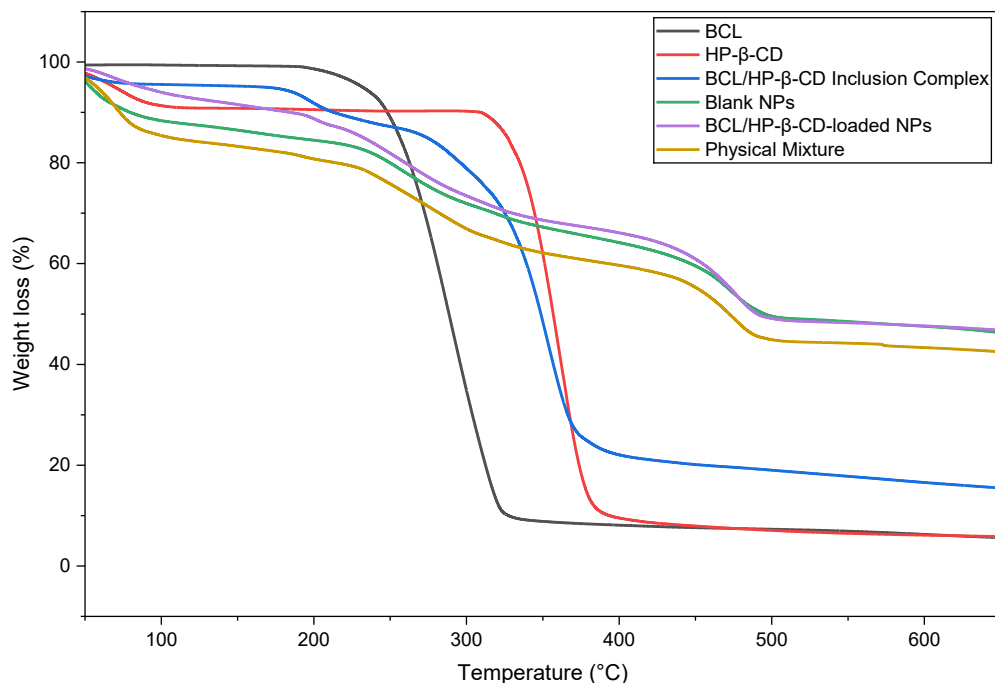


Figure 4.4. TGA profiles of free BCL (black trace), HP-β-CD (red trace), the lyophilized BCL/HP-β-CD inclusion complex (blue trace), blank nanoparticles (green trace), BCL/HP-β-CD-loaded nanoparticles (violet trace), and the physical mixture (orange trace). All samples exhibit characteristic multistage degradation patterns, with enhanced thermal stability observed for both the inclusion complex and the BCL/HP-β-CD-loaded nanoparticles. Analyses were performed over the temperature range of 20-800 °C under a nitrogen atmosphere.

The TGA curve of free BCL (black trace, Figure 4.4) displays the typical thermal instability of an organic compound undergoing progressive decomposition. From room temperature up to approximately 200 °C, only a minimal and gradual mass loss is detected (100 → 98.6 %), consistent with the absence of residual solvents or moisture, as expected for a pure compound. Above ~250 °C ($T_{\text{onset}} = 252.77$ °C), the mass loss becomes more pronounced, reaching its maximum degradation rate

at $T_{\max} = 290.71\text{ }^{\circ}\text{C}$, where decomposition proceeds rapidly and irreversibly. The single-step nature of this transition suggests a concerted molecular breakdown rather than the formation of intermediate stable species. Complete degradation occurs by approximately $325\text{ }^{\circ}\text{C}$, leaving a final residue of $\sim 5.16\%$ at temperatures above $700\text{ }^{\circ}\text{C}$.

