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Abstract

Bone reconstruction after tissue loosening due to traumatic, pathological or surgical causes is in increasing demand. 3D scaffolds are a widely studied solution for supporting new bone growth. Bioactive glass-ceramic porous materials can offer a three-dimensional structure that is able to chemically bond to bone. The ability to surface modify these devices by grafting biologically active molecules represents a challenge, with the aim of stimulating physiological bone regeneration with both inorganic and organic signals. In this research work glass ceramic scaffolds with very high mechanical properties and moderate bioactivity have been functionalized with the enzyme alkaline phosphatase (ALP). The material surface was activated in order to expose hydroxyl groups. The activated surface was further grafted with ALP both via silanization and also via direct grafting to the surface active hydroxyl groups.

Enzymatic activity of grafted samples were measured by means of UV-vis spectroscopy before and after ultrasonic washing in TRIS-HCl buffer solution. In-vitro inorganic bioactivity was investigated by soaking the scaffolds after the different steps of functionalization in a simulated body fluid (SBF). SEM observations allowed the monitoring of the scaffold morphology and surface chemical composition after soaking in SBF. The presence of ALP enhanced the in vitro inorganic bioactivity of the tested material.

Keywords: : surface functionalization, scaffold, bone integration, bioactivity

1. Introduction

Surgical operations for cancer removal, traumatic events and degenerative pathologies are the main causes of bone loosening. These problems cause an increasing demand for bone substitutes that are able to reproduce the physiological functions of natural tissue, both for orthopaedic and dental applications.

The possibility of producing a structure with proper mechanical properties, capable of supporting new bone growth from the biomechanical point of view, and also able to promote chemical bonding of bone and cellular stimulation for the purpose of tissue regeneration, represents an extremely interesting challenge. The ideal scaffold should possess a morphology that is almost identical to that of natural tissue, with large interconnecting macro-pores of about 150-200μm and in amounts above 50%vol. and a Young Modulus and compressive strength close to the physiological one [1, 2]. The evolution of glass-based scaffolds for bone tissue engineering has been recently highlighted in some interesting reviews [3, 4] describing the features, the limitations, the advantages and the future perspectives of the different types of glass-derived scaffolds reported in the literature. To this purpose, glass-ceramic scaffold fabrication using different methods based on the removal of an organic template in the form of particles or 3D cellular sponge, have been described by the authors in several previous papers [5, 6, 7] .

Bioactive glasses and glass-ceramics are known for their ability to react with physiological fluids and modify their surface, favouring the formation of an apatite layer that can strongly bond to bone [8]. These materials present a more efficient mechanism of bioactivity, compared with traditional bioceramics. Their peculiar surface reactivity seems to play a role not only in the osteoconduction process, but also in the first steps of biological mineralization [9, 10] promoting, on their surface, hydroxyapatite precipitation (in-vitro) and bone bonding (in-vivo). Depending on their composition it is possible to tailor the amount and type of released ions and properly stimulate tissue response.

Several strategies have been investigated to enhance the ability of biomaterials to induce bone remodelling and growth. Among them, the development of biomimetic surfaces seems very promising. The opportunity of modifying bone substitutes by surface grafting with biologically active molecules with the aim to stimulate physiological tissue regeneration is widely studied in literature. The possibility of attaching both extracellular matrix proteins [11], or their characteristic peptides, and growth factors [12] have been explored. In the first case the aim is to mimic the

physiological environment for cell adhesion while in the second one the idea is to stimulate cell proliferation and differentiation.

Surface functionalization of implantable biomaterials with proteins and growth factors is an extremely active field in the scientific research community but, on the other hand, few commercial products based on these innovative technologies can be enumerated. They are mainly based on the combination of biological matrices (such as demineralised bone matrix or collagen) and bone morphogenetic proteins or endogenous blood [13, 14, 15].

Significant attention has been dedicated to the surface modification of titanium [16, 17, 18], and polymers [12, 19]. Few papers also consider the combination of the typical inorganic bioactivity of bioactive glasses with a more specific biological one. Silanization of macroporous Bioglass® scaffolds [20, 21] and of bioactive glass nanoparticles [22] has been investigated as a preliminary step for further biomolecular grafting. The opportunity of conjugating osteoproductive characteristics of Bioglass® granules with the periodontal regeneration potentialities of enamel matrix proteins was explored by the coating of glass particles with commercial Emdogain® [23]. A significant enhancement in osteoblast protein production was observed for functionalized particles.

Bioactive glasses and glass-ceramics are particularly prone to surface modifications. Their reactivity is mainly based on ionic exchange between the glass network and surrounding fluids, with the formation of silanols, which, during the subsequent steps of bioactivity, condense to form a silica gel layer and promote the precipitation of hydroxyapatite.

The first step of this mechanism (hydroxylation) can be successfully used to graft biomolecules both by direct anchoring or through functionalization with aminosilanes.

To this purpose, the authors have already investigated the opportunity to surface functionalize bioactive glasses, that have different degrees of bioactivity, with model molecules [24] and the enzyme alkaline phosphatase [25, 26, 27]. In these works an effective functionalization was achieved: ALP maintains its activity after grafting onto the material surface and improves inorganic

bioactivity of the bare scaffold. Moreover preliminary cell culture studies suggest a stimulatory effect on osteoblasts.

In the present research work, the surface functionalization already optimized on bulk bioactive glasses and glass-ceramics was successfully adapted to 3D macroporous glass ceramic scaffolds with moderate bioactive behaviour and excellent mechanical properties [28, 29, 30]. The scaffolds were prepared with the sponge replication method [7, 31] and biologically functionalized with the enzyme ALP, both via silanization and via direct grafting onto properly exposed surface hydroxyls groups.

Enzymatic activity and in vitro bioactivity of functionalized samples were also evaluated.

