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02 September 2025

Alkaline phosphatase grafting on bioactive glasses and glass ceramics

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Abstract

Bone integration of orthopaedic or dental implants and regeneration of damaged bone at the surgical site are still unresolved problems in prosthetic surgery. For this reason, biomimetic surfaces (i.e. bioactive surfaces both from the inorganic and biological point of view) represent a challenge for bone implants.

In this research work, a hydrolase enzyme (alkaline phosphatase) has been covalently grafted to bioactive glass- and glass-ceramic surfaces, which are bioactive from an inorganic point of view, in order to impart also a biological bioactivity.

The functionalized samples have been analyzed by means of XPS in order to verify the enzyme presence on the surface. Enzymatic activity has been measured by means of UV-vis spectroscopy after the reaction of the proper substrate. SEM-EDS observations allow monitoring of materials morphological and chemical modifications during the different steps of functionalization. In vitro inorganic bioactivity has been investigated by soaking samples in simulated body fluid (SBF). Enzymatic activity of samples has been tested and compared before and after soaking. Enzymatic activity of the solution has been monitored at different experimental times.

This study demonstrates that alkaline phosphatase could be successfully grafted to different bioactive surfaces maintaining its activity. Enzyme presence enhances in vitro inorganic bioactivity of tested materials.

Keywords: surface functionalization, biomimetism, bioactivity, bone regeneration, enzyme grafting

Introduction

The improvement of bone implant integration and bone regeneration at the surgical site is still an unresolved problem in orthopaedic and dental surgery. Recently, new strategies to provide an appropriate environment for bone regeneration have been investigated. Among them, the delivery of biomolecules with a specific action on the bone mineralization process has attracted much attention.

From the clinical point of view, it is possible to deliver active molecules systemically or locally at the implant site during surgical procedures. These techniques have not been thoroughly investigated because, through systemic delivery, biomolecules could be rapidly inactivated by serum and, on the other hand, a high amount is required to assure a sufficient level at the damaged site. Moreover, growth factors and proteins locally delivered at surgical site without a carrier immediately diffuse and can be rapidly washed out by drainage. Finally, both these techniques require a high amount of biomolecules and do not allow a gradual release at the damaged site [1,2]. A lot of studies have been developed on polymeric matrices as carrier for osteogenic growth factors [3,4].

On the other hand, it is possible to graft these molecules onto implanted material surface. Functionalization techniques could be classified in terms of type of molecule or strategy of delivery. As far as the first point is concerned, it is possible to graft cell adhesion molecules, such as proteins from the extracellular matrix (ECM proteins) or derived peptides (for example Arg-Gly-Asp sequence - RGD); growth factors and enzymes could be grafted as well. As far as grafting strategies are concerned, there are three main techniques available: simple adsorption, covalent bonding or release from a degradable carrier [5,6].

In literature several solutions have been proposed. ECM proteins or peptides are mainly anchored to biomaterials surfaces in order to improve the biological response of implants. Fibronectin has been grafted to titanium surface via tresyl chloride activation [7], synthetic peptides has been covalently grafted to oxidized titanium substrates after silanization [8].

Other research groups focused their activities on enzymatic grafting onto inert surfaces for analytical purposes, such as sensors [9,10].

Finally, the possibility to anchor growth factors to biomaterials has been considered, for example bone morphogenetic proteins (BMP) have been grafted to titanium surface via plasma surface modification treatment [11] and on bioactive glasses surface by silanization [12].

Biomimetism can be defined as the ability of promoting both inorganic and biological response. The inorganic response refers to the ability of inducing hydroxyapatite precipitation onto material

surface after the exposition to physiological or simulated body fluids (this behaviour is commonly known as "bioactivity", and it was widely studied and documented for bioactive glasses and glass-ceramics [13]). The biological response refers to the material ability of directly stimulating cell behaviour by proper biochemical signals.

It is widely known that bioactive glasses and glass-ceramics are able to induce apatite precipitation in physiological fluids [13] and thus, by anchoring to their surfaces specific biomolecules, it is possible to improve tissue regeneration around implants both from a chemical and biological point of view.

The aim of the present research work is to graft an enzyme involved in bone formation and mineralization (alkaline phosphatase – ALP) to the surface of bioactive glasses and glass-ceramics, in order to obtain stable biomimetic surfaces.

Bioactive glasses and glass ceramics have been chosen because they have high potential as materials for bone defects regeneration.