The TG profile of HP- β -CD (red trace, Figure 4.4.) reflects the characteristic degradation pattern of CD derivatives. In the initial temperature range (room temperature to $\sim 150\text{ }^{\circ}\text{C}$), a slight weight loss is observed ($100 \rightarrow 90\text{--}95\%$), corresponding to the release of both surface moisture and water molecules accommodated within the hydrophilic cavities of the cyclic oligosaccharide [114,115]. This behaviour is typical of CDs and arises from their pronounced hygroscopicity, which is further enhanced in the hydroxypropylated derivative by the presence of additional hydroxyl groups capable of forming hydrogen bonds with water [115]. Beyond this dehydration step, HP- β -CD remains thermally stable up to approximately $300\text{ }^{\circ}\text{C}$, after which a marked decomposition process begins. The main decomposition starts at $T_{\text{onset}} = 331.82\text{ }^{\circ}\text{C}$, reaches $T_{\max} = 365.46\text{ }^{\circ}\text{C}$, and continues up to $450\text{--}500\text{ }^{\circ}\text{C}$, resulting in $>90\%$ mass loss and leaving a minimal residue ($\sim 5.17\%$), attributable to carbonaceous char formation.

The lyophilized HP- β -CD/BCL inclusion complex (blue trace, Figure 4.4.) exhibits a thermal profile indicative of supramolecular interactions between the host CD and the hydrophobic guest drug. In the low-temperature range (room temperature to $\sim 150\text{--}200\text{ }^{\circ}\text{C}$), the mass loss is markedly reduced compared to pure HP- β -CD, suggesting a significant decrease in hygroscopicity. This effect can be ascribed to the inclusion of the hydrophobic BCL molecule within the CD cavity, which partially blocks water access to both the hydroxyl-rich outer surface and the inner

cavity. Upon further heating, the main degradation process begins at $T_{\text{onset}} = 308.90$ °C, an intermediate value between that of free BCL (252.77 °C) and HP- β -CD (331.82 °C). This shift reveals a synergistic thermal behaviour: inclusion within the CD cavity delays BCL degradation while slightly lowering the decomposition onset of HP- β -CD due to modified intermolecular hydrogen bonding and redistribution of electron density upon complexation [116–118]. The maximum decomposition rate ($T_{\text{max}} = 352.68$ °C) occurs at a temperature close to that of HP- β -CD, indicating that the primary degradation event is governed by the host matrix. The process completes around 450 °C, leaving a residual mass of ~12.6 % above 700 °C. This higher char yield, compared with the ~5 % residues of the individual components, suggests the formation of a more stable carbonaceous phase. This comparison highlights the benefits of inclusion: the complex exhibits enhanced thermal stability ($\Delta T_{\text{onset}} = +56$ °C vs. free BCL), which is advantageous for pharmaceutical formulations requiring thermal processing, although a sensitivity to dehydration is observed.

In sharp contrast, the TG curves of the CS/HA NPs exhibit the characteristic multistage degradation profile of crosslinked polysaccharide systems [119].

The blank NPs, obtained by ionotropic gelation of CS with HA in the absence of the complexed drug, exhibit an initial weight loss associated with the hygroscopic nature of the polymeric constituents. From room temperature to approximately 150 °C, the sample mass gradually decreases from 100 % to about 88.4 % at 100 °C. This initial stage mainly corresponds to the evaporation of surface-adsorbed and hydrogen-bonded water molecules, occurring without any structural degradation, in agreement with the dehydration behaviour reported for native CS (49-167 °C, ~20 % mass loss) [119]. The reduced mass loss observed in this context is likely

due to partial stabilization of the polymer network through HA-mediated ionic crosslinking [120]. The main thermal decomposition takes place between approximately 220 and 230 °C, reaching its maximum rate near 254.6 °C. This stage involves depolymerization and dehydration of saccharide units, together with the cleavage of glycosidic and ionic bonds within the CS/HA matrix. At higher temperatures, a secondary degradation phase occurs around 475 °C, attributed to deep oxidative decomposition, carbonization, and volatilization of acetylated or deacetylated fragments, leading to a gradual mass loss up to 500-600 °C [119]. The final residue (~38.5 % above 700 °C) exceeds that typically reported for native CS (~36.4 %), suggesting that HA crosslinking promotes the formation of thermally stable char.