2. Materials and Methods

2.1 Glass preparation

A glass, named SCNA, with the composition (molar %): 57 % SiO_2, 34% CaO, 6% Na_2O and 3% Al_2O_3 was prepared by traditional melt and quenching technique [32].

Starting reagents were melted in a platinum crucible at 1550°C for 1 hour and then poured into water in order to obtain a frit. The frit was subsequently ball milled and sieved up to 32μm, obtaining a powder with particles of below 32μm.

2.2 Scaffold preparation and characterization

3D glass ceramic scaffolds were prepared by the sponge replication method. In brief, a polyurethane sponge were cut into cubic samples and impregnated with a glass slurry following a standardised method described in a previous work [33]. The glass slurry contains (weight %): 30% SCNA powders (less than 32 μm), 6% PVA and 64% bi-distilled water. After the standardised infiltration/compression schedule, the impregnated sponges were thermally treated for 3 hours at 1000°C [29] in order to burn out the polymeric phase and sinter the glassy one, obtaining glass-

ceramic scaffold The sintering temperature was chosen on the base of DTA and hot stage microscopy investigation (data non reported).

The scaffolds were silver coated for scanning electron microscopy (SEM, Philips 525M; accelerating voltage 15KV) to assess the pore size, shape and distribution as well as the overall morphology. The total porosity was evaluated by weight measurements considering the density of bulk SCNA. The volumetric shrinkage S (%) of the scaffold occurring upon the thermal treatment is due both to the polymeric sponge removal and to the glass viscous flow during sintering.

S was calculated as the complement to one of the impregnated sponge vol. ($V_{imp.}$) and the sintered scaffold vol. ($V_{sint.}$) as

$$S = \left(1 - \frac{V_{imp}}{V_{\sin t}}\right) \times 100 \qquad (1)$$

The total scaffold porosity was evaluated on 25 cubic samples through weight measurements considering the density of the glass-ceramic material (GC-SCNA, $\rho_{scaff.}$) and the apparent density of the sintered scaffold (ρ_{bulk})

$$P = \left(1 - \frac{\rho_{scaff}}{\rho_{bulk}}\right) \times 100 \qquad (2)$$

The scaffold compressive strength was evaluated on 5 cubic samples by means of compressive tests (MTS System Corp. apparatus, cross-head speed = $1mm \cdot min^{-1}$ and the failure strength $\boldsymbol{\sigma_f}$ (MPa) was obtained as the ratio of the maximum compressive load registered during the test and the resistant cross section (mm^2).

A solution containing 30%wt. of calf serum and 70%wt of bi-distilled water was prepared and a few drops of red ink (Pelikan 4001, TP/6 Brilliant Red) were added to obtain a strongly coloured solution with an analogous viscosity to human fluids. The glass-ceramic scaffold was then put into contact with 5 ml of the prepared solution to qualitatively observe the capillarity up-take ability of the scaffold and the time required for the complete uptake was registered.

2.3 Hydroxyl exposure

Hydroxyl groups were exposed on the scaffold surface through a simple washing treatment optimized for the bulk glass in previous works [24, 27]. Samples were washed one time for 5 minutes in acetone in order to remove surface contaminants and subsequently 3 times for 5 minutes in bi-distilled water in order to expose reactive groups. All washings were performed in an ultrasonic bath.

Hydroxylated scaffolds are designated simply as SCNA (Table 1). A complete list of sample names and treatments is reported in table 1.

2.4 Silanization

In order to introduce amino groups onto the material surface, 3-aminopropyltriethoxysilane ($H_2N(CH_2)_3Si(OC_2H_5)_3$, APTES, 99% Aldrich) was grafted onto half of the samples, as described in [27] for bulk samples.

In brief, hydroxylated scaffolds were put in a solution containing 150 ml of ethanol and 35µl of 3-aminopropyltriethoxysilane for 6 hours at room temperature. Then the samples were removed from the silane solution and thermally treated for 1 hour at 100°C in order to stabilize silane-surface bonding and then left to cool. 3 washings in ethanol for 5 minutes in an ultrasonic bath were then performed in order to remove unbounded molecules. A final thermal treatment of 1 hour at 100°C was performed to complete the sample drying.

Silanized SCNA scaffolds are designated as SCNA+sil (Table 1).

2.5 Enzyme grafting

A 5 mg/ml solution of ALP was prepared by dissolving bovine alkaline phosphatase (Phosphatase Alkaline from bovine intestinal mucosa, lyophilized powder, 10-30 DEA unit/mg solid – Sigma Aldrich) in phosphate buffered saline solution (PBS - Sigma Aldrich) under magnetic stirring.

Based on the enzyme certificate of analysis it was determined that the activity of the prepared solution is 50 U/ml.

Each sample, both the only hydroxylated and silanized ones, has been immersed in 10 ml of ALP solution for 24 hours at 37°C, as described in [27] for bulk samples.

The samples were then gently washed in TRIS-HCl buffer solution in order to stop the grafting reaction and left to dry at room temperature. Some samples were also washed 2 minutes in TRIS-HCl buffer solution in an ultrasonic bath (these samples are designated as _US washed, table 1) in order to study the bonding stability.

A TRIS-HCl buffer solution was prepared by dissolving 2.42 g of TRIS (Tris(hydroxymethyl)aminomethane – $(HOCH_2)_3CNH_2$, 99,9+% ultrapure grade, Aldrich) in 100 ml of double distilled water; the pH was adjusted to 7.5 by adding 1M HCl.

