## TOF-SIMS APPLICATION FOR EVALUATING THE ATOMIC STRUCTURE OF NEW BONE SUBSTITUTE MATERIAL**

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*The aim of this experimental study is to evaluate, in vitro, the chemical composition and the micromorphological structure of a bone substitute material surface. This material is based on calcium triphosphate and hydroxyapatite microgranules. Some results of a preliminary surface study of the above mentioned bioceramic materials are reported. The study has been carried out by means of time-of-flight secondary ion mass spectrometry (TOF-SIMS), complemented by X-ray photoelectron spectrometry (XPS) measurements. Whereas XPS data supplies the average surface composition of the system, TOF-SIMS supplies laterally and depth resolved information on the sample. This preliminary study confirms the properties of osteoconduction and scaffold features of the material. Moreover, a possible osteoinductive capability could be due to the presence of surface micropores, which could help in the attraction of bone morphogenetic protein (BMP), thus promoting the osteogenesis.*

***Keywords:*** *time-of-flight secondary ion mass spectrometry, bone scaffold, bone tissue.*

## ПРИМЕНЕНИЕ ВРЕМЯПРОЛЕТНОЙ МАСС-СПЕКТРОМЕТРИИ ВТОРИЧНЫХ ИОНОВ ДЛЯ ОПРЕДЕЛЕНИЯ АТОМНОЙ СТРУКТУРЫ НОВОГО МАТЕРИАЛА-ЗАМЕНИТЕЛЯ КОСТНОЙ ТКАНИ

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*Проведено экспериментальное исследование по определению in vitro химического состава и микроморфологической структуры поверхности замещающего костную ткань материала. Основой этого материала служат микрогранулы трифосфата кальция и гидроксиапатита. Приведены некоторые результаты предварительного исследования поверхности указанных биокерамических материалов. Исследование выполнено с помощью времяпролетной масс-спектрометрии вторичных ионов (ВП-МСВИ), дополненной измерениями методом рентгеновской фотоэлектронной спектрометрии (РФЭС). С помощью метода РФЭС получены данные о среднем по поверхности компонентном составе системы, а ВП-МСВИ — о распределении состава вдоль поверхности и по глубине образца. Полученные результаты подтверждают соответствие материала требованиям по остеокондуктивным и поддерживающим характеристикам. Благодаря наличию поверхностных микропор, которые могут способствовать привлечению костного морфологического белка, материал должен обладать остеоиндуктивными свойствами, что способствует остеогенезису.*

***Ключевые слова:*** *времяпролетная масс-спектрометрия вторичных ионов, костный каркас, костная ткань.*

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**Introduction.** Secondary ion mass spectrometry imaging (SIMS imaging) is a technique that allows detailed distribution maps of selected elements/compounds at the surface of a material to be obtained and has been applied to several kinds of biomaterials. Following the recent introduction of bone substitute biomaterials in oral surgery, the clinical use requires choices often related to characteristics such as osteoconductive properties, scaffold features, and stimulation of new bone regeneration. Time of flight secondary ion mass spectrometry (TOF-SIMS) is an analytical surface method capable of recording and imaging, with high mass resolution, all elements including hydrogen (which is problematic for many other analysis devices) and molecular fragments during a single measurement. Commercial TOF-SIMS instruments can scan areas up to 500×500 μm by rastering a focused primary ion beam over the region of interest [1–3]. The author's main proposal is to investigate the surface of a common bone synthetic material by using this powerful tool. Nowadays, great attention is focused on polymer/ceramic, three-dimensional scaffolds for bone tissue regeneration, not only in oral surgery but also in the orthopedic field [4–7]. Although the autologous bone still remains the gold standard, there are several conditions for its use like the second surgery site for grafting, morbidity of patients, and limited quantity that can constitute significant limits [8–11]. Other possibilities of the bone graft material are related to both homologous bone grafts and heterologous ones. All these materials offers the clinicians the opportunity of having large quantities of graft although, at the same time, the physiological properties are not the same as that of the autologous bone. For this reason the trade mission was strongly directed to creating several synthetic bone substitutes aimed at recreating a similar microscopic structure for reproducing micro and macro features of the autologous bone graft [12–14].