The BCL/HP- β -CD-loaded NPs display a similar multistep thermal pattern but with distinct shifts toward higher temperatures and reduced overall mass loss, confirming successful encapsulation and improved stabilization within the hybrid system. The initial dehydration step is less pronounced, with a remaining mass of approximately 94.0 % at 100 °C, denoting lower hygroscopicity, likely caused by the occupation of surface pores by the inclusion complex. The main decomposition begins around 230-240 °C, comparable to that of the blank NPs but occurring slightly later and more gradually, reflecting the overlapping degradation of the polymeric and encapsulated components. A subsequent degradation process is observed around 477 °C, corresponding to oxidative breakdown and carbonization, similar to that of the blank NPs but shifted by about +2-3 °C. The residual mass above 700 °C increases to ~41.4 %, indicating enhanced thermal stability and char formation.

The physical mixture, prepared by manually blending blank CS/HA NPs with the lyophilized BCL/HP- β -CD inclusion complex, exhibits an additive rather than synergistic thermal behaviour, indicating the absence of true inclusion or molecular-level interactions between the components. The initial dehydration is more pronounced, with the mass decreasing to approximately 83.3 % at 150 °C, reflecting higher moisture uptake in the absence of stabilizing interactions. The main decomposition occurs near 275 °C, corresponding to the combined degradation of the CS/HA polymeric matrix (~255 °C) and the BCL/HP- β -CD inclusion complex (~353 °C). The overall mass decreases to about 66.9 % at 300 °C, showing an additive behaviour typical of a simple physical mixture and consistent with the lack of intermolecular interactions between the two components. These findings confirm that physical blending leads to a mere superposition of the individual thermal events, without the stabilizing effects observed in the BCL/HP- β -CD-loaded NPs, where true host–guest interactions occur within the hybrid network.

These results confirm that the BCL/HP- β -CD inclusion complex is effectively incorporated within the CS/HA polymeric matrix. This is evidenced by the reduced water loss observed during the initial stages of the TGA analysis, in contrast to the behaviour of the blank NPs and the physical mixture. Furthermore, the incorporation of the complex leads to an overall improvement in thermal stability, mitigating the typical behaviour of pure BCL, which tends to undergo single-step decomposition at relatively low temperatures. These findings demonstrate the successful formation of a stable hybrid system, in which the inclusion complex interacts strongly with the CS/HA matrix, resulting in greater structural integrity and enhanced thermal resistance.

4.5.3. Controlled bicalutamide release and concurrent HP- β -CD permeation from the nanosystems

For a particulate system to exert its therapeutic effect, it is essential that the encapsulated active compound is released in a controlled and predictable manner. This kinetic profile is pivotal, as it directly influences drug bioavailability and pharmacological outcomes in subsequent *in vitro* evaluations [113]. In polymeric nanosystems such as those based on CS and HA, drug release typically occurs through a combination of mechanisms: initial desorption from the NP surface, diffusion through the porous polymeric matrix or walls, and eventual erosion or dissolution of the polymer network [114]. These processes are affected by several factors, including NP composition, environmental pH, and the physicochemical properties of the drug, such as solubility and affinity for the carrier [115,116].

To elucidate the release profile of BCL from the hybrid nanosystem developed in this study, an *in vitro* release assay was conducted in comparison with the BCL/HP- β -CD inclusion complex, which serves as a water-soluble reference form of the otherwise lipophilic drug. The experiments were carried out using the dialysis bag method over a 120-hour period, with PBS (pH 7.4) as the release medium. All samples were analyzed in triplicate to ensure statistical reliability.

It is reasonable to assume that BCL is initially released in the form of the inclusion complex, which subsequently dissociates, allowing diffusion of free BCL across the semi-permeable membrane.