The SCNA scaffolds functionalized with ALP are designated as SCNA+ALP, when enzyme grafting was performed directly to exposed hydroxyls, and SCNA+sil+ALP when silanization was employed (Table 1)

2.6 Enzymatic activity tests

In order to verify if ALP maintains its activity after grafting onto the scaffold, the reaction between alkaline phosphatase and p-nitrophenilphosphate was monitored. Functionalized samples were introduced in multi-well cell culture plates with a reactive mixture containing equal volumes of $MgCl_2$ 2mM, p-nitrophenilphosphate 2 mM and 2-amino-2-methyl-1-propanol (AMP) 2 mM (all reagents were purchased from Sigma Aldrich Fluka). The pH value of the reactive mixture is 10.5. The reaction was stopped after 2 min by the addition of NaOH 0.1 N. After removing the scaffolds from the wells the intensity of the yellow colour produced was quantified by measuring the UV absorbance at 405 nm (GENIUS Spectra FLUOR plus TECAN).

All tests were performed in triplicate.

For each plate, 3 measurements on the reactive mixture alone were carried out to get a reference. Reference values were subtracted from the sample absorbance measurements in order to obtain pure enzymatic activity values.

To compare the activity of different samples, the specific enzymatic activity of the scaffolds was calculated by dividing the absorbance by the scaffold volume.

The enzymatic activity of ALP solutions at defined concentrations was evaluated in order to have a standard reference curve for ALP activity.

2.7 Statistical analysis

The data was represented as mean ± standard deviation. All tests were performed in triplicate.

The data was analyzed by means of one-way ANOVA, with a significance level $p<0.05$.

2.8 In vitro bioactivity test

In order to evaluate the in-vitro bioactivity of functionalized scaffolds, samples were soaked in simulated body fluid (SBF) at 37°C for periods of up to 2 months. SBF was prepared according to the Kokubo description [34].

Solution refresh was carried out every two days to mimic the physiological turnover of body fluids. The pH was measured before each refresh.

Hydroxyapatite precipitation after SBF soaking was investigated by means of SEM and EDS analysis (EDS - EDAX PV 9900) after silver sputter coating of the samples. The comparison between different samples was performed by qualitatively observing the amount of hydroxyapatite particles on the surface. Moreover a semi-quantitative evaluation was carried out by comparing several EDS spectra of significant areas (200x magnification) and by quantifying the Calcium and Phosphorous content.

3. Results and discussion

3.1 Scaffold characterization

After the thermal treatment of the impregnated sponges, glass-ceramic scaffolds with the wollastonite unique crystalline phase were obtained. The average porosity of the structures P, determined by density measurements, was 40.5 ± 2.2 volume %. and the volumetric shrinkage S was 66.9±1,4%; Figure 1a qualitatively shows the shrinkage of the impregnated sponge after the thermal treatment and the scaffold morphology.

Figure 1c qualitatively report the scaffold ability to uptake fluids with an analogous viscosity to human ones, the complete uptake took less then 2 seconds, demonstrating the high ability of the scaffold to be quickly impregnated by physiological fluids through its network of micropores.

As far as the scaffold structure is concerned, the pores are well interconnected and the struts are dense and well sintered as can be seen in Figure 1b. The bare scaffolds present very high mechanical properties (18 ± 5.6. MPa in compression) for porous bioactive glass-ceramics with trabecular structure, as can be seen in figure 2 where an example of registered plot is reported.

The mechanical competence of the obtained scaffold is very high if compared with similar porous structures reported in literature [7, 28, 31, 33].

3.2 Enzymatic activity test

ALP was successfully grafted onto scaffolds while maintaining its activity, as shown in figure 3 (first and third bars). There are no significant differences between the silanized (SCNA+sil+ALP) and the only hydroxylated (SCNA+ALP) ALP-grafted samples ($p > 0.05$). These results are in accordance with previous observations on bulk glassy SCNA [27]. The activity of a functionalized scaffold (about 250 mm^3 in volume) is comparable with the one of a 2,5 mg/ml ALP standard solution.

Looking at the second and fourth bars of figure 3 it is possible to observe that a severe ultrasonic washing removes a significant amount of ALP from scaffolds. Also in this case no significant difference was detected between silanized and directly grafted samples ($p > 0.05$). The percentage of reduction of ALP activity after severe ultrasonic washings is about 39% for SCNA+ALP scaffolds and about 50% for SCNA+sil+ALP ones. These values are lower than the ones observed on plane SCNA glass slices reported in [27]. As far as the scaffolds are concerned, the results show that ALP is firmly grafted onto the surface; an additional amount of the enzyme is probably trapped through macro and micro pores and cannot be completely washed off.

Finally, it must be underlined that the proposed washings are extremely aggressive and that in physiological conditions a gradual release of the enzyme can be supposed. A gradual release of ALP from functionalized plane bioactive glasses was documented in a previous work [27]. It is interesting to note that the removed ALP maintains its activity in solution, so it can be assumed that the enzyme released in the host tissues after the implant can also effectively stimulate cells.

3.3 In vitro bioactivity

The scaffolds soaked in SBF for up to 2 months were analysed by SEM and EDS to observe the hydroxyapatite precipitation. A reproducible trend in the hydroxyapatite precipitation was observed for the samples at different experimental times.

After one week of soaking in SBF the pure SCNA scaffold did not show any hydroxyapatite particles on its surface (fig. 4a) but a certain reduction in the surface calcium content (observed with EDS analysis) and a slight pH increase of the SBF indicated a certain reactivity of the material. An analogous behaviour was observed for silanized scaffold (figure 4b). This result indicates that silanization procedure does not significantly alter the early surface reactivity of the glass ceramic material, as previously observed on SCNA slices [27]

Small hydroxyapatite crystals were observed on the surface of SCNA +ALP (figure 4c) and SCNA+sil+ALP (figure 4d) scaffolds after 1 week in SBF. EDS analyses confirmed an enrichment in Ca and P ions.

