New collagen-coated calcium phosphate synthetic bone filler (Synergoss®): a comparative surface analysis

Giorgio Iviglia¹, Marco Morra¹, Clara Cassinelli¹, Elisa Torre¹, Ruggero Rodriguez Y Baena²

¹Nobil Bio Ricerche srl, Via Valcastellana 26, 14037 Portacomaro, Italy
²Department of Clinical, Surgical, Diagnostic and Pediatric Science, School of Dentistry, University of Pavia, Viale Brambilla 74, 27100 Pavia, Italy

Abstract
Replacement of bone loss or reconstruction of bone defect is still a clinical challenge. Synergoss® is a recently developed synthetic bone filler that exploits biomolecular surface engineering to deliver directly to the filler-implant interface the signaling properties of type I collagen. In this article we compared Synergoss® bone-filler with the most used materials present on the market derived from animal source (Bio-Oss®, Gen-Os®) or synthetic source (BoneCeramic®). All tested bone-fillers were analyzed by ATR-IR, providing information concerning the chemical composition and the main functional groups. Surface zeta potential analysis shows a positive surface charge for Synergoss®, confirming that the material exposes a collagen nanolayer to the surrounding environment, unlike typical synthetic and bone-based fillers. The chemical composition of the first few nanometers of the surface of...
tested bone fillers was evaluated by XPS, and Synergoss® shows a significant amount of nitrogen (9.8 at%), which is not detected in any of the remaining materials. Morphological structure of Synergoss® (analyzed by SEM) shows a uniformly distributed micro- and macro- porosity, which plays an essential role in influencing cell adhesion and blood vessel infiltration, nutrient transportation and clot formation to stimulate the overall healing process.

**Introduction**

Dental replacement using titanium implants is nowadays a quite common procedure in oral surgery\(^1\). Millions of dental implants are placed per year worldwide, and this number is expected to increase\(^2\). The success rate of dental implant installation, depends on the quality and quantity of alveolar bone which is present in the extraction site\(^3,4\). If sufficient bone is not available, an augmentation strategy is required, using bone substitutes, such as autografts, xenografts, allografts and osteoactive agents\(^5\). The common use of autografts or allografts is affected by the morbidity and the risk of diseases transmission associated with the donor site, thus the use of xenograft and synthetic material is more and more attractive\(^6-11\). Xenografts, from porcine or bovine source, are widely used in clinics since they have a fine microarchitecture and properties of the original bone tissue\(^12,13\). Depending on the purification process from the animal source they could preserve some biomolecular structure in the mineralized matrix which makes the material highly biocompatible, making them a reliable alternative to autografts. Synthetic bone fillers, mostly based on calcium phosphate materials, have been widely used due to their good reproducibility, biocompatibility, non-immunogenicity, and also because they offer the opportunity of advanced material engineering\(^14,15\). Structural, chemical and morphological properties could be customized varying the synthesis parameters, the Ca/P ratio, the starting materials to maximize the rate of new bone formation and tune the degradation rate, miming the behavior of natural bone\(^16-20\).
Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) are widely used in bone tissue engineering as bone filler particles. HA is the most abundant component in native bone (around 65% of the inorganic phase) but despite the excellent biocompatibility and mechanical properties, the slow degradation rate limits its use as bone filler only. Usually HA is combined with β-TCP, since the latter has a degradation rate 3 – 12 times faster than HA. The ratio of HA and β-TCP is the key point in order to obtain a bone filler with good mechanical properties and degradation rate, maintaining the biocompatibility and the osseointegration capability.

While most research papers deal with bulk properties of bone fillers, surface properties are an important tool offered by material science to stimulate osseointegration. The solid-liquid (body fluids) interface dominates the biological response of the host tissue, adhesion and differentiation of cells. Great efforts are currently addressed at the development of coating or surface treatments to encourage tissue growth, since it is supposed that tissue-biomaterials interactions are regulated by surface properties and that the “primary interaction zone”, where the important interaction occurs, is generally between 0.1 - 1 nm of the biomaterial surface. Many surface modification approaches have been implemented by research groups, physiochemical (surface charge), morphological (roughness) or biochemical (adsorption or immobilization of biomolecules).

Simply trapping cells on the surface, is not enough, for an effective interaction with host tissue, is mandatory encourage the cell to migrate to the material surface, and stimulate them to differentiate. In particular biomolecular modification of surface is an effective strategy to improve bone-material interaction, and could be combined with morphological modification (such as porosity), which act as an anchorage for the cells. Many study has been conducted on the mechanism by which cells adhere to substrates, and great advances have been made in understanding how biomolecules attached on the surface could influence the...
differentiation and remodeling process of the host tissue. The effect of extracellular matrix peptide is fundamental to promote cell adhesion and to promote cell interaction with the biomaterials, in particular certain sequence found in a number of different extracellular matrix, Arg-Gly-Asp (RGD) is the key founding which serves as a primary cell attachment cue. In orthopedic and dental application, the use of a biochemical surface modification, allow to control the initial bone-implant interaction through an organic molecules which is a components of bone. In those fields, a number of paper underline the role of collagen type I in bone regeneration, since it is the most abundant protein in bone, where it makes around 85% of the organic phase. Our research group has recently developed a new synthetic biomimetic bone filler, Synergoss® (SC+) that exploits the novelty concept of biomolecular surface engineering, to deliver directly to the filler-implant interface the signaling properties of type I collagen. Contrary to the clinically available bone fillers from animal source, where the collagen is embedded in the inorganic matrix, Synergoss® contains just a surface cross-linked collagen nanolayer, with a thickness of the order of nanometers, on top of biphasic calcium phosphate (200 – 1000 µm) granules. Collagen type I is the most abundant protein in bone, where it makes around 85% of the organic phase. It contains RGD sequence and it plays an important role in osteoblast cells behavior, promoting not only cell adhesion, but also osteoblastic differentiation of bone marrow cells and controlling the osteogenic pathway. If the collagen is exposed on the surface, it interacts also with growth factor and other biomolecules, making the surface of the bone filler an osteoinductive matrix. Furthermore, collagen shows well-known hemostatic properties, and it activates platelets in an unique way. Since, the blood-implant interaction is very important in the early stage of bone healing, the pro-coagulant properties of collagen enhance the osseointegration of the bone filler. The properties mentioned above are fundamentals for a more rapid and
healthy bone regeneration in particular in dental field, where the presence of bacteria could interfere with tissue regeneration.