Alkaline phosphatase has been chosen as biomolecule for the biological functionalization since it could be a good model enzyme: it is well known and widely used as a marker of osteoblast differentiation for *in vitro* tests and it is quite simple and relatively inexpensive. At the same time, ALP is of potential interest as it is involved in bone forming and mineralization processes. A study in literature [14] underlines that higher ALP activity is detected in mineralization area of alveolar bone and cellular cementum, and in particular it shows that there is a relationship between enzyme activity and cementum thickness. Another research work [15] describes that alkaline phosphatase application in conjunction with titanium implants improves new bone formation and mineralization around materials. Moreover, it has been suggested [16] that ALP coatings onto surfaces could favour mineralization and attachment of collagen sheets to functionalized substrates. The active role of ALP in the mineralization process was also evidenced by E.E. Golub et al. [17] and investigated by L.T. de Jonge at al. [18] who reported experimental evidences of the ability of ALP grafted surfaces of providing local enrichment of Ca^{2+} and PO_4^{3-} ions.

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Materials and methods

Materials preparation

Two different biomaterials have been considered in this research work. The first one is a glass with a simple composition and a low bioactivity index, belonging to the system SiO_2-CaO-NaO-Al_2O_3, named SCNA from now on. The second one is a more complex glass with a high degree of bioactivity, named CEL2 (belonging to the system SiO_2-P_2O_5-CaO-MgO-Na_2O-K_2O).

Glasses has been prepared by melting the precursors in a platinum crucible and quenching the melt on a brass plate obtaining glass bars, which have been subsequently annealed in furnace to relax residual stresses. Detailed compositions of glasses, melting temperatures and annealing conditions are reported in Table1.

Annealed bars have been cut in a mechanical cropper and polished with SiC abrasive papers and diamond pastes.

SCNA was chosen for its simple composition and high stability, as demonstrated by previous works concerning silver surface enrichment on bulk and coatings [19,20] as well as surface functionalization with bone morphogenetic proteins [12]. It was investigated only in it glassy form since the foreseen applications (i.e. bioactive coatings) do not need a devitrification treatment. On the contrary, CEL2 has been investigated both in its glassy and glass-ceramic forms. This second step has been carried out because CEL2 has been employed for the preparation of highly bioactive glass-ceramic scaffolds as described in previous works [21,22]. Functionalization of glass-ceramic scaffolds will be a possible future application of the process described in this paper. In order to prepare CEL2 glass ceramic samples (CEL2-cer), CEL2 samples, have been subjected to a thermal treatment, 1 h at 950°C, before polishing. After this process CEL2-cer samples have been polished in the same way of glass samples.

Hydroxyls exposition

The first step of functionalization process is a cleaning treatment able to promote both the surface removal of contaminants and the exposition of reactive hydroxyls. The cleaning procedure has been chosen from a previous study on functionalization of bioactive glasses [12]. In the cited reference, different treatments were compared through contact angle measurement and XPS analysis in order to study surface modification (hydroxyls exposition and bioactivity reactions). The optimal treatment has been chosen as the one that allows high hydroxylation in a controlled and reproducible way. On this basis, a first wash in acetone for 5 min in an ultrasonic bath has been carried out and subsequently the samples have been washed three times in bi-distilled water for 5 min in ultrasonic bath to favour -OH groups exposition.

After this treatment, samples have been air dried at room temperature as reported in ref 12. Hydroxylated samples are indicated, from now on, as GLASS_wash (Table 2).

The presence of hydroxyls groups onto samples surface has been investigated by means of contact angle measurements (Drop Shape Analysis System DSA10, Kruss), since their exposition makes surface hydrophilic.

Silanization

The second step of the process is the introduction of specific functional groups in order to promote and stabilize the bonding between material and organic molecule.

In this research work, silanization has been chosen because it is a well known technique for traditional glass functionalization. In our experiments this technique has been transferred to reactive glasses and glass-ceramics, as previously described [12].

3-amminopropyltriethoxysilane ($H_2N(CH_2)_3Si(OC_2H_5)_3$, APTES, 99% Aldrich) has been employed in order to introduce amino groups as functionalities for enzyme grafting.

Silanization has been carried out using a method analogue to the one described by Ma et al. [23] and already applied by Verné et al. in ref. [12]. Glass and glass-ceramic samples have been immersed in a solution containing 150 ml ethanol and 35 µl of silane for 6 h at room temperature

and then dried 1h at 100°C in a furnace in order to stabilize the silane-surface bond. After cooling the samples have been washed three times in ethanol in an ultrasonic bath for 5 min in order to remove not-bonded molecules and dried 1 h at 100°C in a furnace. Silanized samples are indicated, from now on, as GLASS+sil (Table 2).