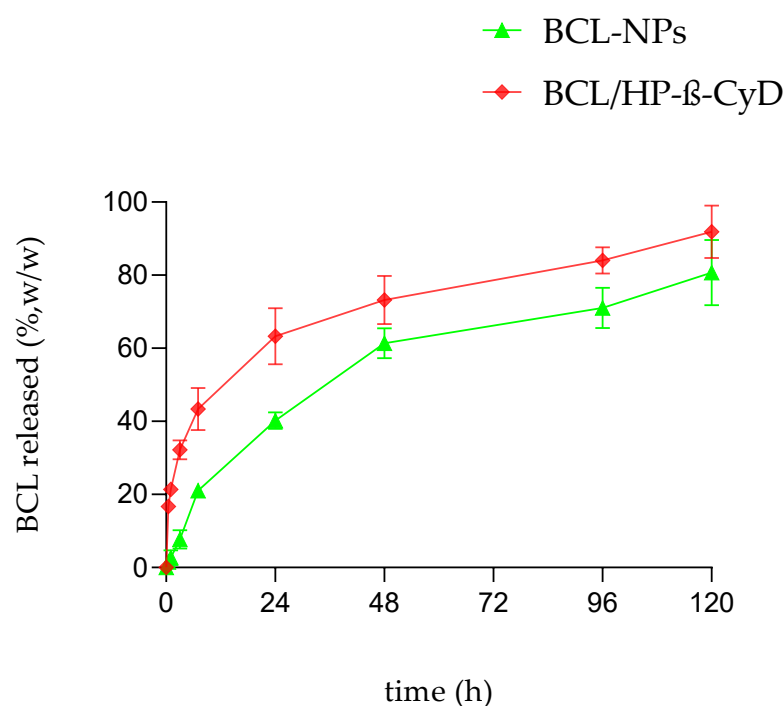


Figure 4.5. *In vitro* release profiles of BCL from the BCL/HP-β-CD inclusion complex (red trace) and from chitosan nanoparticles (green trace) over 120 hours. Data represent the mean $\pm$ SD of three independent experiments.

Figure 4.5. shows the cumulative release profiles of the free BCL/HP-β-CD inclusion complex (red trace) and BCL encapsulated in CS/HA NPs (green trace). Both formulations exhibit a gradual release of BCL; however, notable differences highlight the role of the NPs in prolonging drug delivery. The free BCL/HP-β-CD complex exhibits a rapid initial release, reaching approximately 40% cumulative release within the first 24 hours, increasing to around 60% at 48 hours, and approaching near-complete release by 120 hours. In contrast, the NP-encapsulated BCL displays a markedly slower release profile: approximately 20% at 24 hours, 40% at 48 hours, 50% at 72 hours, 60% at 96 hours, and stabilizing at around 70% by 120 hours, leaving an estimated 30% of the drug retained within the nanosystem.

This delayed release profile indicates that the CS/HA matrix effectively retards diffusion, likely due to electrostatic interactions between the positively charged CS and negatively charged HA, which form a compact gel-like network. Importantly, the absence of a pronounced burst release suggests homogeneous entrapment of the BCL/HP- β -CD complex throughout the polymer matrix [117].

Table 4.3. Kinetic parameters and correlation coefficients (R^2) for the *in vitro* release of BCL from BCL/HP- β -CD-loaded nanoparticles.

<i>BCL/HP-β-CD-loaded NPs</i>	<i>Zero order</i>		<i>First order</i>	
	R^2	K_0 (d⁻¹)	R^2	K_1 (d⁻¹)
	0.7103 ± 0.0008	0.7537	0.9213 ± 0.0075	0.0318
	<i>Higuchi</i>		<i>Korsmeyer-Peppas</i>	
	R^2	K_H (d^{-1/2})	R^2	n
	0.9464 ± 0.0006	9.3723	0.9341 ± 0.0194	0.6826

To further elucidate the release kinetics, the experimental data were then fitted to established mathematical models, as summarized in Table 4.3. The superior correlation coefficient obtained for the Higuchi model ($R^2 = 0.9464 \pm 0.0006$) indicates that drug release from the NPs is predominantly governed by diffusion, consistent with Fickian kinetics, where the release rate is proportional to the square root of time (Figure 4.6.). Complementarily, analysis using the Korsmeyer-Peppas model yielded an exponent $n = 0.6826$ ($R^2 = 0.9341 \pm 0.0194$), which falls within the 0.45-0.89 range indicative of anomalous (non-Fickian) transport. This suggests

that, in addition to diffusion, polymer chain relaxation or minor erosion also contributes to the release. In contrast, the poorer fits observed for the zero-order ($R^2 = 0.7103 \pm 0.0008$) and first-order ($R^2 = 0.9213 \pm 0.0075$) models reinforce that release is not simply constant or concentration-dependent but modulated by the nanoparticulate architecture.