The scope of this paper is to compare, by a combination of advanced surface analysis techniques, the surface properties of Synergoss® and the most used bone fillers present on the market.

This work emphasizes the surface properties of the most used bone-filler in dental field, since the biomolecular surface engineering could upgrade the properties of implant devices, by promoting more specific and targeted implant-host cells interactions, we compared those well-known bone-filler with Synergoss® which has already reported in a previously paper an excellent biocompatibility in vitro and in vivo\textsuperscript{48}. The general aim is to show that biomolecular surface engineering can be at the basis of a novel generation of granulated bioceramics, combining mechanical and structural properties of calcium phosphates with specific surface signaling.

**Experimental procedures**

**Materials**

Tests were performed on materials in the “on the shelf” conditions, that is they were taken from commercially available vials, not expired, sealed and sterile, as follows. Tested materials were chosen to encompass bone fillers of different origins (xenograft from bovine and porcine, synthetic alloplastic), including different biomimetic factors preserved during purification process from animal bone tissue, namely:

Bio-Oss® (Geistlich Pharma AG, Wolhusen, Switzerland) code 30643.3/500079, lot 81500471, 0.5 g vial; is spongiosa granules from bovine source, produced by Geistlich Pharma AG. The granules (250 – 1000 µm) are purified through a proprietary extraction
process (heat treatment, up to 300 °C, using alkalis and organic solvent) which makes the material not antigenic and protein free.

Gen-Os (Osteobiol® by Tecnoss Dental srl, Pianezza, Italy) code M1010FS, lot 160158, 1 g vial; is a xenograft material constituted as a mixture of cancellous and cortical bone (80% - 20%) from porcine source. It is provided as granules (250 – 1000 µm) and it is obtained through a low temperature process which allows to eliminate any pathogenic elements, preserving the structure and the composition of natural collagen and hydroxyapatite.

BoneCeramic® (Straumann USA LLC, USA) code 070.205, lot KE369, 0.5 g vial; is a granules biphasic calcium phosphate alloplastic (500 – 900 µm). It is composed of hydroxyapatite and β-tricalcium phosphate, in the percentage of 60% - 40%.

Synergoss®, (henceforth SC+), bio-enhanced synthetic bone filler, biphasic calcium phosphate composed by 25% Hydroxylapatite and 75% β-Tricalcium phosphate⁴⁴, bearing a surface coating of collagen type I, code P11, lot 15009, 1 g vial

Furthermore, as a comparison, Synergoss® without the collagen surface layer (SC-, lot 15008), that is a biphasic calcium phosphate composed by 25% hydroxylapatite and 75% β-Tricalcium phosphate, was obtained from the producer.

**Surface Analysis Investigation:**

**Morphological Characterization (SEM)**

Scanning Electron Microscopy (SEM) analysis was performed to observe the surface morphology of bone fillers. Samples were mounted on aluminum stubs and sputtered with gold at 15 mA for 2 min using Agar Sputter Coater. The morphology of granules was captured using EVO MA10 system (Zeiss).
**ATR-IR**

ATR-IR spectra were obtained using a Nicolet iS10 ATRIR spectrometer, produced by Thermo Scientific and equipped by a diamond crystal. Samples were gently placed on the crystal and kept in place by the specific crimping tool. The experimental set up involves acquisition of 32 scans in the range 500–4000 cm\(^{-1}\), both of sample and background, at a resolution of 4 cm\(^{-1}\).

**XPS**

XPS analysis was performed using a Perkin Elmer PHI 5600 ESCA system. The instrument is equipped with a monochromatized Al anode operating at 10 kV and 200 W. The diameter of the analyzed spot is approximately 500 micrometers, the base pressure 10\(^{-8}\) Pa. The angle between the electron analyzer and the sample surface was 45°. Measurements were performed by pressing granules, in order to make a complete layer on a double sided adhesive tape, one side of which was fixed to the instrument sample holder. Analysis was performed by acquiring wide range survey spectra (0–1000 eV binding energy) and detailed high resolution peaks of relevant elements. Quantification of elements was accomplished using the software and sensitivity factors supplied by the manufacturer.

**Zeta potential measurement (ζ-potential)**

ζ-potential measurement was performed using SurPass 3 equipped with a cylindrical cell for granulate materials (Anton-Paar GmbH, Graz, Austria). Measurement were performed using the Cylindrical Cell, and for each bone filler material, 1.5 g of granules were transferred in the cylindrical compartment of the cylindrical cell and mounted between support disk and filters (with 25 µm mesh) on both sides of the granular sample plug. 0.001 M KCl was used as electrolyte solution. Analysis was performed according to pH scan method. It consists in the measurement of the streaming potential at different pH, between 9 and 5. The pH of electrolyte solution was modified automatically by the instruments using a
0.05 M HCl and 0.05 M KCl. At each pH point three measurements were performed in order to condition the sample, then the fourth value was taken and reported. To calculate the ζ-potential, the Helmholtz-Smoluchowski equation was used:

\[
\zeta = \frac{dI_{str}}{d\Delta p} \ast \frac{\eta}{\varepsilon \ast \varepsilon_0} \ast \frac{L}{A} \quad (eq. 1)
\]