Silanization effectiveness has been verified by means of contact angle measurements as the silane presence onto materials surfaces induces hydrophobicity.

Some samples have been ultrasonically washed for 2 minutes in TRIS-HCl solution after silanization process and then analyzed in order to study the stability of silane-surface bonding. TRIS-HCl has been chosen because it is one of the media involved in the following steps of functionalization.

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

A second technique employed to investigate this step of the process has been XPS analysis (Al source, Surface Science Instruments, M-Probe) as it allows the detection of nitrogen, characteristic of amino groups, onto samples surface.

Alkaline phosphatase grafting

The final step of the functionalization process is biomolecule anchoring.

In order to covalently graft alkaline phosphatase to glasses and glass-ceramics, the samples have been immersed in a solution 5 mg/ml of ALP in phosphate buffered saline solution (PBS - Sigma Aldrich). ALP solution has been prepared by dissolving bovine alkaline phosphatase (Phosphatase Alkaline from bovine intestinal mucosa, lyophilized powder, 10-30 DEA unit/mg solid – Sigma Aldrich) in PBS, in a recipient under magnetic stirring.

Silanized samples and only hydroxylated ones have been immersed into 5 ml of ALP solution for 20 hours. Different grafting temperatures have been tested: 4°C in a refrigerator, 25°C in a

thermostatic bath and 37°C in an incubator. At the end of incubation period samples have been washed, without stirring or shaking, in TRIS-HCl solution buffered at pH 7.5 and air dried at room temperature.

Functionalized samples are indicate, from now on, as GLASS+sil+ALP and GLASS+ALP respectively (Table 2). Anchoring temperature is specified at each time.

As far as grafting temperature is concerned, the two glasses (SCNA and CEL2) and the glass ceramic (CEL2-cer) have been grafted at 4°C, while glassy SCNA and CEL2 have been studied also at different temperatures, in order to compare a more stable glass and a more reactive one. Temperatures have been selected on the basis of the functionalization procedures described in literature [7,24]. The three temperatures mentioned above have been chosen in order to maximize reaction effectiveness, preserving at the same time ALP from inactivation and denaturation.

ALP is sensitive to both temperature and pH alterations: it has been shown that for pH below 5 and temperatures higher than 40°C inactivation occurs [25,26]. All treatments have been performed in PBS in order to keep pH constant around 7.5. Temperature has been kept below 37°C.

In order to verify the effect of temperature on both grafting and protein activity, also samples of pure ALP solution have been stored at the different temperatures and then analyzed to verify their activity, as described in the following paragraph.

In order to study the bonding stability between grafted ALP and the material's surface, some functionalized samples (both by directly anchored and silanized) have been washed for 2 minutes in ultrasonication apparatus [12] in 10 ml TRIS-HCl solution and then analyzed for enzymatic activity. Also washing solutions have been analyzed to determine the amount of ALP washed away from the samples.

Grafting efficacy has been investigated by means of enzymatic activity tests (described in the following paragraph) and by means of XPS analysis. As far as the last technique is concerned, in the survey spectra an enrichment in carbon and nitrogen and a decrease in signals characteristic of glass/glass-ceramic substrate are expected as a consequences of ALP presence onto materials

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surface. The detailed study of carbon region allowed to investigate the characteristics peaks of the enzyme at about 286 eV (C-O and C-N bonds) and 292-293 eV (aromatic rings).

Enzymatic activity study

In order to improve bone formation and mineralization ALP grafted onto materials surface has to be biologically active. Enzymatic activity has been studied by monitoring the reaction between ALP and p-nitrophenilphosphate in alkaline environment. P-nitrophenilphosphate is hydrolyzed by ALP and produce p-nitrophenol, characterized by a yellow colouring. The amount of p-nitrophenol produced is proportional to the amount of ALP present and could be quantified by means of UV-vis spectroscopy.

In order to determine enzymatic activity, functionalized samples have been put into 1 ml of 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 have been purchased from Sigma Aldrich Fluka). The pH value of the reactive mixture is 10.5. The reaction between ALP grafted onto samples surface and p-nitrophenilphosphate in the solution has been stopped after 2 min by the addition of 1 ml of NaOH 0.1 N. After removing solid samples from the wells the intensity of the produced yellow colour has been quantified by measuring the UV absorbance at 405 nm (GENIUS Spectra FLUOR plus TECAN).