It was recently documented that, by choosing the correct polymer and ceramic, it was possible to create a useful scaffold material. A possible choice is a composite based on hydroxyapatite (HA) due to its excellent osteoconductivity, biocompatibility, and bioactivity properties. The ideal composite material should be highly compatible with the surrounding biological systems (the bone tissue) and also be biodegradable. The scaffold 3D structure should be characterized by several interconnected porous structures capable of promoting cell adhesion, proliferation, and vascularization, and enabling a controlled supply of bioactive substances that may influence the behavior of incorporated or ingrown cells [15–19].

A synthetic bone grafted material offers a number of advantages. Usually it is an abundant, renewable, biocompatible, nontoxic, and biodegradable polymer. Moreover, it has good mechanical properties. However, it has no bioactivity within the bone tissue. Several research studies have been carried out to establish a direct bond between developed material and natural bone tissue: this consists of the development of a hydroxyapatite layer by means of biomimetic mineralization [20].

Bone regeneration for the treatment of deep bone defects without the application of large bone grafts, exogenous growth factors, or cells still remains a challenge for clinicians and surgeons. Numerous bone substitutes are available to be used as growth factors, carriers, or scaffolds. Practitioners and surgeons need to harvest bone from body sites when performing oral and maxillofacial atrophic ridge reconstruction, and the patient's pain and discomfort related to these procedures are to be avoid [2, 16, 19, 21].

The aim of this study is to evaluate the surface of a common bone graft material using mass spectrometry in order to obtain a significant knowledge of the possible interaction between the surface material and the surrounding bone tissue. The results of this research will allow clinicians to evaluate the biomaterial surface and structure. Particles of the material and its micropore surface, which appears to be crucial for the attraction of bone morphogenetic protein (BMP) and for the activation of osteoblastic cells, favor new bone tissue formation (osteoinduction). On the basis of these premises, an experimental *in vitro* study has been performed on the analysis and characterization of a common biomaterial applied as a bone substitute in oral surgery.

**Experimental.** The biomaterial investigated in the present study is a fully synthetic bone substitute; the product is in the form of biphasic calcium phosphate and hydroxyapatite crystals (TCP and HA) chemically synthesized to ensure a homogeneous distribution of the two phases. The source of the material is polycrystalline aluminum oxide, hydroxyapatite (a mineral of calcium phosphate, which is also the major component of vertebrate bones), partially stabilized zirconium oxide, bioactive glass or glass-ceramics, and polyethylene. The biomaterial is 90% porous, with pore diameter ranging from 100 to 500 μm; it seems that the pores act as a scaffold for the BMP deposition on the surface, stimulating the migration of osteoblasts and therefore the formation of new bone.

This bone substitute is characterized by a dual activity. Firstly, it supports the formation of new bone while maintaining stability: the material is resorbed and replaced by newly formed bone. Secondly, it promotes bone replacement with newly-formed mature bone. The surface of the material was analyzed using the

TOF-SIMS (time-of-flight secondary ion mass spectrometry) method (phase 1) in combination with XPS (X-ray photoelectron spectrometry) measurements (phase 2).

The TOF-SIMS is a highly sensitive analytical technique that can provide information on the composition of the outermost layers of the analyzed surface, either from elemental and/or molecular points of view. The uniqueness of this technique is in the possibility of knowing, with high mass resolution and lateral resolution, the distribution of elements and compounds on the surface. This, in the present study case, allows the shape, size, and chemical nature of the particles present in the investigated composite biomaterial to be highlighted.

The technique involves the bombardment of the sample by a beam of primary ions ($Ga^+$, $Cs^+$, $Ar^+$, or others). Due to the well-known phenomenon of sputtering (Fig. 1), atoms, clusters of atoms, and molecular fragments are emitted from the surface, with a part of these carrying a charge. The charged parts of the sputtered material, the secondary ions, are collected and analyzed by mass with a mass spectrometer (in the present study case, a time-of-flight type) producing a mass spectrum. By using a focused scanning ion beam over the sample area of interest, a mass spectrum of the sputtered material can be obtained from each pixel. Then, the intensity distribution of any peak present in the spectrum can be reconstructed, thereby obtaining a chemical map of the sample surface.