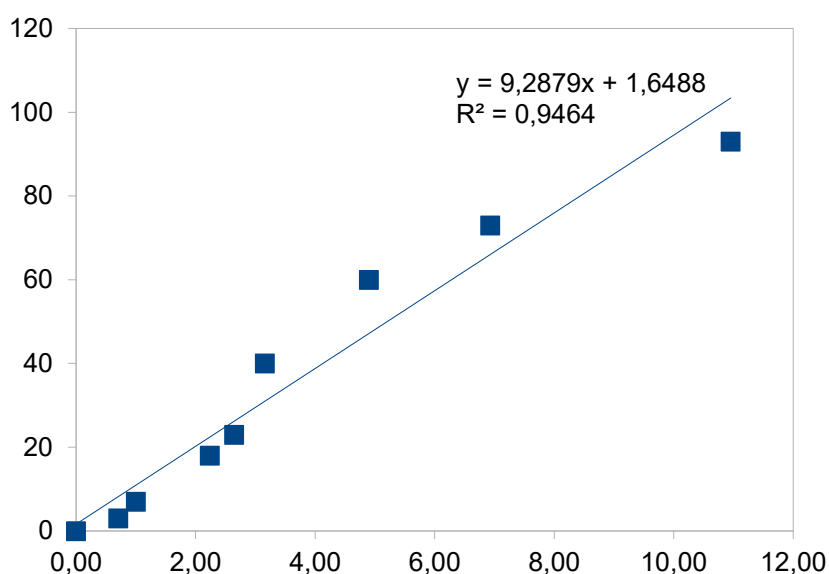


Figure 4.6. Cumulative percentage of BCL released from the BCL/HP- β -CD-loaded nanoparticles as a function of the square root of time, fitted according to the Higuchi kinetic model. The linear regression equation is $y = 9.2879x + 1.6488$, with a correlation coefficient $R^2 = 0.9464$, indicating a diffusion-controlled release mechanism.

To further probe the dynamics of the hybrid system, the permeation of HP- β -CD itself across the dialysis membrane was monitored in parallel, as depicted in Figure 4.7. The profile exhibits a gradual ascent, achieving approximately 35% cumulative permeation within the first 7 hours and progressively escalating to around 70% by the 120-hour endpoint, closely mirroring the sustained release kinetics observed for BCL from the NPs. This parallelism suggests that BCL and HP- β -CD are co-

released predominantly as an intact inclusion complex, with HP- β -CD facilitating BCL solubilization during transit through the CS/HA matrix. Upon release into the medium, the complex dissociates, allowing both components to diffuse independently across the membrane. This synchronized release highlights the stability of the inclusion complex within the NPs, preventing early dissociation and promoting a controlled and harmonized delivery, an attribute potentially advantageous for sustaining therapeutic drug concentrations *in vivo*.