Values of ALP activity for bulk samples have been related to samples surface area in order to minimize data dispersion due to differences in samples shape and dimensions. In order to obtain enzymatic specific activity absorbance values have been divided by samples surface area, only the surface exposed to functionalization has been considered. Roughness has not been contemplated because samples have all been mirror polished.

As far as the washings solutions are concerned, 0.5 ml of the solution has been added to 0.5 ml of reactive mixture; reaction has been stopped adding 0.5 ml of NaOH after 2 minutes and then measurements have been carried out in the same way of solid samples.

Enzymatic activity of ALP solutions at defined concentrations from 0.01 mg/ml to 5.00 mg/ml has been evaluated in order to have a standard reference curve for ALP activity.

Tests have been carried out using multi-well plates.

In order to get a reference, for each test plate 3 measurements on wells containing only reactive mixture and NaOH have been carried out. Reference value has been subtracted to samples absorbance in order to obtain standard and comparable results.

Analysis of surface morphology and chemistry during functionalization procedure

In order to understand surface modifications during the functionalization process, samples at different steps of the treatment (hydroxylation, silanization, enzyme grafting) have been investigated by means of scanning electron microscopy and EDS analysis (SEM – FEI, QUANTA INSPECT 200, EDS - EDAX PV 9900).

In-vitro bioactivity tests

Samples of both glasses at different stages of functionalization process (hydroxylation, silanization, enzyme grafting) have been soaked in simulated body fluid (SBF) in order to evaluate whether biological modification affects also inorganic bioactivity. SBF has been prepared as described by Kokubo et al. [27]. For this test ALP grafting has been performed at 4°C.

Samples of the most reactive glass (CEL2) have been maintained in SBF up to 1 week because at this time material is already able to trigger and expose its bioactive behaviour [21]. As far as SCNA is concerned 2 weeks of dipping have been considered as shorter time because its kinetics are slower [19].

Solution refresh has been performed every two days in order to mimic natural turnover of physiological fluids, at each refresh time solution pH has been registered.

At the end of soaking period samples have been washed, without stirring or shaking, in double distilled water and observed by means of scanning electron microscopy (SEM – FEI, QUANTA INSPECT 200, EDS - EDAX PV 9900).

Before SEM observation all samples have been sputter coated with a thin metal layer in order to make them conductive. Silver has been employed for CEL2 samples while Chromium for SCNA samples.

As far as the most reactive glass (CEL2) is concerned, enzymatic activity test have been carried out before and after SBF soaking in order to determine whether ALP has been released or still remains onto glass surface in active state. SBF solution has also been analyzed at different experimental times in order to individuate ALP presence.

Statistical analysis

All tests have been performed in triplicate.

Data have been analyzed by means of one-way ANOVA, with a significance level $p<0.05$.

Data have been represented as mean ± standard deviation.

Results

Hydroxyls exposition

After acetone and water washings a significant reduction in water contact angle could be observed (Figure 1). The difference between contact angle before and after washing procedure is statistically significant ($p<0.05$) for all tested materials. This result indicates the high wettability induced by hydroxyl exposition and proves the efficacy of the first step of the functionalization process

Silanization

This step has been firstly investigated by means of contact angle measurement, since a silane layer onto material surface would change its wettability and in particular would make the surface hydrophobic.

Contact angle measurements after silanization are described in Figure 1. There is an evident increase in contact angle for all tested materials, due to the anchoring of a hydrophobic molecule on the glass surface, and the difference between angles is statistically significant in all cases ($p<0.05$). This means that silanization is effective on all the investigated bioactive materials, despite their intrinsic reactivity. These data are in agreement with previous studies [12].

Silane-surface bonding results stable as there is not a significant decrease ($p>0.05$) in contact angles values after ultrasonic TRIS washings of the silanized samples (see last series in figure 1).

XPS analysis shows an enrichment in nitrogen (characteristic of silane amino groups) after silanization for all materials tested (Table 3, row 3).

Alkaline phosphatase grafting

XPS analysis of functionalized samples shows an enrichment in carbon and nitrogen after the grafting and also a decrease in substrate constituents for all the samples (Table 3, rows 2 and 4). XPS spectra (carbon region details) of SCNA samples after washing procedure (for control purpose) and after functionalization (direct ALP grafting and silanization and ALP grafting) are reported, as examples, in Figure 2.