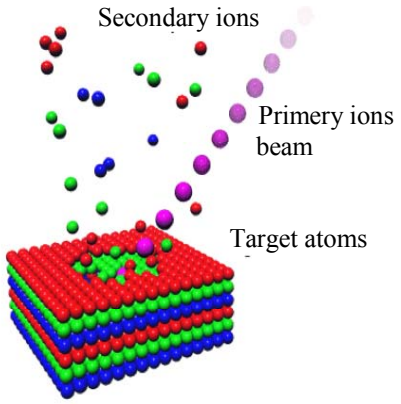


Fig. 1. Sample of sputtering. By using this system, it is possible to know the atomic structure of the analyzed material surface.

In general, the analysis provides a qualitative, not quantitative, evaluation of the elements present at the surface. Also, by operating in the so-called "static SIMS" mode, this technique provides information on the molecular composition of the surface. As electrons are emitted from the surface during ion bombardment, it is possible to obtain secondary electron images by using a suitable electron detector that provides topographic information on the sample similar to those obtained in scanning electron microscopy.

Due to the characteristics of modern time-of-flight mass spectrometers, the technique is very sensitive (the limit of detection being in many cases well below the order of picomoles) and, in virtue of the high mass resolution, it is characterized by its ability to distinguish very small differences between the species on the basis of mass defects. Finally, because the secondary ions originate only from a few (≈1–5) atomic-molecular layers of the outer material, secondary ion mass spectrometry is a surface technique.

X-ray photoelectron spectroscopy (XPS) is a surface analysis technique based on the measurement of the kinetic energy of electrons (photoelectrons) emitted from a material when irradiated with electromagnetic radiation. In the XPS analysis the energy of soft X-rays used is typically within the range of 1000–1500 eV. XPS allows information on the electronic structure of the core levels of atoms to be obtained and hence to identify which atoms are present at the surface and also their chemical environment. In fact, it is possible to identify the elements present on the surface of a sample with atomic number >2, and their oxidation state. Due to the short depth of origin of photoelectrons, XPS is also a surface technique, and the typical analyzed depth is of the order of 5–50 interatomic distances. To obtain information on deeper layers, the sample is subjected to sputtering with argon ions.

**Results and discussion.** A mass spectrometer is today considered the smallest weighing scale in the world ever used. Mass spectrometry (MS) is a unique technique that has an interdisciplinary nature, which freely crosses the borders of physics, chemistry, biology, and medicine. Mass spectrometry application helps scientists to specifically establish the mass of large biomolecular complexes, individual biomolecules, small organic molecules, as well as single atoms and their isotopes [22, 23]. Leadley et al. first applied time-of-flight secondary ion mass spectrometry (TOF-SIMS) and X-ray photoelectron spectroscopy (XPS) to investigate the dissolution of hydroxyapatite in the presence of titanium chloride, suggesting the substitution of titanium ions for calcium in the hydroxyapatite structure [24].

Leonard and Mathieu published a novel investigation in 1999 in which they illustrated the advantages and possible limitations of TOF-SIMS in the field of biochemistry, in the characterization of engineered heterogeneous bioactive surfaces (including biosensors), the combinatorial synthesis of peptides, the molecular imaging of cells, and the quantification of biomolecules in real biological samples [25].

Because of its high sensitivity, TOF-SIMS was used in combination spectrometry XPS forits capability of assessing the outer atomic-molecular layers, thus providing enough reliable information on the presence of compounds barely detectable with XPS. Although more quantitative, it has a sensitivity which is, in order of magnitude, lower than TOF-SIMS. In addition, TOF-SIMS has the ability to provide even more detailed information on organic molecules. This information is, however only qualitative or semi-quantitative due to the effects of the chemical matrix. Belu et al. the flexibility of the TOF-SIMS technique and that the wealth of data produced has generated much interest in its use for biomaterial characterization. Moreover, the authors highlighted the ability to determine the composition, structure, orientation, and spatial distribution of the molecules and chemical structures on the surface [26].