In this equation the streaming current coefficient \(dI_{str}/d\) is related with the cell constant \(L/A\), where \(L\) is the length of the slit channel formed between the two samples forming the capillary, and \(A\) is the area cross-section of the capillary. \(\eta\) and \(\varepsilon\) are the viscosity and dielectric coefficient of the solution forced to pass through the capillary. If the Ohm’s law is used, it is possible to substitute the streaming current with the streaming potential as follows:

\[
\zeta = \frac{dU_{str}}{d\Delta p} \ast \frac{\eta}{\varepsilon \ast \varepsilon_0} \ast \frac{L}{A} \ast \frac{1}{R} \quad (eq. 2)
\]

This equation requires to know the geometry of the channel. In case of flat surfaces, it is possible to take \(L\) as the sample length, and the gap between the two surfaces is calculated from the measured volume flow rate of liquid passing through the channel and the generated differential pressure. In the case of irregular shaped samples, such as granulates material, this approach is not suitable. In this case, the cell constant \(L/A\) and the resistance \(R\), are substitute by the electrical conductivity \(\kappa\)

\[
\kappa = \frac{1}{R} \ast \frac{L}{A} \quad (eq. 3)
\]

and the eq. 2 became:

\[
\zeta = \frac{dU_{str}}{d\Delta p} \ast \frac{\eta}{\varepsilon \ast \varepsilon_0} \ast \kappa \quad (eq. 4)
\]

The electrical conductivity inside the streaming channel cannot be measured directly, however just for non-conducting samples it is possible to replace it by the conductivity of the bulk electrolyte solution. Thus, in the present case of irregularly shaped ceramic granulated materials, it is possible to use the Helmholtz-Smoluchowcki approximated equation:
\[
\zeta = \frac{dU_{str}}{d\Delta p} \ast \frac{\eta}{\varepsilon * \varepsilon_0} \ast \kappa_B \quad (eq. 5)
\]

All calculations were performed by the instrument software.

**Results**

**SEM**

Morphology and surface area play a fundamental role in the interaction of osteogenic cells with biomaterials surfaces\textsuperscript{5,49}. Micro-/macro-surface pores and roughness influence cell and tissue response to implants\textsuperscript{50-52}. Porous surfaces promote mechanical interlocking, bone ingrowth and increase fixation and stability of the implant. Roughness and porosity act as guide for bone cells providing support and stimulating proliferation and differentiation\textsuperscript{53}.

Figure 1 shows SEM images of surfaces of granules of tested bone-fillers, at two different magnifications (2000 X and 10000 X). Surface morphology of xenograft materials, Gen-Os® and Bio-Oss® is quite similar, with a classical stratified structure, similar to the natural bone, low micro-porosity and a quite uniform roughness more developed on Bio-Oss® surface. As to synthetic bone fillers (SC+, SC- and Bone-Ceramic®), some interesting remarks can be made. Bone-Ceramic® bone filler shows a compact surface, with high grain size, which is the result of a sintering process conducted at high temperature, or for long time\textsuperscript{54,55}. This process creates a rough surface due to the fusion of the grain each other, but without micro-porosity on the surface\textsuperscript{55}. On the other hand, SEM images show a highly porous surface for Synergoss® (SC+) and SC- material, reflecting a different manufacturing process, which enhances the micro-porosity of the granules, and consequently the surface area. Sintering is conducted in a way that the single grain grows and bridges with the others, creating a network, providing mechanical stability but preserving the fundamental micro-porosity. Some collagen fibers are clearly visible in the SEM image at 10000X of SC+, compared to
SC-. Fibers are a few micrometers long, while the width is of the order of the tens of nanometers, in good agreement with AFM findings of collagen fibers on mica\textsuperscript{43}.

**ATR-IR.**

All tested bone-fillers were analyzed by ATR-IR, to provide information concerning the chemical composition and the main functional groups. In figure 2a are reported the spectra of the material analyzed from 500 to 4000 cm\textsuperscript{-1}, figure 2b and c focus on the peaks related to the inorganic phase (650 – 1500 cm\textsuperscript{-1}) and the organic phase (1200 – 3700 cm\textsuperscript{-1}), the material’s spectra were compared with the pure HA, β-TCP and collagen type I from porcine source. All bone-fillers are calcium phosphates, so they all show the typical bands originated by this mineral in the range between 950 – 1140 cm\textsuperscript{-1}\textsuperscript{144,56,57}. However, subtle differences can be detected depending on details of the mineral composition percentage, source, processing, etc.. Figure 2b is a focus on triply degenerated asymmetric stretching mode of P-O bond of phosphate group. SC+ shows peaks from both hydroxyapatite and tricalcium phosphate. The 1125 cm\textsuperscript{-1} peak is due to tricalcium phosphate, while peaks 1025 cm\textsuperscript{-1} and 1010 cm\textsuperscript{-1} are typically associated with HA, overlapped with the component due to the β-TCP\textsuperscript{3,58}. The intensity of the peak due to the β-TCP phase (90 cm\textsuperscript{-1} and 1000 cm\textsuperscript{-1}), is higher than that of HA, according with the composition of the material (75% of β-TCP and 25% of HA). Furthermore, the main peak is more shifted forward 1010 cm\textsuperscript{-1} similar to the β-TCP spectrum. The SC- spectrum is definitely identical to that of SC+, as shown in figure 2a and b. BoneCeramic® yields spectra very similar to those of SC+ and SC-, since it is composed of a mixture of HA and β-TCP. However it contains a higher percentage of HA (60% compared with the 25% of Synergoss®), and this is clearly shown in figure 2b, which is quite similar to the pure HA spectra. Peaks, at 1125 cm\textsuperscript{-1} and 1025 cm\textsuperscript{-1} are associated to the P-O stretching bond (ν3) of HA phase. Peaks between 950 and 1000 cm\textsuperscript{-1} associated to the β-TCP are not visible in the spectrum. Bone-fillers from animal source, Bio-Oss® and Gen-Os®,
show again the typical bands of calcium phosphates in the range of 950 cm\(^{-1}\) and 1025 cm\(^{-1}\). Bio-Oss\(^{®}\), shows a more intense peak associated to the HA mineral constituent, HA, gives the most intense peaks at 1025 cm\(^{-1}\), and 1125 cm\(^{-1}\), while Gen-Os\(^{®}\) spectrum shows features more similar to \(\beta\)-TCP. There is a shift in the principal peak from 1025 cm\(^{-1}\) to 1015 cm\(^{-1}\) for Gen-Os\(^{®}\), which shows also a lower intensity of the inorganic peak compared to the Bio-Oss\(^{®}\). This is probably due to the process used to obtain Bio-Oss\(^{®}\) bone-filler, where high temperature and the use of organic solvent make the material completely protein free\(^5\).