The detailed analysis of carbon region shows a peak characteristic of hydrocarbons contaminants (284.6 eV) always present, but on ALP grafted samples two other peaks appear: peak B (286 eV) typical of C-O and C-N bonds present into the enzyme and also a peak E (292-293 eV) due to aromatic rings flattened on the surface .

Enzymatic activity

Figure 3 reports a graph of the specific enzymatic activity for the tested materials after 4°C grafting showing that ALP has been successfully grafted on all the considered substrates.

It could be noted that the amount of ALP is quite lower on CEL2 glass and CEL2-cer than on SCNA, nevertheless, it is important to note that a significant amount of ALP could be grafted in an active state also onto very bioactive materials such as CEL2 and CEL2-cer.

In order to evaluate the bonding stability between ALP and materials surface, functionalized samples have been ultrasonically washed for 2 minutes in TRIS solution. Figure 4 shows the comparison between enzymatic activity before and after washings of SCNA and CEL2 functionalized at different temperatures with and without silanization. Table 4 reports the percentage decrease of the specific enzymatic activity after washing. It can be observed that silanization does not increase the amount of ALP grafted to both glasses (SCNA and CEL2) at 4°C and 37°C. The difference between enzymatic activity of Glass+ALP and Sil-Glass+ALP is not statistically significant (p>0.05). On the other hand, the amount of ALP that remains onto materials surface after washing is higher for silanized samples for both glasses if anchoring has been performed at 4°C, but not at 37°C (Figure 3 and 4, Table 3).

The situation is similar for CEL2-cer: it seems that direct anchoring could be slightly more effective than silanization to graft ALP at 4°C.

Considering the grafting temperature, it could be noted that there is not a significant difference in the amount of ALP grafted to both glasses (SCNA and CEL2) at 4°C or at 37°C, except in the case of direct anchored samples after washing. In this case the amount of ALP maintained onto glasses surfaces is higher if grafting has been performed at 37°C.

It has been verified that ALP solution maintains its activity after storage at all tested temperatures. The mean value of enzymatic activity of standard solution after 20 hours storage at 4°, 25° and 37° are 1.4, 1.9 and 2.0 respectively. It seems that low temperature storage could reduce ALP activity.

As far as the type of glass is concerned, it could be observed that the amount of ALP grafted onto the most reactive glass (CEL2) is slightly lower than the one grafted onto the more stable one

(SCNA) both at 4°C and 37°C, with or without silanization. Differences are not statistically significant and so we can state that ALP could be successfully grafted onto bioactive glasses despite their reactivity. On the other hand, the obtained data underline that after washing, CEL2 samples are able to maintain an higher amount of ALP onto their surface (both silanized and not at 4°C and only not silanized at 37°C).

Considering the washing solutions (Figure 5), it could be observed that a significant amount of ALP has been washed off from samples and it is present in the solution in an active state. Data of ALP activity for samples are related to surface area (specific activity) while data for solutions are measured on the whole solution volume, so they could not be quantitatively compared. After washing there is no significant difference ($p > 0.05$) between the amount of ALP found in the solution for directly grafted and silanized samples for every materials and anchoring temperatures.

Analysis of surface morphology and chemistry during functionalization procedure

Figures 6-8 show SEM images and EDS spectra for SCNA, CEL2 and CEL2-cer at different steps of functionalization process. In the EDS analysis the presence of Ag is due to the metallization process and the presence of Cl is due to the use of Tris-HCl. It could be seen that for the most reactive glass (CEL2), the bioactivity reactions (hydroxyl condensation to form silica gel) already begins into the functionalization medium (see Figure 6 c and d). In fact in this case, a silica rich gel-like layer is observed in the SEM micrograph and its presence can be confirmed through EDS analysis showing a high Si peak. This behaviour is not shown by CEL2-cer (Figure 7) and SCNA (Figure 8).

In-vitro bioactivity of functionalized samples

Figure 9 shows SEM micrographs of CEL2 samples (at different step of functionalization process) after one week in simulated body fluid. It could be observed that an evident silica gel layer is present on all samples. EDS analysis underline an enrichment in calcium and phosphorous for this gel. Looking at ALP grafted samples (figure 9c and 9d – ALP grafted directly to hydroxyls groups,

figure 9e and 9f – ALP grafted to silanized surface) it is clear that a significant amount of particles, with the typical morphology of hydroxyapatite, start to precipitate all over the gel. EDS spectra confirm that they are constituted mainly of Ca and P.