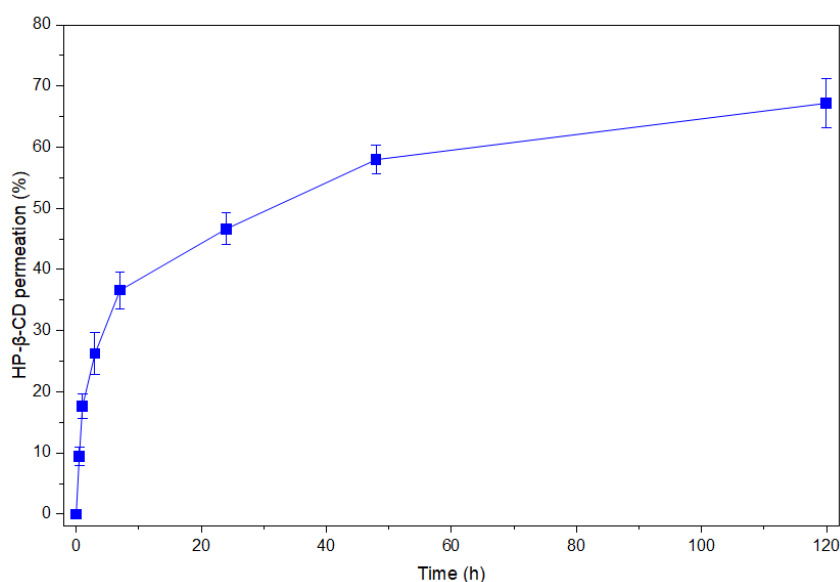


Figure 4.7. Cumulative permeation of HP- β -CD from BCL/HP- β -CD-loaded nanoparticles across the dialysis membrane over 120 hours. Data represent the mean $\pm$ SD of three independent experiments.

4.5.4. *In vitro biological studies*

To evaluate the cytotoxic effect of the antiandrogenic drug-loaded NPs on MDA-MB-231 BC cells, commonly used as a model of TNBC LAR subtype, a comparative study was carried out using the free drug (BCL) and its inclusion complex (BCL/HP- β -CD). Cells were treated with free BCL, the BCL/HP- β -CD

inclusion complex, or the NP formulation at concentrations of 25, 50, 75, and 100 μM for 72 hours.

As shown in Figure 4.8, exposure to free BCL resulted in a concentration-dependent reduction in cell viability, with an LC_{50} value of 98.9 μM (95% C.L. 80-122 μM). The incorporation of BCL into HP- β -CD inclusion complexes moderately enhanced its cytotoxic effect, lowering the LC_{50} to 75.7 μM (95% C.L. 66-87 μM). Notably, encapsulation of BCL within CS/HA NPs produced a more pronounced effect, with an LC_{50} of 56.5 μM (95% C.L. 49-65 μM), corresponding to roughly half the value observed for the free drug.

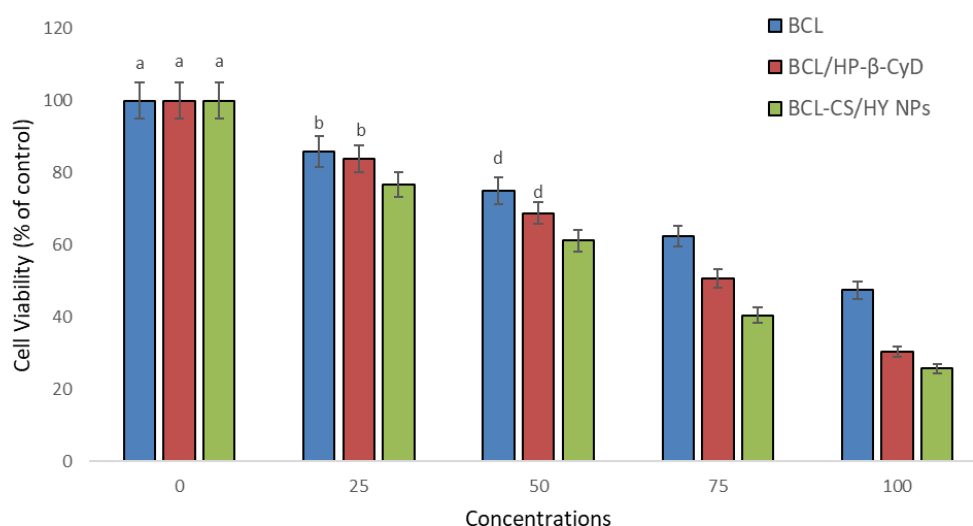


Figure 4.8. Cytotoxicity evaluation of MDA-MB-231 cells treated with different bicalutamide formulations. Cells were exposed for 72 h to BCL (25-100 μM), BCL/HP- β -CD inclusion complex (25-100 μM , expressed as BCL equivalent), and BCL/HP- β -CD-loaded nanoparticles (25-100 μM , expressed as BCL equivalent). Data are expressed as cell viability (%) relative to untreated control (CTR). Each point represents the mean $\pm$ SD of three independent experiments. Means sharing the same letter within the same concentration are not significantly different from each other ($p > 0.05$); the absence of letters indicates significant differences among treatments at the same concentration.