Spectra of both Bio-Oss\(^{®}\) and Gen-Os\(^{®}\) show, in addition, a low intensity double-band at 1410 -1460 cm\(^{-1}\) that corresponds to stretching vibration of \(\text{CO}_3^{2-}\)\(^{5,14}\). However, Bio-Oss\(^{®}\) have an higher intensity compared to Gen-Os\(^{®}\), indicating an higher amount of carbonate compounds\(^{59,60}\).

As to the organic phase, in figure 2 c, the spectra of collagen type I is reported for comparison with the investigated materials. Collagen shows a characteristic IR spectrum, with absorption bands of amide I at \(~1650\) cm\(^{-1}\), amide II at \(~1560\) cm\(^{-1}\) and a set of three weaker bands that represent amide III vibration modes centered at \(~1245\) cm\(^{161}\). A further absorption peak between \(3100 - 3400\) cm\(^{-1}\) is present, due to the \(-\text{OH}\) bond and NH stretching. Gen-Os\(^{®}\) goes under a low temperature process which allow to maintain the collagen component\(^5\), inside the matrix, and its presence is clearly visible in the spectrum reported in figure 1 c, where peaks above 1400 cm\(^{-1}\) are all associated at collagen matrix.

Collagen on the SC+ surface is not detected. The sampling depth of ATR-IR, using a diamond crystal, is several hundred nanometers. Moreover, the irregular shape of bone filler granules and the ensuing uncomplete contact between crystal and sample lower the sensitivity of this technique. Thus, the collagen layer is very thin as compared to the technique sampling-depth and its intensity is very low as compared to that of the inorganic phase, to the point that it is not detected.
XPS analysis

The chemical composition of the surface of tested bone fillers was evaluated by XPS. This technique is much more surface-sensitive than ATR-IR, the sampling depth is a few nanometers. Spectra of all analyzed samples are obviously dominated by the peaks of oxygen, calcium and phosphorus, beside ubiquitous adventitious carbon contamination due to adsorption from the atmosphere. Interesting hints are provided by the detected surface stoichiometry, as reported in Table 1.

As expected, the surface composition of all samples is very similar, except for SC+. The latter shows significantly more carbon and significantly less calcium and phosphorous as compared to the remaining bone fillers. Moreover, it shows a significant amount of nitrogen, which is not detected in any of the remaining materials. The obvious reason for these data is that a thin (to the point to be undetected by ATR-IR) proteinaceous layer covers the outer surface of SC+, yielding the C and N contribution to surface stoichiometry and attenuating the signal from “core” elements Ca and P14,56.

A further interesting observation is that the surface composition of GenOs®, whose ATR-IR spectrum clearly shows collagen-related bands, does not contain N: this suggests that the proteinaceous component of this material is embedded within the bone matrix and it is not exposed on the material surface.

Zeta potential measurement (ζ-potential)

The ζ-potential generated at the material-aqueous interface by the surface charge of the tested materials as a function of pH in 1mM KCl is shown in figure 3 a,b. The graph in figure 3 c shows the ζ-potential values at physiological pH 7.4.

Data can be, broadly speaking, divided into three groups: BoneCeramic®, SC- and Gen-Os® show negative values in the whole tested pH range: this means that in every case negative charges dominate the aqueous interface; Bio-Oss® is still mostly negative, yet its ζ-
potential is much lower (in absolute value) as compared to materials of the previous group and it shows an isoelectric point at low pH; SC+ shows an amphoteric behavior and from slightly below pH 7 towards the acidic range its \(\zeta\)-potential turns positive, meaning that at the material/aqueous interface positive charges establish a positive interfacial potential. Insights from these results, on the light of information provided by the different techniques adopted, will be discussed in the next section.

**Discussion**

Five different bone fillers, both from animal and synthetic origin, were analyzed by a range of surface-sensitive techniques. Starting from morphological properties, it has been shown that micro-porosity plays an essential role in influencing cell adhesion to the biomaterial surface, while macro-porosity promotes blood vessel infiltration, nutrients transportation and clot formation, which is important to stimulate the overall healing process, besides allowing cell infiltration and new bone growth\(^{35,62-65}\). Micro-porosity is generated by the manufacturing process (sintering, purification, temperature), it affects together with the particle size the surface area. Usually, increasing these parameters, osseointegration increases\(^66\). Macro-porosity is formed between one particle and another. It is affected by the particle size, because particle size affects the packing characteristics of the material\(^{35,62}\). The tested materials show widely different porosities, reflecting both the source (natural bone Vs synthetic) and the process (BoneCeramic® Vs SC+, SC-). Existing clinical data show ample variability and suggest that, in general, all detected morphologies are suitable on the light of the intended use\(^{67-70}\).

Chemical data obtained from this work, that is ATR-IR and XPS measurements, itemize the tested materials into two different categories: those that are completely inorganic (Bio-Oss®, BoneCeramic®, and SC-) and those that contain also organic (proteinaceous) material (Gen-Os® and SC+). As to the first category, while chemical differences exist in terms of
relative amount and nature of calcium phosphate phases contained in each of them, the most striking result from this study is the different behavior shown by \( \zeta \)-potential measurement.