Figure 10 shows SEM micrographies of SCNA (at different stages of functionalization process) after 2 weeks in SBF. As far as washed and silanized samples are concerned no silica gel is present (figure 10a and 10b) and only few small hydroxyapatite particles could be detected and their dimensions are lower than 3 μm. Looking at ALP grafted samples (figure 10c– ALP grafted directly to hydroxyls groups, figure 10e– ALP grafted to silanized surface) a significant number of particles with dimensions comprised between 5 and 20 μm could be detected. EDS (figure 10d– ALP grafted directly to hydroxyls groups, figure 10f– ALP grafted to silanized surface) analysis confirms that they are constituted mainly of Ca and P.

pH of SBF solution shows small variation (7.4 – 7.6) in the dipping period for both glasses.

Enzymatic activity tests after SBF soaking

Figure 11a reports enzymatic activity measured on functionalized samples, functionalized and ultrasonically washed samples and functionalized samples after 1 week in SBF. It could be noted the SBF soaking significantly reduces ALP activity of samples but less than ultrasonic washing.

Figure 11b underline that ALP, gradually released in SBF, maintains its activity both on material surface and in solution, for at least 1 week. Since tests have been carried out with solution refresh, it could be observed that enzyme release continues through the entire dipping period even if it is higher in the first days.

Discussion

Surface cleaning, hydroxyls exposition and silanization have been successfully carried out applying a procedure optimized in previous works [12].

In this research the enzyme alkaline phosphatase has been grafted to two bioactive glasses and a glass-ceramic, with different degree of bioactivity, both via silanization and also through direct

anchoring to hydroxylated surfaces. In both cases a significant increase in carbon and nitrogen has been detected in XPS analysis. At the same time the decrease in elements characteristic of glass substrate has been observed. These observations suggest the presence of an organic layer on the surface. Enzyme grafting has been confirmed by detailed analysis of carbon region, in fact specific signals for C-O and C-N bonds (286 eV) and for aromatic rings flattened onto the surface (292-293 eV) has been detected. The first one is related to typical bonds in proteins while the second could be attributed to phenylalanine, an amino acid present in ALP [28]

It is recognised that a grafted enzyme should be in an active conformation in order to fulfil its tasks and so, in order to verify the ALP activity after anchoring, the reaction between ALP grafted samples and p-nitrophenylphosphate has been monitored.

From enzymatic activity study it could be noted that the amount of grafted ALP is quite lower on CEL2 glass and CEL2-cer than on SCNA, this is likely due to their reactivity in PBS (that has pH similar to physiological fluids – 7.00) that could interfere with bonding reactions reducing the total amount of enzyme finally attached. Hydroxyl condensation could interfere with bonding reactions as described in Figure 12 in which the presence of well exposed or condensed hydroxyl group is shown. In fact, after the washing treatment, on the less reactive glass (SCNA), hydroxyls are well exposed and prone to silanization or biomolecule anchoring, while onto the most reactive surface (CEL2) hydroxyls partially condense to form hydrated Si – O – Si species (silica gel). The situation is more complex for CEL2-cer that is a glass-ceramic: in fact, in this case only the amorphous phase exposes hydroxyl groups, whereas, as far as the exposition of -OH group is concerned the crystalline phases are inert. Also in the case of CEL2-cer, the residual amorphous phase is reactive and the exposed -OH can partially condense, as for amorphous CEL2, leading to a lower surface reactivity toward both silanes and ALP.

This hypothesis is confirmed by SEM observation of functionalized samples, since CEL2 samples show an evident reaction layer after 1 day incubation in PBS for ALP grafting, while this phenomena did not appear for SCNA samples treated in the same conditions (Figures 6 and 8).

Surface reactivity (silica gel formation) could also explain the CEL2 ability to retain a higher amount of ALP after washings, as the silica-gel is porous and could adsorb and retain ALP.

Looking at washing solutions (Figure 5) it could be observed that the released ALP maintained its activity. This is indeed a very positive feature, as it would induce osteoblasts differentiation and bone mineralization combining this effect with the one due to the grafted ALP that is retained at the materials surface after washings.