In the present investigation, the secondary electron maps generated in the TOF-SIMS instrument show the presence of a microstructure on the surface of bone ceramic with dimensions of the order of 1–2 μm (Fig. 2). The TOF-SIMS spectra of the positive and negative ions of the resulting bone ceramic exhibit a high amount of Na, Ca, and phosphate. Other masses such as $C_2H$– originating from the organic material present at the surface seem to behave like impurities due to the cutting power of TOF-SIMS (Fig. 3). Several published papers underlined how the atomic constitution of the biomaterial used as scaffold or bone substitute plays a fundamental role in the integration of the graft and for the healing of the treated site. The structure of the biomaterial strongly influences the adhesion of the growth factors related to the new bone formation [27–30]. The spectra in Fig. 3 provide information on the atomic features of the investigated material. In particular, the high presence of calcium and phosphate ions in the spectra are representative of the ideal characteristic for any bone graft material.

In the present investigation, the TOF-SIMS spectra were acquired in a static mode with a spectrometer (Ion TOF-SIMS IV) using a pulsed beam of $^{69}Ga^+$ ion primary (25 keV, 0.1 pA) referring to an area of about 300×300 mm. These results confirm that hydroxyapatite (50–70%) and tricalcium phosphate (30–50%) are the main constituents of this biomaterial (Fig. 4). Also, with TOF-SIMS a two-dimensional map of the surface chemistry of the bone ceramic was created, and chemical images show the presence of $Na^+$ and $Ca^+$.

The surface chemical composition and the contamination of a biomaterial may alter its biological performance. Therefore, techniques allowing a detailed analysis of the surface, such as XPS and TOF-SIMS, can be used in combination with the surface chemical characterization.

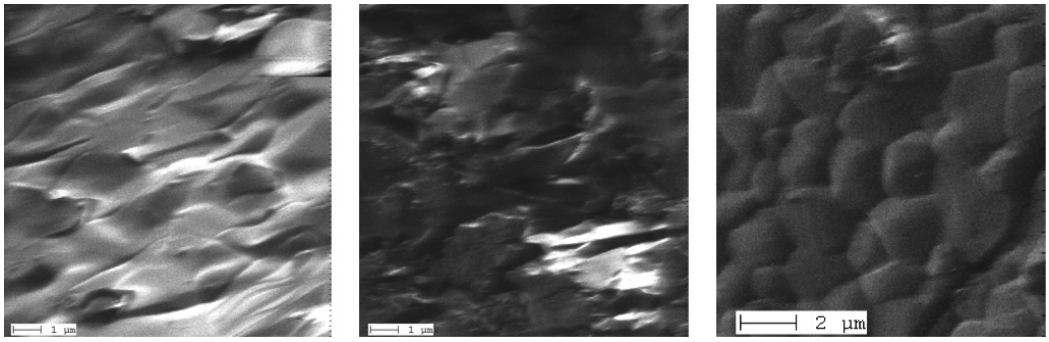


Fig. 2. Ion-induced secondary electron maps showing the topography of the surface of the bone substitute material.