Calcium phosphate materials usually show a negative charge on the surface and an acidic behavior due to the dissociation of ceramic compounds in phosphate ions \( \text{PO}_4^{3-} \), which bind with \( \text{H}_3\text{O}^+ \) in solution to form acidic species (\( \text{HPO}_4^{2-} \), \( \text{H}_2\text{PO}_4^- \), \( \text{H}_3\text{PO}_4 \))\(^{71,72} \). In the present case, while all three materials are, generally speaking, acidic, that is they show negative \( \zeta \)-potential values along almost the whole range of \( \text{pH} \) tested, BioOss\( \text{®} \) is definitely less acidic than the others. It is also definitely less acidic than the other tested bone filler from animal source, that is Gen-Os\( \text{®} \). It reaches an Isoelectric Point (IEP, the point where the \( \zeta \)-potential is equal to zero, and where the surface charge is balanced\(^73 \)) at \( \text{pH} \) of 5.74. A possible explanation of this behavior is that the composition of Bio-Oss\( \text{®} \), analyzed through IR , reveals the presence of hydroxyapatite and calcium carbonate, and a lower amount, compared to GenOs\( \text{®} \) of \( \beta \)-TCP\(^{59} \). This is probably the reason of the different magnitude of the \( \zeta \)-potential, between the two xenograft materials analyzed and among BioOss\( \text{®} \) and the other wholly inorganic bone fillers tested. The acidity resulting from the combination of \( \text{CO}_3^{2-} \) dissociated ions from the surface of BioOss\( \text{®} \) with \( \text{H}_3\text{O}^+ \) from the solution, is lower than that formed by GenOs\( \text{®} \) which generates more phosphoric acids, due to the high dissolution of \( \beta \)-TCP\(^{71} \). Many research groups have demonstrated that HA is stable in a body fluid, while \( \beta \)-TCP is rather soluble\(^{23,74} \). BioOss\( \text{®} \) shows a \( \zeta \)-potential curve with a long plateau and no buffer behavior, neither at acidic \( \text{pH} \). Zeta potential analysis supported by spectroscopic technique yields very specific information on surface chemistry, since just the surface affects the streaming potential.

A further interesting observation within the “inorganic” category is that BoneCeramic\( \text{®} \) shows a much more negative \( \zeta \)-potential value than SC\( \text{®} \), with a long flat curve resulting in a strong acidic behavior of the surface in solution. Literature shows that the surface charge
depends on the sintering temperature and time\textsuperscript{72,75}. Analyzing the microstructure of the BoneCeramic\textsuperscript{®}, through SEM, figure 1, it is possible to observe a much lower microporosity, as compared to SC-, meaning an higher level of sintering\textsuperscript{76}.

A particularly interesting result from this study involves the different contribution of proteinaceous material to surface properties of Gen-Os\textsuperscript{®} and SC+. The collagen layer on the SC+ surface is not detected in the IR spectrum, while typical amide bands are clearly detected in the analysis of Gen-Os\textsuperscript{®}. On the other hand, when surface sensitivity is enhanced by adopting a technique with lower sampling depth (XPS), SC+ shows a clear nitrogen signal while the surface of Gen-Os\textsuperscript{®} is completely inorganic. Basically, this is due to the fact that, in bone, collagen is embedded within the inorganic matrix, and it does not contribute to interfacial interactions\textsuperscript{77}. Many studies demonstrated that the primary interaction between implant and tissue occurs in the first nm (0.1 – 1 nm) from the surface of the material\textsuperscript{28}.

Following this way, many efforts have been made in order to optimize the surface of existing materials rather than incorporating biomolecules into the matrix, without exposing the important sites to the surrounding cells. Along this line, SC+ bears a collagen nano-layer on the surface which is directly exposed to the host tissue and can interact with cells in order to stimulate bone regeneration. Collagen as fibrous protein is the major component of mammalian connective tissue\textsuperscript{78}. It is involved in many fundamental biological processes, and in particular it is involved in cell attachment\textsuperscript{41}. Among the many types of collagen, Type-I collagen is the most abundant and building up of a thin collagen nano-layer on implanted medical devices could enhance and improve the host tissue response\textsuperscript{73}. Collagen, is a typically amphoteric protein, containing amino groups in the side chains of basic amino acid residues (such as lysine and arginine) an carboxylic groups on the acidic side chains (such as glutamic and aspartic acid). This chemical structure allows collagen to bind and release H\textsuperscript{+} and to be ionized in aqueous solution, and it makes the surface amphoteric, that is
positively/negatively charged depending on the pH. The collagen layer adsorbed on the Synergoss® (SC+) surface is crosslinked, to make the bimolecular surface more stable during the implantation-time, the cross linking step leads to the consumption of some of the acidic/basic groups of the side chain. Therefore, depending on the cross linking procedures, the ζ-potential curve and the isoelectric point could change. In the present study, the presence of collagen nano-layer, is clearly visible, in figure 3 a, comparing SC+ and SC-(that is, the same SC+ inorganic base without collagen) behavior. SC+ shows almost a completely positive curve, due to the dissociation of NH$_3^{2+}$ which attract the OH$^{-}$ from the liquid, showing a slightly acidic surface behavior. The formation of a plateau during a ζ-potential measurement, and a single value of pKa of the surface, means that interfacial charge behavior is dominated by chemical functionalities on the surface of the material rather than ions from the solution. An initial slightly negative ζ-potential value was recorded at basic pH, with an inversion of ζ-potential sign at pH 6.7, which is the IEP off SC+, followed by a positive plateau. Pure collagen shows, usually, a IEP around 10. The decrease of IEP in SC+ is due to the presence of ceramic surface partially exposed to the solution, in particular due to the microporosity of the bone filler, which allow the infiltration of the solution. Gen-Os®, which keeps the collagen phase inside the matrix, shows a classical negative ζ-potential curve. The proteinaceous component of Gen-Os® is thus not available for interfacial interactions at the host tissue/biomaterial interface and it does not affect surface properties of the material. The surface-bound collagen layer on SC+, instead, affects surface properties and it changes the way the phosphate ceramics interacts at the aqueous interface in the crucial early phases of host cell colonization and beginning of the bone regeneration process.
Conclusion

Consistently with the scope of this paper, the comparison, by a combination of surface analysis techniques, of surface properties of several commercially available bone fillers shows that the surface properties of SC+ are different from those of conventional animal/synthetic calcium phosphate bone fillers. SC+ is based on a process based on bio-design concept\textsuperscript{44}, and it presents a collagen layer to the surrounding environment, combining biomolecular signaling properties to scaffolding effects of bioceramics. In particular, data show that the collagen layer is exposed to and affects the properties of the material interface, as detected by the surface composition of the first few nanometers (XPS) and by the amphoteric surface charge behavior. Bone fillers containing collagen embedded within the inorganic matrix shows instead surface chemical composition and interfacial electrical behavior similar to those of wholly inorganic bone fillers\textsuperscript{72}.