Bioactive glasses are able to induce hydroxyapatite precipitation into simulated body fluid solutions with a mechanism described by Hench et al. [13] that could include, during the early stages of immersion in simulated body fluids, a silica gel layer formation. As described in previous works [19] SCNA presents a slower kinetic in bioactive behaviour, in fact this glass contains 3% molar of Al_2O_3 that, as reported in literature [13], inhibits bioactivity. If compared with CEL2 it is clear that surface reactivity is lower even after 2 weeks dipping.

In vitro bioactivity tests on modified samples underline that biological functionalization even if it includes the deposition of a double organic layer does not inhibit reactivity of bioactive glasses Figures 9 and 10). In fact, for the most reactive glass, silica gel formation is allowed both on only silanized and silanized and grafted with ALP samples.

Besides, the presence of the enzyme alkaline phosphatase quickens reaction kinetics both for the most reactive glass but also for the most stable one. In fact hydroxyapatite particles precipitate on both functionalized surfaces in number and dimensions higher than the ones that could precipitate onto non functionalized samples.

Finally, it could be noticed that direct anchoring of ALP (without silanization) seems to be more effective for inorganic bioactivity enhancement, in fact an higher amount of Ca and P rich particles of bigger dimensions (figure 9d and 10c) could be observed on these samples.

Alkaline phosphatase is a homodimeric metalloenzyme which catalyzes the hydrolysis of almost any phosphomonoester with release of phosphates and alcohol, its properties has been associated to mineralization process and phosphates homeostasis of bone tissue [29]. ALP acts as a nucleation

centre for hydroxyapatite because it is able to release phosphates and captures calcium ions, in this way the presence of alkaline phosphatase and of its substrate (β-glycerophosphate) or simply of a solution rich in calcium ions induces the deposition of HAp crystals and amorphous calcium phosphates. This property has been applied in order to realize hydroxyapatite layers onto organic substrates [16, 30]. In its physiological activity alkaline phosphatase binds the phosphate group of the phosphate ester to form a transient phosphoenzyme which is subsequently hydrolyzed [31]. The active site of ALP is characterized by two zinc ions and is a phosphate binding site of high affinity. It has been observed that it is able to bind phosphates also at neutral pH [32, 33]. In this way it could be suggested that enzyme, present onto glass surface soaked in SBF, could attract phosphates from the solution to material surface and subsequently calcium ions favouring hydroxyapatite precipitation.

It has also been observed that ALP concentration and activity are particularly high in cellular mineralized tissue such as cellular cementum in the rat molar periodontum [14].

Finally in vivo experiments in rats [15] have demonstrated that alkaline phosphatase adsorption onto plasma-sprayed titanium implants increases bone production around the implant.

Comparing these literature observations with our experimental results it could be noted that ALP grafting onto the surface of bioactive glasses firstly does not reduce surface reactivity but enhance the ability to induce hydroxyapatite precipitation. So the bioactive behaviour of a biocompatible and slightly reactive glass, such as SCNA, could be improved by biological functionalization. Moreover enzyme presence could also promote new bone formation by stimulating osteoblast cells. This last hypothesis will be investigated in a future work with specific cellular tests on functionalized materials. Further investigations should be also planned in order to asses the role of the silane layer on the early stages of bioactivity, but this is not the first aim of the present work.

Conclusions

ALP has been successfully attached to bioactive glasses and glass-ceramics with different degree of bioactivity. Modification has been carried out with good results despite the differences in the surface reactivity. ALP maintains its activity after grafting on all investigated surfaces.

Enzyme anchoring has been performed effectively both on silanized surfaces and also by direct anchoring to exposed hydroxyls. A fraction of the grafted biomolecule is strongly bonded and remains attached to surface also after severe (ultrasonic) washings; besides the released ALP maintains its activity in solution. This is an interesting result as, in vivo, the functionalized materials will be in contact with physiological fluids and it is expected that a certain amount of ALP can be released while the remaining one will be strongly bonded to surface. Both fractions of ALP will be in their active state, so they could stimulate bone tissues for both regeneration and implant integration. The amount of ALP released in vivo will be related to fluids aggressiveness but it is foreseen that their effect will be less aggressive than ultrasonic treatment.

Finally it has been observed that ALP is able to enhance and accelerate bioactive kinetics in simulated body fluid, so it seem that biological functionalization has a positive synergic interaction with inorganic bioactivity. Cellular tests are in progress in order to evaluate how the conjunction between inorganic and biological activity is able to stimulate cell behavior.