After 2 weeks in SBF calcium and phosphorous enrichment (in a ratio close to 1.67) was recorded on the SCNA scaffold together with the appearance of small hydroxyapatite crystals (figure 5a). Also on SCNA+sil, HAp particles were observed after 2 weeks in SBF (figure 5b). Two weeks soaking allowed the observation of a significant amount of hydroxyapatite on SCNA+ALP (figure 5c) and of several crystals on the SCNA+sil+ALP (figure 5d).

An example of EDS analyses after 14 days in SBF for the different scaffold typologies is reported in figure 6.

After 1 month in simulated body fluid many small hydroxyapatite crystals were observed on the SCNA in accordance with its slow bioactive behaviour (figure 7a). HAp particles were also detected also on SCNA+sil (figure 7b). A higher amount of hydroxyapatite precipitated on both SCNA+ALP (figure 7c) and SCNA+sil+ALP (figure 7d) scaffolds compared with the not functionalized ones .

After two months soaking in SBF hydroxyapatite particles were detected on SCNA scaffolds (figure 8a), this is an indication that the material presents a slow but effective bioactive behaviour. Similarly HAp was observed on the scaffold functionalized with ALP directly to surface hydroxyls (SCNA+ALP, figure 8c). On the other hand no hydroxyapatite crystals were detected on silanized and silanized and ALP grafted scaffolds after two months soaking (SCNA+sil and SCNA+sil+ALP, figure 8b and 8d respectively). These observations suggest that hydroxyapatite can detach from silanized surfaces after prolonged soaking times. A possible explanation for this phenomenon could be the poor stability of silanized interfaces in humid environments reported in literature [35]. However this point should be investigated in more depth.

To summarize, the bare scaffold presents low bioactivity kinetics, in fact 2 weeks are necessary to initially observe the precipitation of apatite crystals. In spite of this, a moderate reactivity can be

underlined when observing a pH increase of the SBF and changes in the ionic composition on the surface, indicating that an ionic exchange between the scaffold surface and SBF solution occurs. Silanization does not significantly alter material reactivity.

On the other hand ALP functionalization significantly modifies the material response to physiological solutions, since both silanized and ALP grafted samples (SCNA+sil, SCNA+sil+ALP) and, in a more pronounced way, also directly functionalized ones (SCNA+ALP) showed hydroxyapatite precipitation after only 1 week of soaking.

For the longest soaking time (2 months) it seems that silanization can somehow favour hydroxyapatite detachment from the material surface, in vitro.

SCNA presents a low bioactivity index because of Al_2O_3 presence in its composition. Alumina significantly improves the material's mechanical properties but, as reported in literature [8], it reduces glass and glass-ceramic bioactivity; the introduction of an amount of Al_2O_3 higher than 3% mol. can completely hinder surface reactivity. 3D macroporous scaffolds present an increased surface area, useful for ionic exchange with physiological media, that allows higher reactivity compared with traditional bulk materials [27]. In fact in this research work the precipitation of few hydroxyapatite crystals was observed on the bare scaffold after 2 weeks of soaking in SBF, while on the bulk SCNA the bioactivity was much lower [27]. However, the faster reactivity seems to be related not only to the higher surface area, but also to the action of ALP. It was also observed that ALP absorption on the scaffold surface can improve SCNA bioactivity and fasten hydroxyapatite precipitation. These results are in accordance with previous observations on bulk glasses of different reactivity functionalized with the same enzyme [27] and also on Ti6Al4V alloy grafted with ALP [36, 37].

Alkaline phosphatase is a homodimeric metalloenzyme and its physiological function is the catalysis of the hydrolysis of phosphate monoesters with the release of inorganic phosphate and alcohol. The ALP active site contains two Zn cations that are able to tightly bind phosphates in order to catalyze their hydrolysis [38, 39, 40]. In a solution (such as SBF) free of organic

phosphates but rich in inorganic ones, it can be hypothesized that ALP, grafted onto the material surface, is able to attract phosphate ions and consequently accelerate calcium ions precipitation and hydroxyapatite nucleation.

Conclusion

Glass-ceramic porous scaffolds belonging to the system SiO_2-CaO-NaO-Al_2O_3 were prepared by the sponge replication method, obtaining glass-ceramic scaffolds with open interconnecting pores and a mechanical strength of up to 18MPa. Their surface was modified firstly by a washing treatment, aimed at exposing hydroxyl groups, and subsequently with the grafting of an aminosilane (3-APTES). The enzyme alkaline phosphatase was bound in an active state both to silanized structures and also to only hydroxylated ones.

It was observed that a strong ultrasonic washing can remove a significant part of the ALP from scaffolds but a considerable amount remains active on the surface. Moreover the washed-off ALP maintains its activity in solution. It seems that the silane presence does not affect the effectiveness and stability of ALP grafting, in fact no significant difference was observed between silanized and direct grafted scaffold for both enzyme activity and its resistance to washings. On the basis of these results, we can infer that silanization does not significantly improve ALP immobilization on scaffold surface.

Finally it was observed that ALP functionalization can improve and fasten bioactivity kinetics in terms of hydroxyapatite precipitation after soaking in SBF. Since in vitro bioactivity results also indicate that hydroxyapatite-surface bonding is weaker for silanized samples, we conclude that direct grafting could be better for this kind of structure.

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Figures/Tables legend.

Table 1: Names and characteristics of analysed samples.