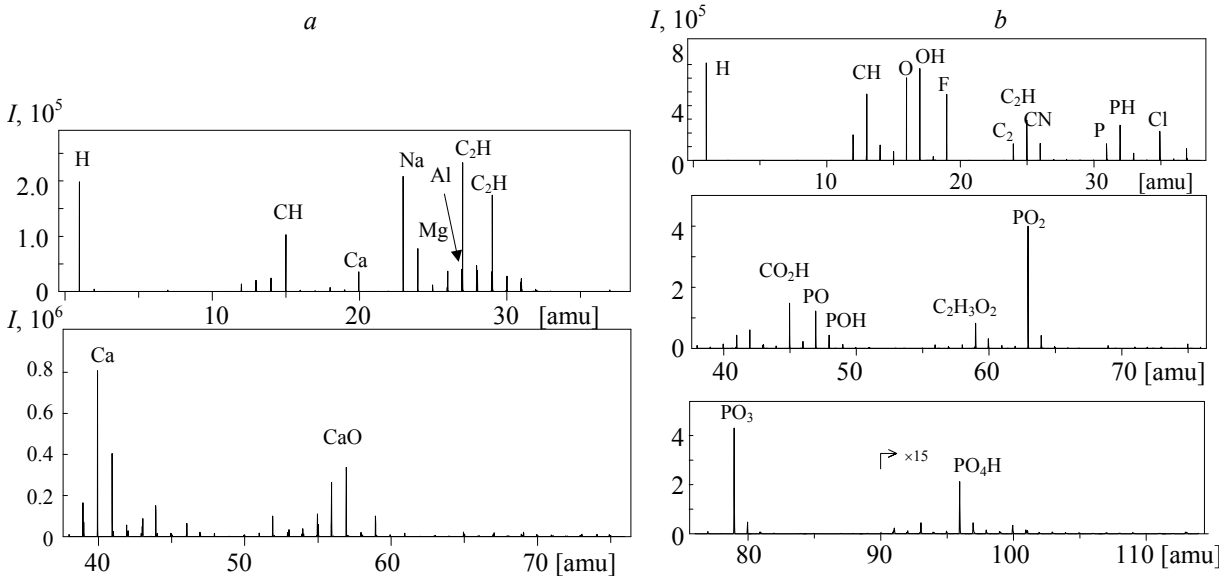


Fig. 3. Positive (a) and negative (b) ion spectrum obtained from the surface of the investigated material. For the sake of simplicity the +(–) sign is omitted in the labels indicating the main ions.

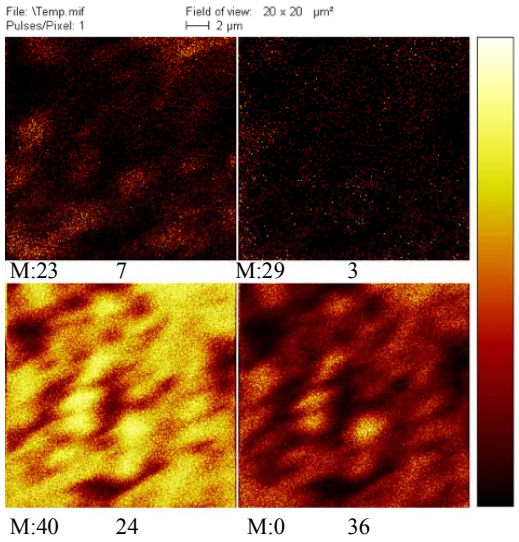


Fig. 4. TOF-SIMS chemical maps of positive ions emitted from the bone substitute under investigation.

However, from another point of view, Wagner et al. demonstrated the limits of detection of TOF-SIMS for plasma protein fibrinogen. While TOF-SIMS was able to determine some qualitative trends in the composition of the plasma protein films as a function of adsorption time, the detection limits of minor components in the multicomponent adsorbed protein films ultimately limit the ability of TOF-SIMS to quantify the composition of these films [31]. This preliminary study confirms the quality of the hydroxyapatite bone substitute and its osteoinductive properties due to the presence of micropores on the surface that are able to recruit BMP in the bloodstream and so favoring deposition on porous surfaces and stimulating the next growth of bone by osteoblastic cells.

**Conclusion.** The present work had the objective of analyzing the TOF-SIMS spectrometry method in combination with XPS on the surface of a biomaterial recently used as a bone substitute. The preliminary study has provided information on the chemical and micromorphological composition of the material.

The results seem to confirm the alleged osteoinductive capacity of the material related to its ability to act as a decoy for bone morphogenetic proteins (BMP) and to stimulate bone growth in conjuction with the work of osteoblastic cells.

Further development of the research is oriented towards the analysis of the surface of other bone substitutes such as that of animal origin (bovine or equine) or sourced beef. The goal is to make comparative characterizations, evaluate the affinity for BMPs, and compare the results in order to identify which material best meets the needs of the clinician and the patient.

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