Surface Functionalisation of biomaterials with alkaline phosphatase

Surface Functionalization of Biomaterials with Alkaline Phosphatase

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Abstract

Two different glasses, one biocompatible but with a low bioactivity index (G1) and the other with an higher bioactivity index (G2), the ceramic version of the second glass and a titanium alloy (Ti6Al4V) have been functionalizated by anchoring alkaline phosphatase (ALP) on their surfaces. The enzyme has been chosen because it is involved in mineralization processes of hard tissues and is a model for more complex ones. ALP has been grafted on glasses and glass-ceramics surfaces both with and without samples silanization and on metallic surfaces with and without tresyl chloride activation. Samples have been analyzed at each step of the functionalization process in order to verify it .

Introduction

The success of prosthetic surgery is strictly connected to integration between implant and tissue and the rapid healing of bad quality bone. In this way an interesting solution is the realization of biomimetic surfaces for implants, this means that surface is able to send signals directly to cells in order to promote tissue regeneration. A so characterized material is bioactive not only from a physicochemical point of view (hydroxyapatite precipitation in simulated body fluid), but also from a biological one.
Bioactive glasses have been widely studied for orthopaedic and dental applications, as coatings on metallic substrates or for the realization of small bone substitutes. They have an excellent

biocompatibility and promote osteoinduction proportionally to their bioactivity index. Titanium alloys are extensively employed in prosthetic surgery (orthopaedic and dental implants) because of their biocompatibility and good mechanical properties. It could be very interesting for clinical application that these materials also be able to send cells signals to promote their adhesion, proliferation, differentiation, migration and matrix mineralization. So the aim of this research work is the grafting of a biomolecule involved in bone regeneration processes (alkaline phosphatase) to their surface.

Materials and Method

A glass belonging to the system SiO_2-CaO-Na_2O-Al_2O_3 (G1) and one to the system SiO_2-P_2O_5-CaO-MgO-Na_2O-K_2O (G2) were prepared through traditional melting and quenching technique. Glasses have been poured on brass plate in order to obtain bars then annealed in furnace to relax residue tensions. Bars have then been cut and polished to prepare samples. G2 has also thermally treated to promote crystallization (G2cer). Ti6Al4V samples have been prepared cutting and polishing a metallic bar.

The firs step in functionalization process is the cleaning of surfaces to remove any contaminants and the exposition of hydroxyl groups. As for glasses samples have been washed for 5minutes in acetone in an ultrasonic bath for contaminant removing, then they have been washed three times in distilled water for 5 minutes in ultrasonic bath. Contact angle measurements have been carried out in order to verify OH groups exposition as an increase in surface hydrophilicity.

As for titanium samples an acid attack and a thermo-chemical treatment have been realized. Hydroxyls groups have been observed by means of XPS and FTIR analysis.

The second step is the introduction of specific functional groups or a surface activation in order to promote and stabilize the bonding with enzyme molecule. In this research work the activation method selected for glasses is silanization [1] with 3-amminopropyltriethoxysilane, which is characterized by amino groups that support biomolecule bonding. Samples have been immersed for 6 hours in a solution of silane in ethanol, thermally dried in a furnace (1 hour at 100°C) to stabilize bonding between surface hydroxyls and silane molecule, then washed three times in ethanol in ultrasonic bath to remove not-bound molecule and finally thermally dried in furnace 1 hour at 100°C. In order to verify silane grafting on glass surface contact angle measurements and XPS analysis have been carried out. In order to analyze the stability of silane-surface bonding some samples have been washed in TRIS and then observed.

As for titanium an activation with Tresyl Chloride has been selected [2,3].

The last step is alkaline phosphatase grafting. Alkaline phosphatase is a glycoprotein containing zinc and magnesium, it is employed in in-vitro tests as a marker for osteoblast differentiation and some studies underline that it promote new bone forming and mineralization [4,5].

Samples have been incubated in a solution of alkaline phosphatase in PBS for 20 hours at 4°C. Reaction has been stopped by a TRIS washing. As for control also samples without silanization (treated only for hydroxyl exposition) have been grafted with ALP. Some samples have been washed in TRIS in ultrasonic bath after ALP anchoring, in order to investigate bond stability. The same grafting process has been applied to both glasses and metal.

Samples have been then analyzed in order to examine enzyme grafting, by means of XPS and UV-vis spectroscopy. The first technique studies the chemical elements present on the surface and their state, while the second allows to determine enzyme activity. This last test has been carried out by dipping functionalized samples in a solution containing p-nitrophenilphosphate, which is hydrolyzed by ALP to produce p-nitrophenol which has a yellow coloration, this has been quantified by UV-vis spectroscopy.

Results and Discussion

As for hydroxyls exposition tests show that, as expected, surface became hydrophilic. The initial value of contact angle is about 70° and after the different washings it was comprised between 20°

and 30° for all glasses and glass ceramics studied. Titanium samples show OH characteristic peaks both on XPS detailed analysis of oxygen region and on FTIR spectra.
As for silanization contact angle measurements exhibit that surface became hydrophobic, values change to 70°-80°. Results show also that the bonding is resistant to TRIS washings, in fact contact angle values remains unvaried after washings (Fig. 1).