Sample name	**Material**	**Treatment**
SCNA	SCNA glass ceramic scaffold	Hydroxyls Exposition
SCNA+sil	SCNA glass ceramic scaffold	Hydroxyls Exposition and Silanization
SCNA+ALP	SCNA glass ceramic scaffold	Hydroxyls Exposition and Enzyme Grafting
SCNA+sil+ALP	SCNA glass ceramic scaffold	Hydroxyls Exposition, Silanization and Enzyme Grafting
SCNA+ALP_US-washed	SCNA glass ceramic scaffold	Hydroxyls Exposition, Enzyme Grafting and ultrasonic washing in TRIS buffer solution
SCNA+sil+ALP_US-washed	SCNA glass ceramic scaffold	Hydroxyls Exposition, Silanization, Enzyme Grafting and ultrasonic washing in TRIS buffer solution

Figure 1: a) Impregnated polymeric sponge (left)- sintered glass-ceramic scaffold (right), b) SEM image of the macroporous glass-ceramic scaffold (SCNA)

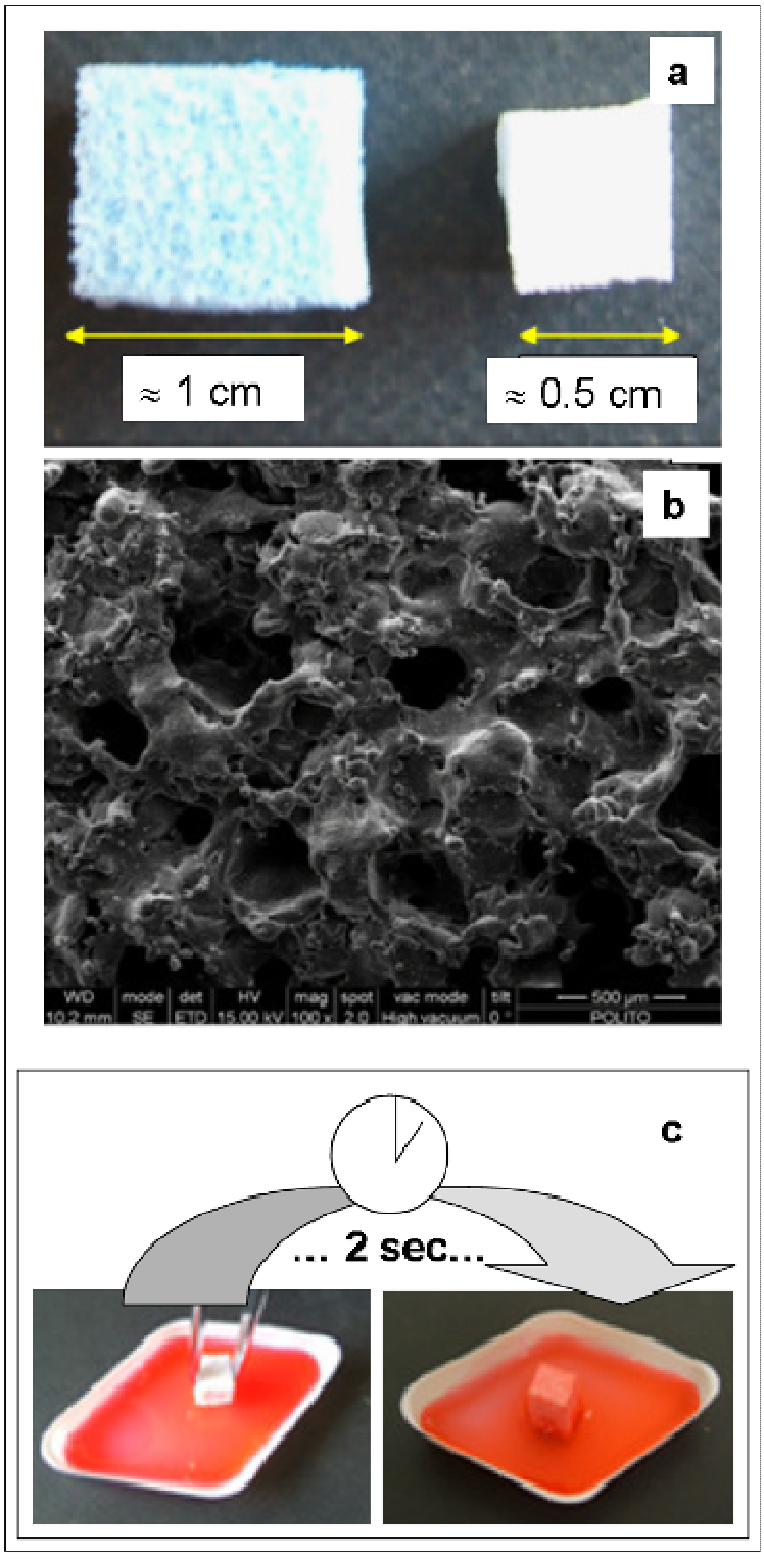

Figure 2: Scaffold ability to uptake fluids having an analogous viscosity as human one