References

1. Gaviria L, Salcido JP, Guda T, \textit{et al.} Current trends in dental implants. \textit{J Korean Assoc Oral Maxillofac Surg.} 2014; 40: 50.

2. Gupta A, Dhanraj M, Sivagami G. Status of surface treatment in endosseous implant: a literary overview. \textit{Indian J Dent Res.} 2010; 21: 433–8.

3. Iviglia G, Cassinelli C, Torre E, \textit{et al.} Novel bioceramic-reinforced hydrogel for alveolar bone regeneration. \textit{Acta Biomater.} 2016; 44: 97–109.

4. Tonelli P, Duvina M, Barbato L, \textit{et al.} Bone regeneration in dentistry. \textit{Clin Cases Miner Bone Metab.} 2011; 8: 24–8.

5. Figueiredo M, Henriques J, Martins G, \textit{et al.} Physicochemical characterization of biomaterials commonly used in dentistry as bone substitutes - Comparison with human bone.
6. Kumar G, Narayan B. Morbidity at bone graft donor sites. In: Classic Papers in Orthopaedics., 2014, 503–5.

7. November O. Update: allograft-associated bacterial infections--United States, 2002. MMWR Morb Mortal Wkly Rep. 2002; 51: 207–10.

8. Arrington ED, Smith WJ, Chambers HG, et al. Complications of iliac crest bone graft harvesting. Clin Orthop Relat Res. 1996; 329: 300–9.

9. Giannoudis P V, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury. 2005; 36 Suppl 3: S20–7.

10. Chiapasco M, Casentini P, Zaniboni M. Bone augmentation procedures in implant dentistry. Int J Oral Maxillofac Implants. 2009; 24 Suppl: 237–59.

11. Sheikh Z, Sima C, Glogauer M. Bone Replacement Materials and Techniques Used for Achieving Vertical Alveolar Bone Augmentation. Materials (Basel). 2015; 8: 2953–93.

12. Buser D, Dahlin C, Schenk RK. Guided Bone Regeneration in Implant Dentistry. 1994: 31–96.

13. Chen F-M, Jin Y. Periodontal tissue engineering and regeneration: current approaches and expanding opportunities. Tissue Eng Part B Rev. 2010; 16: 219–55.

14. Tadic D, Epple M. A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone. Biomaterials. 2004; 25: 987–94.

15. Samavedi S, Whittington AR, Goldstein AS. Calcium phosphate ceramics in bone tissue engineering: A review of properties and their influence on cell behavior. Acta Biomater. 2013; 9: 8037–45.

16. Wang H, Lee J-K, Moursi A, et al. Ca/P ratio effects on the degradation of hydroxyapatite in vitro. J Biomed Mater Res A. 2003; 67: 599–608.

This article is protected by copyright. All rights reserved.
17. Rablee SM, Moztarzadeh F, Kenari HS, et al. Preparation and properties of a porous calcium phosphate bone graft substitute. *Mater Sci Pol*. 2007; 25: 1019–27.

18. LeGeros RZ. Calcium phosphate materials in restorative dentistry: a review. *Adv Dent Res*. 1988; 2: 164–80.

19. LeGeros RZ. Properties of osteoconductive biomaterials: Calcium phosphates. *Clin Orthop Relat Res*. 2002; 395: 81–98.

20. LeGeros RZ, Lin S, Rohanizadeh R, et al. Biphasic calcium phosphate bioceramics: preparation, properties and applications. *J Mater Sci Mater Med*. 14: 201–9.

21. Yuan H, Van Den Doel M, Li S, et al. A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats. *J Mater Sci Mater Med*. 2002; 13: 1271–5.

22. Zhang L, Hanagata N, Maeda M, et al. Porous hydroxyapatite and biphasic calcium phosphate ceramics promote ectopic osteoblast differentiation from mesenchymal stem cells. *Sci Technol Adv Mater*. 2009; 10: 25003.

23. Schaefer S, Detsch R, Uhl F, et al. How Degradation of Calcium Phosphate Bone Substitute Materials is influenced by Phase Composition and Porosity. *Adv Eng Mater*. 2011; 13: 342–50.

24. Alcaide M, Serrano MC, Pagani R, et al. Biocompatibility markers for the study of interactions between osteoblasts and composite biomaterials. *Biomaterials*. 2009; 30: 45–51.

25. Kwon S-H, Jun Y-K, Hong S-H, et al. Synthesis and dissolution behavior of B-TCP and HA/B-TCP composite powders. *J Eur Ceram Soc*. 2003; 23: 1039–45.

26. Jones FH. Teeth and bones: Applications of surface science to dental materials and related biomaterials. *Surf Sci Rep*. 2001; 42: 75–205.

27. Barrère F, Blitterswijk CA Van. Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. 2006; 1: 317–32.

This article is protected by copyright. All rights reserved.
28. Puleo D a, Nanci a. Understanding and controlling the bone implant-interface. *Biomaterials*. 1999; 20: 2311–21.

29. Kasemo B, Lausmaa J. Surface science aspects on inorganic biomaterials. *J Name CRC Crit Rev Clin Neurobiol; (United States); J Vol 4*. 1986: Medium: X; Size: Pages: 335-380.