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Figures captions

Figure 1: Contact angles before and after washings and silanization process. * and **indicates a statistically significant difference between values marked with the respective symbols (p<0,05)

Figure 2: Carbon region XPS spectra for: a) SCNA washed, b) SCNA directly grafted with ALP without silanization and c) SCNA silanized and grafted with ALP

Figure 3: Enzymatic specific activity - comparison between direct anchoring and silanization for the different materials after grafting at 4°C.

Figure 4: Enzymatic activity - comparison between direct anchoring and silanization before and after washing, a) 4°C grafting, b) 37°C grafting. * or ** indicates a statistically significant difference between values marked with the respective symbols (p<0,05)

Figure 5: Enzymatic activity in washing solutions, a) 4°C grafting, b) 37°C grafting

Figure 6: SEM micrographs of CEL2 at different steps of functionalization process

Figure 7: SEM micrographs of CEL2cer at different steps of functionalization process

Figure 8: SEM micrographs of SCNA at different steps of functionalization process

Figure 9: SEM micrograph of CEL2 samples after 1 week in SBF. a) washed sample, b) silanized sample, c) directly ALP grafted sample, d) directly ALP grafted sample, higher magnification, e) EDS of the particle in d, f) silanized and ALP grafted sample, g) silanized and ALP grafted sample, higher magnification, h) EDS of the particle in g.

Figure 10: SEM micrograph of SCNA samples after 2 weeks in SBF. a) washed sample, b) silanized sample, c) directly ALP grafted sample d) EDS of the particle in c, e) silanized and ALP grafted sample, f) EDS of the particle in e.

Figure 11: Enzymatic activity on CEL2 samples after functionalization, functionalization and ultrasonic washing, functionalization and SBF soaking (a) and in SBF solution after sample soaking (b). ALP grafting has been performed at 4°C.

Figure 12: Hydroxyls exposition on biomaterials with different reactivity

Table 1: glasses composition (molar percentages), melting temperatures and annealing conditions.

Glass	\multicolumn{7}{c}{Glass composition (molar %)}	$T_{melt}[°C]$	Annealing
	SiO_2	P_2O_5	CaO	MgO	Na_2O	K_2O	Al_2O_3		
SCNA	57	-	34	-	6	-	3	1550	10h@600°C
CEL2	45	3	26	7	15	4	-	1400	12h@500°C

Table 2: Names and characteristics of prepared and analysed samples

Sample name	Material	Treatment
SCNA_wash	SCNA	Hydroxylation[a]
SCNA+sil	SCNA	Hydroxylation and silanization
SCNA+sil+ALP	SCNA	Hydroxylation, silanization and ALP grafting
SCNA+ALP	SCNA	Hydroxylation and ALP grafting
CEL2_wash	CEL2	Hydroxylation[a]
CEL2+sil	CEL2	Hydroxylation and silanization
CEL2+sil+ALP	CEL2	Hydroxylation, silanization and ALP grafting
CEL2+ALP	CEL2	Hydroxylation and ALP grafting
CEL2cer_wash	CEL2cer	Hydroxylation[a]
CEL2cer+sil	CEL2cer	Hydroxylation and silanization
CEL2cer+sil+ALP	CEL2cer	Hydroxylation, silanization and ALP grafting
CEL2cer+ALP	CEL2cer	Hydroxylation and ALP grafting

[a]5min acetone and (3*5min) water ultrasonic washings

Table 3: N/Si and C/Si ratios for SCNA, CEL2 and CEL2cer at different steps of functionalization process. n.a. indicates that ratio could not be calculated because silicon is not detectable on the surface

	\multicolumn{2}{c}{SCNA}	\multicolumn{2}{c}{CEL2}	\multicolumn{2}{c}{CEL2-cer}
	N/Si	C/Si	N/Si	C/Si	N/Si	C/Si
Glass_wash	0	4,86	0,11	1,92	0,12	1,95
Glass+ALP	n.a.	n.a	5,75	35,81	0,70	4,82
Glass+sil	0,21	1,67	0,54	14,56	0,27	3,58
Glass+sil+ALP	n.a	n.a	3,60	87,30	1,01	6,20

Table 4: Decrement in ALP specific activity after washings

Decrement in ALP specific activity after washings [%]
	\multicolumn{2}{c}{4°C anchoring}	\multicolumn{2}{c}{37°C anchoring}
	ALP	Sil+ALP	ALP	Sil+ALP
SCNA	98,3	93,5	95,8	90,0
CEL2	93,0	73,7	83,6	86,2