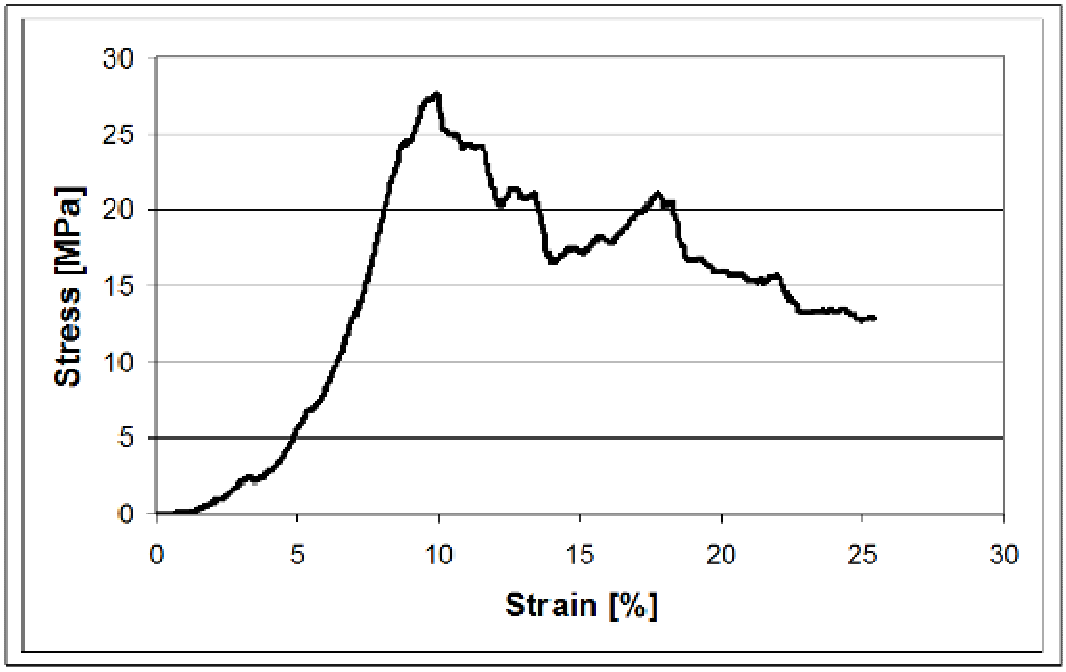

Figure 3: Example curve for compressive strength of SCNA scaffolds

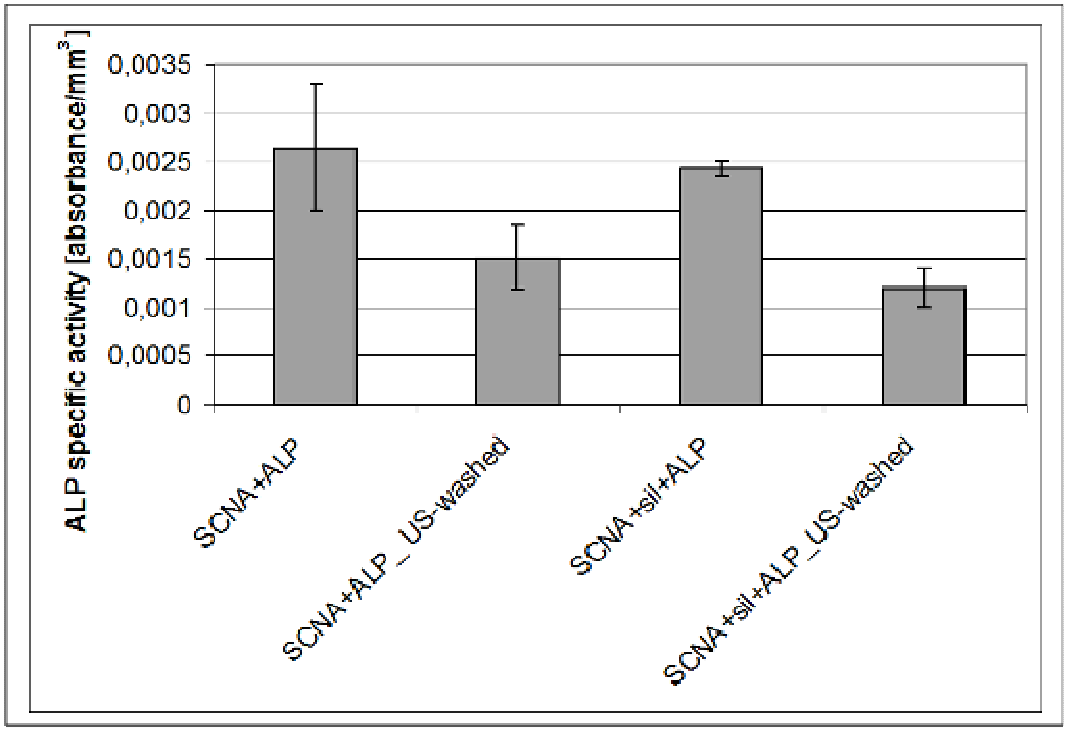

Figure 4: ALP activity of SCNA scaffolds before and after ultrasonic washings

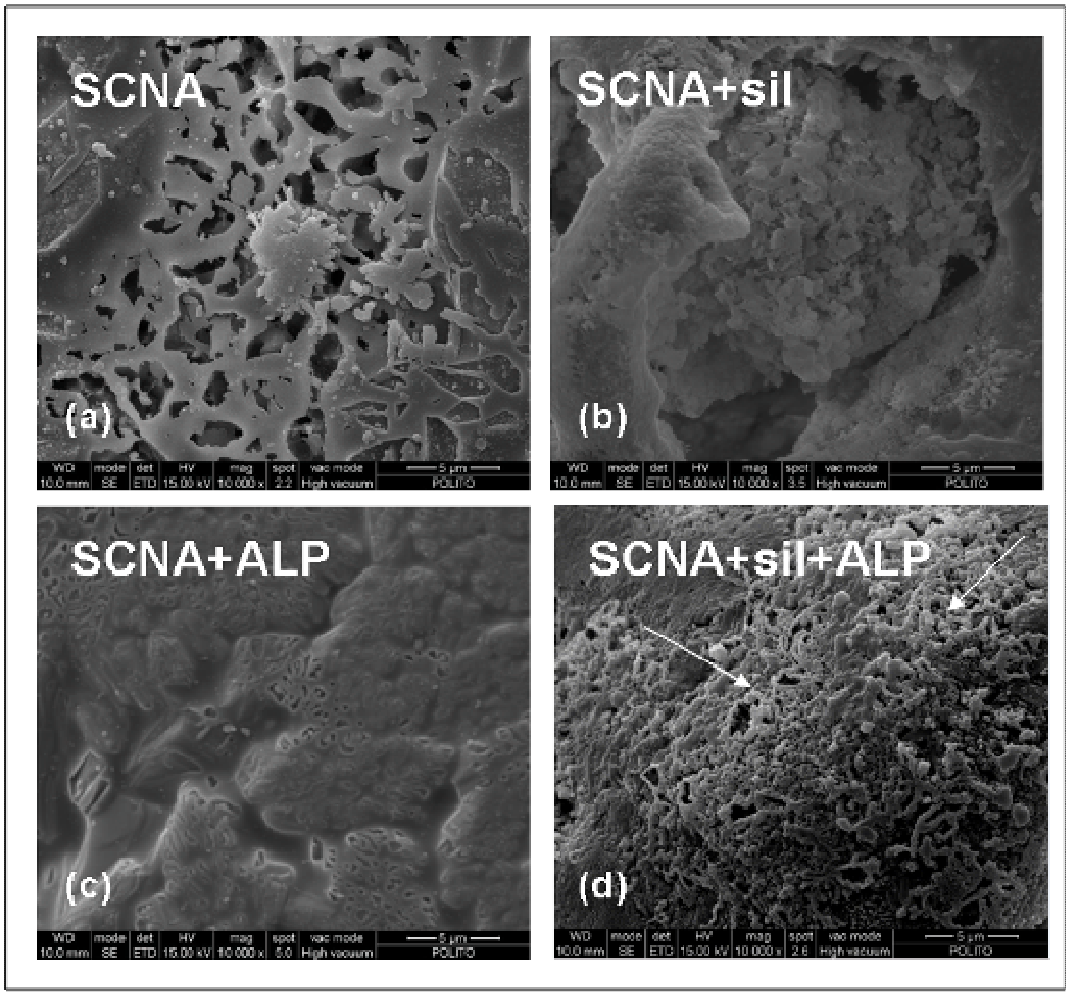