30. Hamamoto N, Hamamoto Y, Nakajima T, *et al.* Histological, histocytochemical and ultrastructural study on the effects of surface charge on bone formation in the rabbit mandible. *Arch Oral Biol*. 1995; 40: 97–106.

31. Brunette DM. The effects of implant surface topography on the behavior of cells. *Int J Oral Maxillofac Implants*. 1988; 3: 231–46.

32. Ito Y, Kajihara M, Imanishi Y. Materials for enhancing cell adhesion by immobilization of cell adhesive peptide. *J Biomed Mater Res*. 1991; 25: 1325–37.

33. Morra M. Biomolecular modification of implant surfaces. *Expert Rev Med Devices*. 2007; 4: 361–72.

34. Blawas AS, Reichert WM. Protein patterning. *Biomaterials*. 1998; 19: 595–609.

35. Woodard JR, Hilldore AJ, Lan SK, *et al.* The mechanical properties and osteoconductivity of hydroxyapatite bone scaffolds with multi-scale porosity. *Biomaterials*. 2007; 28: 45–54.

36. Khalili AA, Ahmad MR. A Review of cell adhesion studies for biomedical and biological applications. *Int J Mol Sci*. 2015; 16: 18149–84.

37. Wang C, Duan Y, Markovic B, *et al.* Proliferation and bone-related gene expression of osteoblasts grown on hydroxyapatite ceramics sintered at different temperature. *Biomaterials*. 2004; 25: 2949–56.

38. Rouahi M, Champion E, Hardouin P, *et al.* Quantitative kinetic analysis of gene expression during human osteoblastic adhesion on orthopaedic materials. *Biomaterials*. 2006; 27: 2829–44.

This article is protected by copyright. All rights reserved.
39. Jikko A, Harris SE, Chen D, et al. Collagen Integrin Receptors Regulate Early Osteoblast Differentiation Induced by BMP-2. *J Bone Miner Res*. 1999; 14: 1075–83.

40. Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction. *J Cell Physiol*. 2000; 184: 207–13.

41. Heino J. The collagen family members as cell adhesion proteins. *BioEssays*. 2007; 29: 1001–10.

42. Ferreira AM, Gentile P, Chiono V, et al. Collagen for bone tissue regeneration. *Acta Biomater*. 2012; 8: 3191–200.

43. Morra M, Cassinelli C, Cascardo G, et al. Surface engineering of titanium by collagen immobilization. Surface characterization and in vitro and in vivo studies. *Biomaterials*. 2003; 24: 4639–54.

44. Morra M, Giavaresi G, Sartori M, et al. Surface chemistry and effects on bone regeneration of a novel biomimetic synthetic bone filler. *J Mater Sci Mater Med*. 2015; 26: 159.

45. LeBaron RG, Athanasiou KA. Extracellular Matrix Cell Adhesion Peptides: Functional Applications in Orthopedic Materials. *Tissue Eng*. 2000; 6: 85–103.

46. Gungormus M, Kaya O. Evaluation of the effect of heterologous type I collagen on healing of bone defects. *J Oral Maxillofac Surg*. 2002; 60: 541–5.

47. Heemskerk JW, Vuist WM, Feijge M a, et al. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca2+ responses. *Blood*. 1997; 90: 2615–25.

48. Morra M, Giavaresi G, Sartori M, et al. Surface chemistry and effects on bone regeneration of a novel biomimetic synthetic bone filler. *J Mater Sci Mater Med*. 2015; 26.
49. Abagnale G, Steger M, Nguyen VH, et al. Surface topography enhances differentiation of mesenchymal stem cells towards osteogenic and adipogenic lineages. *Biomaterials*. 2015; 61: 316–26.

50. Cassinelli C, Morra M, Bruzzone G, et al. Surface chemistry effects of topographic modification of titanium dental implant surfaces: 2. In vitro experiments. *Int J Oral Maxillofac Implants*. 18: 46–52.

51. Morra M, Cassinelli C, Bruzzone G, et al. Surface chemistry effects of topographic modification of titanium dental implant surfaces: 1. Surface analysis. *Int J Oral Maxillofac Implants*. 18: 40–5.

52. Davis SS. An introduction to tissue–biomaterial interactions. *J Control Release*. 2003; 93: 85.

53. Faia-Torres AB, Guimond-Lischer S, Rottmar M, et al. Differential regulation of osteogenic differentiation of stem cells on surface roughness gradients. *Biomaterials*. 2014; 35: 9023–32.

54. Zhang D, Jain H, Hupa M, et al. In-vitro Degradation and Bioactivity of Tailored Amorphous Multi Porous Scaffold Structure. *J Am Ceram Soc*. 2012; 95: 2687–94.

55. Daculsi G, LeGeros RZ, Grimandi G, et al. Effect of Sintering Process of HA/TCP Bioceramics on Microstructure, Dissolution, Cell Proliferation and Bone Ingrowth. *Key Eng Mater*. 2008; 361–363: 1139–42.

56. Tavares DDS, Castro LDO, Soares GDDA, et al. Synthesis and cytotoxicity evaluation of granular magnesium substituted β-tricalcium phosphate. *J Appl Oral Sci*. 2013; 21: 37–42.

57. Koutsopoulos S. Synthesis and characterization of hydroxyapatite crystals: a review study on the analytical methods. *J Biomed Mater Res*. 2002; 62: 600–12.

58. Iviglia G, Cassinelli C, Bollati D, et al. Engineered porous scaffolds for periprosthetic infection prevention. *Mater Sci Eng C*. 2016; 68: 701–15.
59. Rombouts C, Jeanneau C, Camill J, et al. Characterization and angiogenic potential of xenogeneic bone grafting materials: Role of periodontal ligament cells. Dent Mater J. 2016; 35: 900–7.

60. Accorsi-Mendonça T, Conz MB, Barros TC, et al. Physicochemical characterization of two deproteinized bovine xenografts. Braz Oral Res. 2008; 22: 5–10.

61. De Campos Vidal B, Mello MLS. Collagen type I amide I band infrared spectroscopy. Micron. 2011; 42: 283–9.

62. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials. 2005; 26: 5474–91.