Importantly, neither free HP- β -CD (in its uncomplexed form) nor blank CS/HA NPs induced any cytotoxic effects compared with control cultures (data not shown), confirming that the observed decrease in cell viability was attributable to BCL and not to the carrier materials. These findings demonstrate that both inclusion systems enhance the cytotoxic efficiency of BCL, with a more significant improvement achieved through NP encapsulation.

It is reasonable to assert that the BCL/HP- β -CD inclusion complex improves the therapeutic index of BCL by modulating both its pharmacokinetic and pharmacodynamic properties. The complex enhances BCL solubility and stability, facilitating diffusion through cellular layers and promoting accumulation in target tissues. Additionally, the β -CD units exposed on the polymer surface may interact with membrane components, such as cholesterol and phospholipids, partially destabilizing the lipid bilayer and thereby promoting cellular uptake [118,119]. These combined effects likely contribute to the observed increase in cytotoxicity compared to free BCL.

Furthermore, when BCL is encapsulated within CS/HA NPs, its biological activity is further enhanced. This can be attributed to several concurrent mechanisms: first, the nanoscale size and mucoadhesive properties of the CS/HA acid matrix facilitate cellular internalization through endocytic pathways; second, the HA component promotes selective interaction with CD44 receptors, which are overexpressed in TNBC cells, enabling a more efficient and targeted delivery of the drug. In addition, the presence of the HP- β -CD inclusion complex within the NP core ensures sustained drug solubilization and controlled release, thus maintaining cytotoxic concentrations over prolonged periods.

Although numerous delivery systems for BCL have been described in the literature, a critical comparison is necessary to highlight the conceptual and functional advances introduced by the present approach.

4.5.6. Critical comparison with previously reported bicalutamide delivery systems

As discussed in Section 4.3, several formulation strategies have been explored to overcome the main biopharmaceutical limitations of BCL. While these approaches have demonstrated specific advantages, most of them address individual formulation challenges in a reductionist manner, focusing on a single performance parameter (most commonly solubility or dissolution) rather than on the broader requirements of effective anticancer drug delivery. This aspect is particularly relevant in the context of antitumor therapy, which imposes stricter demands in terms of drug release control, tumor selectivity, and biological responsiveness compared with other therapeutic settings.

Nanodispersions and nanocrystalline formulations developed by Li *et al.* and Meer and co-workers primarily enhance BLC dissolution by increasing surface area and, in some cases, by partially reducing crystallinity, resulting in improved systemic exposure [89,90]. Despite their effectiveness from a biopharmaceutical standpoint, these systems mainly function as bioavailability-enhancing formulations and remain essentially passive carriers. They lack control over drug release kinetics and do not provide mechanisms for preferential tumor accumulation. Consequently, their therapeutic efficacy is largely dependent on systemic exposure and does not address issues related to off-target distribution or insufficient drug concentrations at the tumor site, offering limited innovation from a drug delivery perspective.

Polymeric NPs based on PLGA, such as those proposed by Pokharkar *et al.* (2012) and Ray *et al.* (2016), represent a further advancement by enabling sustained drug release and enhanced cellular uptake [91,95]. However, these systems do not intrinsically overcome the poor aqueous solubility of BCL and do not incorporate active targeting strategies. Although more sophisticated than simple nanodispersions, they still operate as relatively generic delivery platforms, not specifically designed for a defined tumor phenotype.

CD inclusion complexes, described in detail in Section 4.3, offer an elegant supramolecular solution to the solubility issue of BCL and have demonstrated clear benefits in terms of dissolution rate and *in vitro* biological activity [81]. Nevertheless, when administered as standalone systems, they generally result in rapid drug release and provide limited control over spatial and temporal drug distribution within solid tumors. Magnetic nanoparticle-based systems described by Lee *et al.* (2012) rely on external magnetic fields for targeting and have so far been evaluated mainly in preclinical studies, with limited translational applicability [92].