Figure 5: SEM images of a) SCNA, b) SCNA+sil, c) SCNA+ALP, d) SCNA+sil+ALP scaffolds after 1 week in SBF

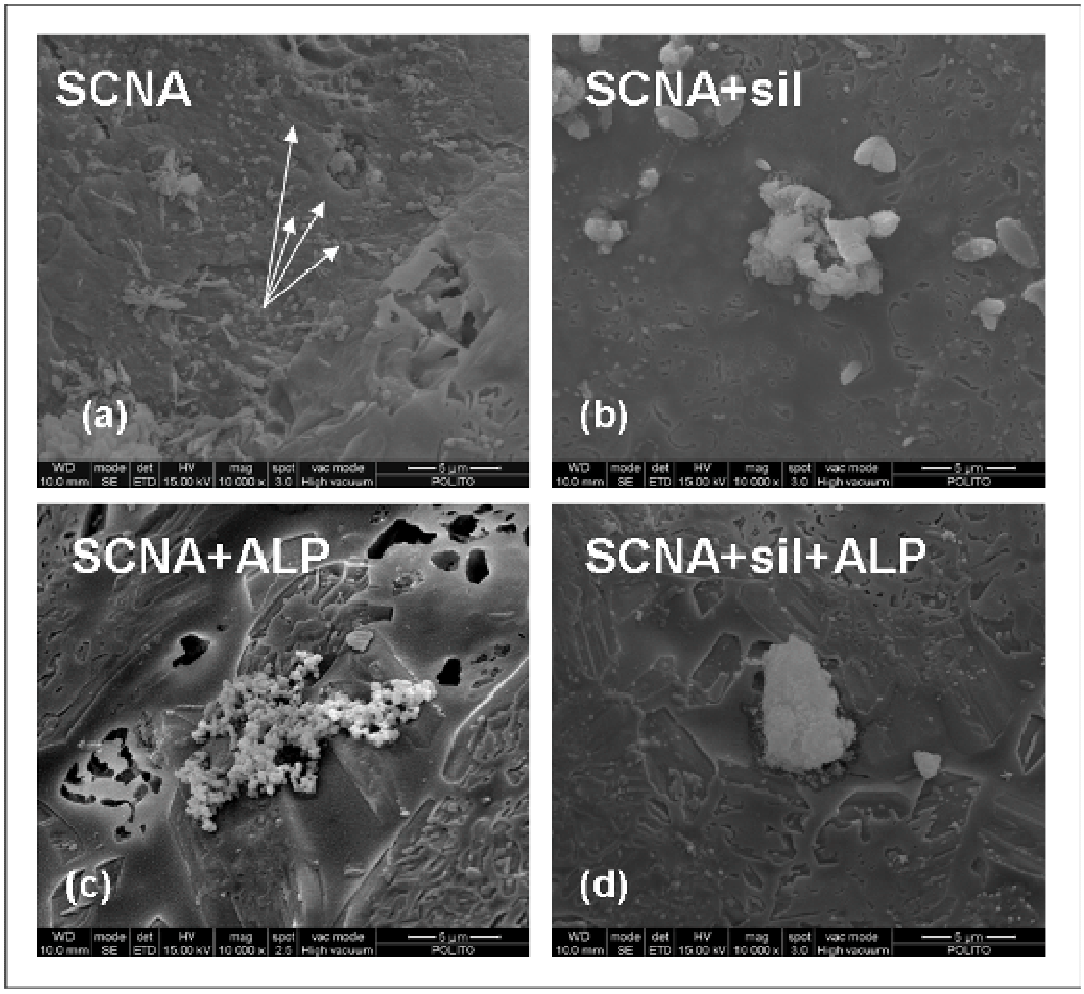

Figure 6: SEM images of a) SCNA, b) SCNA+sil, c)SCNA+ALP, d) SCNA+sil+ALP scaffolds after 2 week in SBF