63. Baino F, Vitale-Brovarone C. Three-dimensional glass-derived scaffolds for bone tissue engineering: current trends and forecasts for the future. J Biomed Mater Res A. 2011; 97: 514–35.

64. Scabbia A, Trombelli L. A comparative study on the use of a HA/collagen/chondroitin sulphate biomaterial (Biostite) and a bovine-derived HA xenograft (Bio-Oss) in the treatment of deep intra-osseous defects. J Clin Periodontol. 2004; 31: 348–55.

65. Matsuno T, Nakamura T, Kuremoto K, et al. Development of beta-tricalcium phosphate/collagen sponge composite for bone regeneration. Dent Mater J. 2006; 25: 138–44.

66. Ryu H-S, Namgung C, Lee J-H, et al. The influence of thread geometry on implant osseointegration under immediate loading: a literature review. J Adv Prosthodont J Adv Prosthodont. 2014; 5476: 547–54.

67. De Lange GL, Overman JR, Farré-Guasch E, et al. A histomorphometric and micro-computed tomography study of bone regeneration in the maxillary sinus comparing biphasic calcium phosphate and deproteinized cancellous bovine bone in a human split-mouth model. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014; 117: 8–22.

This article is protected by copyright. All rights reserved.
68. Cordaro L, Bosshardt DD, Palattella P, et al. Maxillary sinus grafting with Bio-Oss® or Straumann® Bone Ceramic: Histomorphometric results from a randomized controlled multicenter clinical trial. *Clin Oral Implants Res.* 2008; 19: 796–803.

69. Caubet J, Ramis JM, Ramos-Murguialday M, et al. Gene expression and morphometric parameters of human bone biopsies after maxillary sinus floor elevation with autologous bone combined with Bio-Oss® or BoneCeramic®. *Clin Oral Implants Res.* 2015; 26: 727–35.

70. Schmitt CM, Doering H, Schmidt T, et al. Histological results after maxillary sinus augmentation with Straumann® BoneCeramic, Bio-Oss®, Puros®, and autologous bone. A randomized controlled clinical trial. *Clin Oral Implants Res.* 2013; 24: 576–85.

71. Xiangdong Z, Hongsong F, Dongxiao L, et al. Protein Adsorption and Zeta Potentials of a Biphasic Calcium Phosphate Ceramic Under Various Conditions. *J Biomed Mater Res B Appl Biomater.* 2007; 83: 340–4.

72. Smeets R, Kolk A, Gerressen M, et al. A new biphasic osteoinductive calcium composite material with a negative Zeta potential for bone augmentation. *Head Face Med.* 2009; 5: 13.

73. Wang Y, Zhang H, Tian L, et al. A novel method for determining surface charge and isoelectric point of leather. 2015: 1–8.

74. Dias a. G, Lopes M a., Gibson IR, et al. In vitro degradation studies of calcium phosphate glass ceramics prepared by controlled crystallization. *J Non Cryst Solids.* 2003; 330: 81–9.

75. Tanimoto Y, Shibata Y, Kataoka Y, et al. Osteoblast-like cell proliferation on tape-cast and sintered tricalcium phosphate sheets. *Acta Biomater.* 2008; 4: 397–402.

76. Dietze S, Bayerlein T, Proff P, et al. The ultrastructure and processing properties of Straumann Bone Ceramic and Nanobone. *Folia Morphol (Warsz).* 2006; 65: 63–5.

77. Florencio-Silva R, Sasso GRDS, Sasso-Cerri E, et al. Biology of Bone Tissue: Structure,
Function, and Factors That Influence Bone Cells. Biomed Res Int. 2015; 2015.

78. Pachence JM. Collagen-based devices for soft tissue repair. J Biomed Mater Res. 1996; 33: 35–40.

79. Caldwell JW, Arsura EL, Kilgore WB, et al. Layer-by-layer assembly of collagen thin films: Controlled thickness and biocompatibility. Biomed Microdevices. 2001; 3: 301–6.

80. Charulatha V, Rajaram A. Influence of different crosslinking treatments on the physical properties of collagen membranes. Biomaterials. 2003; 24: 759–67.

81. Andrade ÂL, Ferreira JMF, Domingues RZ. Zeta potential measurement in bioactive collagen. Mater Res. 2004; 7: 631–4.

**TABLE:**

| Sample        | O [at%] | N [at%] | Ca [at%] | C [at%] | P [at%] |
|---------------|---------|---------|----------|---------|---------|
| Bio Oss       | 39.6    | -       | 27.2     | 18.6    | 14.6    |
| Gen Os        | 39.1    | -       | 25.0     | 22.9    | 13.0    |
| Bone Ceramic  | 36.2    | -       | 23.6     | 27.4    | 12.8    |
|               | 35.7    | -       | 22.0     | 28.9    | 13.4    |
|               | 26.6    | 9.8     | 11.1     | 45.9    | 6.6     |

Table 1. Surface composition (% at.) of tested bone fillers analyzed through XPS.
Figure Captions

Figure 1. Images of the morphological structure obtained by scanning electron microscopy, of the surface of different bone-fillers (Bio-Oss®, BonCeramic®, Gen-Os®, SC- and SC+), at different magnifications 2000x and 10000x.

Figure 2. ATR-IR spectra of analyzed bone-fillers (Bio-Oss®, BonCeramic®, Gen-Os®, SC- and SC+) between 500 – 4000 cm\(^{-1}\) (a). Focus on the peaks related to the inorganic phase (650 – 1500 cm\(^{-1}\)) (b) and the organic phase (1200 – 3700 cm\(^{-1}\)) (c).

Figure 3. Graphical images of the principle of zeta potential measurement for solid surfaces (Bio-Oss®, BonCeramic®, Gen-Os®, SC- and SC+) (a). pH scan curve from 9 to 5.5 (b), and zeta potential value at physiological pH 7.4 for all bone-filler tested (c).