In contrast, the delivery system developed in the present work is distinguished by the rational integration of multiple complementary functionalities within a single nanoplatform. The initial complexation of BCL with HP- β -CD ensures effective solubilization and compatibility with aqueous processing, while subsequent encapsulation into CS NPs provides protection of the drug and enables controlled and sustained release. Importantly, unlike previously reported systems that primarily aim to optimize the general pharmaceutical performance of BCL, the CS/HA NPs incorporating the BCL/HP- β -CD inclusion complex were specifically designed for application in antitumor therapy. This approach is particularly relevant

for tumors potentially responsive to anti-androgen treatment and characterized by overexpression of HA receptors. By combining solubility enhancement, controlled release, and receptor-mediated targeting within a single nanoplatform, the proposed system addresses both formulation-related and disease-specific challenges. From a translational perspective, this multifunctionality represents a critical step toward maximizing the therapeutic potential of BCL in clinical contexts where conventional delivery strategies are unlikely to be sufficient.

4.6. Conclusions

In this work, CS NPs cross-linked with HA were successfully developed as a novel delivery system for BCL, an antitumor drug with established use in PC therapy and promising activity against TNBC. To overcome BCL's intrinsic poor aqueous solubility and enable its encapsulation, the drug was first incorporated into a highly water-soluble HP- β -CD inclusion complex, which proved to be a key strategy for achieving stable loading into the polymeric nanosystem. The resulting CS/HA NPs exhibited high production yields and a narrow size distribution, with mean D_H below 200 nm, a feature particularly relevant for cellular internalization and tumor accumulation. Their strongly negative surface charge (~ -30 mV) ensured good colloidal stability over time, preventing aggregation and preserving reproducible physicochemical properties essential for reliable biological evaluation. EE was notably high ($\sim 90\%$), with consistent drug loading ($\sim 7\%$), highlighting the suitability of combining ionotropic gelation with inclusion complexation for the formulation of water-dispersible nanosystems containing lipophilic drugs. *In vitro* release studies demonstrated a sustained and controlled release of BCL over more than 120 hours. The release kinetics indicate that diffusion through the polymeric

network is the predominant mechanism, with minimal initial burst release, suggesting homogeneous incorporation of the BCL/HP- β -CD inclusion complex throughout the NP matrix. Parallel monitoring of HP- β -CD permeation confirmed that the inclusion complex remains largely intact during release, supporting its stabilizing role. Thermogravimetric analysis confirmed the successful incorporation of the inclusion complex into the CS/HA NPs and revealed a clear improvement in thermal stability compared to free BCL and to simple physical mixture. The reduced initial water loss and the shift of the main degradation events toward higher temperatures indicate strong interactions between the inclusion complex and the polymeric matrix, resulting in enhanced structural integrity and resistance to thermal degradation. Biological studies on MDA-MB-231 cells showed that both inclusion and encapsulated formulations significantly enhanced the cytotoxic activity of BCL compared with the free drug. While inclusion complexation improved drug solubility and facilitated cellular uptake, the CS/HA nanoparticle system produced an even greater cytotoxic response. This effect can be attributed to the combined contribution of nanoscale size, controlled release behavior, and the presence of HA, which promotes selective interactions with CD44 receptors overexpressed in TNBC cells, leading to enhanced intracellular accumulation and prolonged drug exposure.

Further investigations are currently underway to deepen the structural and morphological characterization of the nanosystem. In particular, SEM analyses are planned to directly visualize NP shape, size, and surface morphology, as well as to gain insight into the spatial organization of the HA chains at the NP interface. In parallel, FTIR studies are being conducted to elucidate the molecular interactions

among CS, HA, HP- β -CD, and BCL and to further clarify the internal architecture of the hybrid system.

In conclusion, this study shows that CS/HA NPs loaded with the BCL/HP- β -CD inclusion complex represent a promising strategy for delivering this NSAA. This approach improves the drug's solubility and stability, allows controlled and sustained release, and could enhance its activity at the cellular level. Moreover, the presence of HA offers potential for targeted delivery to CD44-expressing cancer cells, including aggressive TNBC. The findings of this study lay a solid foundation for future *in vivo* investigations aimed at validating the therapeutic potential of this nanosystem and advancing its development toward clinical application.

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