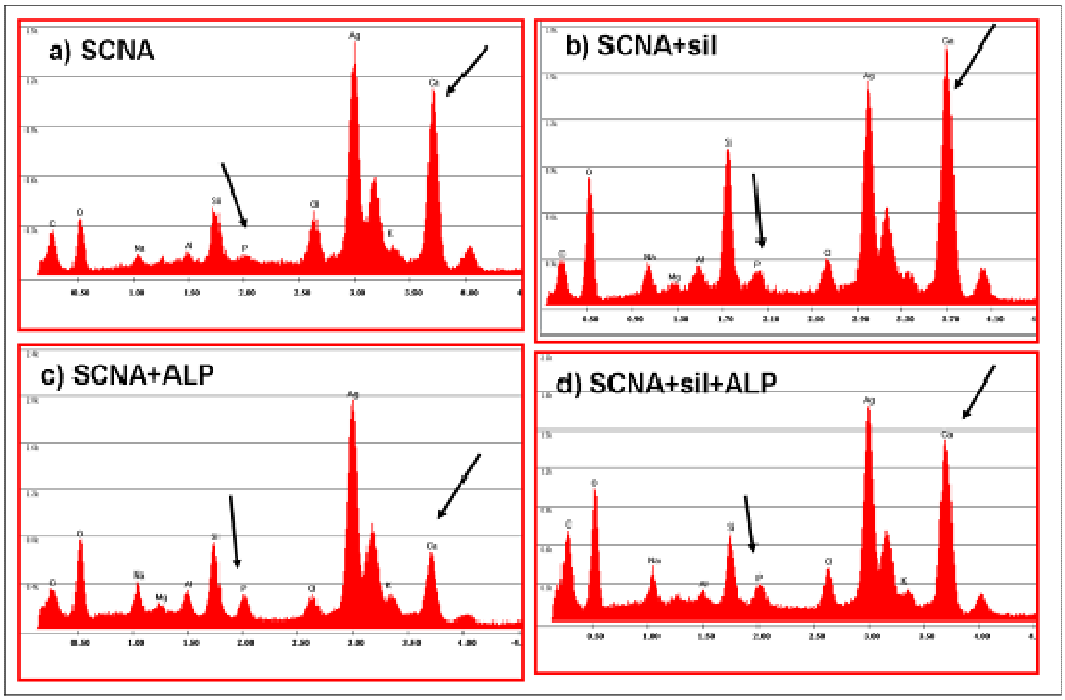

Figure 7: SEM images of a) SCNA, b) SCNA+sil, c)SCNA+ALP, d) SCNA+sil+ALP scaffolds after 1 month in SBF

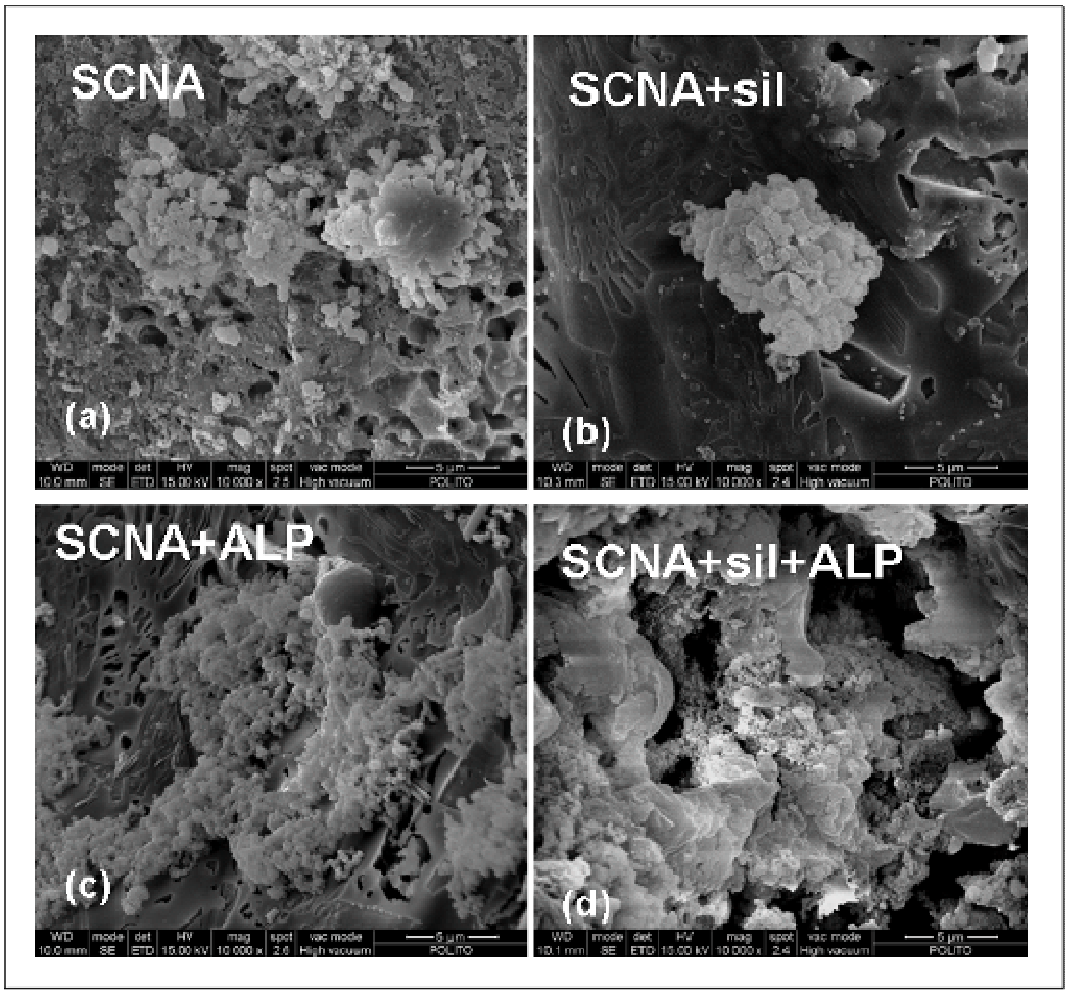

Figure 8: SEM images of a) SCNA, b) SCNA+sil, c)SCNA+ALP, d) SCNA+sil+ALP scaffolds after 2 months in SBF