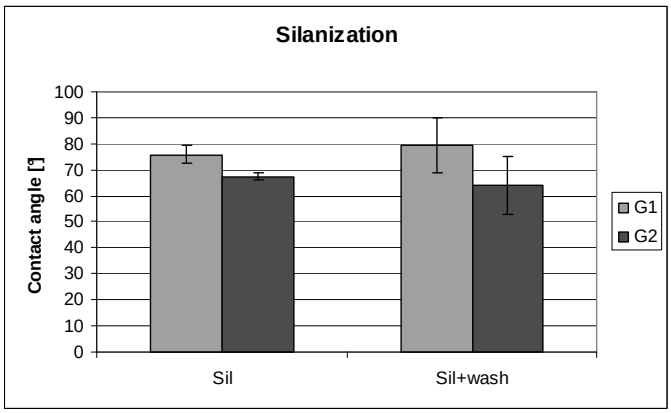

Fig. 1: Contact angle measurement on silanized samples

XPS spectra on samples surfaces show the appearance of nitrogen peak, characteristic of silane amino groups (Table 1).
FTIR spectra of tresylated titanium exhibit characteristic peaks for C-F vibrations.
As for enzymatic activity evaluations graphs show that the molecule is anchored in active state to the surface of all glasses and glass ceramics tested. After washing the weakly bonded portion of ALP is taken off from sample but a significant part remains on surface (Fig. 2). An aliquot of enzyme could be grafted also onto not-silanized samples (exploiting OH groups), but the bond is weaker, in fact after washings the amount of enzyme that remain on sample's surface is lower.

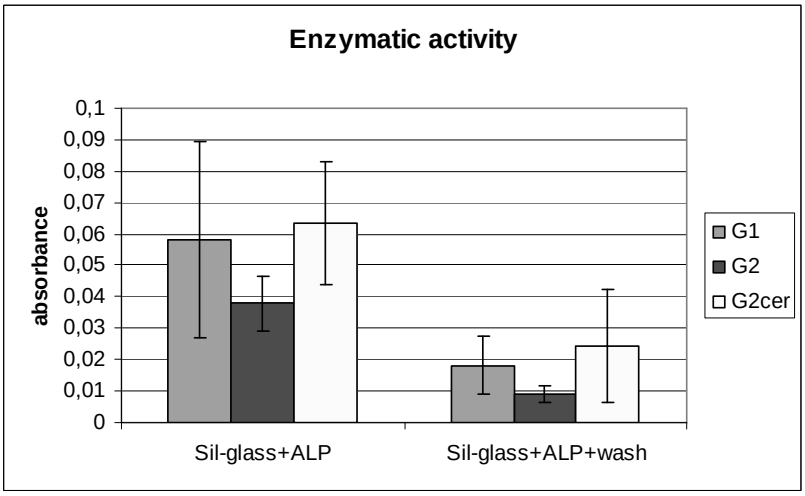

Fig. 2: Enzymatic activity on silanized samples

XPS spectra show an enrichment in carbon and nitrogen that hide glass constituents on surface of functionalized samples (Table 1).

Table 1: XPS data for samples at different step of functionalization process

Sample	O	Na	N	Ca	C	Si	Mg	Cl	S	P
G1+sil	40.1	0.8	4.1	0.9	32.6	19.5	-	-	-	-

G1+sil+ALP	23.8	0.8	7.8	-	61.7	-	-	4.5	0.3	1.1
G1	37.1	1.9	-	1.8	49.1	10.1	-	-	-	-
G1+ALP	23.7	0.8	10.6	-	57.7	-	-	6.5	-	0.8
G2	39.4	1.9	1.8	1.7	32.1	16.7	5.7	-	-	-
G2+sil	18.0	2.6	2.6	-	69.9	4.8	1.8	-	-	-
G2+ALP	26.5	0.7	9.2	-	57.3	1.6	-	3.6	-	1.1
G2cer	41.3	1.5	2.0	3.4	31.9	16.4	3.1	-	-	-
G2cer+ALP	31.2	0.7	6.7	2.0	46.3	9.6	-	2.3	-	1.3
G2cer+sil	32.7	2.0	3.5	2.1	46.1	13.0	-	-	-	-
G2cer+sil+ALP	25.8	0.2	8.7	0.9	53.3	8.6	-	1.3	1.2	-

Besides a detailed analysis of carbon region exhibit a peak characteristic of C-O and C-N bonds (286 eV) and a peak related to aromatic rings (292-293 eV) that are a mark of ALP presence.
It could be noted that on less reactive materials (G1 and G2cer) the bonding with silane and with ALP is stronger and more stable, this happens because in this case bioactivity reactions doesn't interfere with bonding processes.
Similar results have been obtained for metallic samples. Enzymatic activity tests underline that pure metallic samples didn't anchor ALP, hydroxylated ones are able to graft an aliquot of biomolecule but a significant amount is anchored only to tresylated samples. As for washing tests results are analogous to glasses ones.

Conclusions

It is possible to anchor alkaline phosphatase to the surface of glasses and glass-ceramics with different degrees of bioactivity, and to titanium alloy (Ti6Al4V). As for glasses the grafting is realizable both with and without surface silanization, but silane presence on sample's surface strengthens and stabilizes the bond.
Enzyme activity is preserved on all materials after anchoring.
In order to evaluate biocompatibility of functionalized materials and investigate cellular response to biological stimuli, in-vitro tests are in progress.
Both for cellular test and for future clinical applications of this processes, is in progress a study of the possibility of sterilize functionalized samples without reducing enzyme activity.

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