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BIOFUNCTIONALIZATION OF ZIRCONIA BASED MATERIALS BY IMMOBILIZATION OF ALP IN TISSUE ENGINEERING APPLICATIONS

Master of Science Thesis

Examiner: Professor Minna Kellomäki
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ABSTRACT

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The aim of this project is to modify Zirconia surface to perform as a bioactive material in contact with biological tissue with high stability and activity. Alumina was also modified in order to compare the properties with Zirconia in the same laboratory condition. The protein used in this test is alkaline phosphatase (ALP) which improves mineralization and creation of hydroxyapatite in bone formation process. Adsorption and covalent bonding immobilization were investigated on both ceramics. The modified ceramic surfaces also tested in contact with and without human body cells, in biological condition in vitro.

This project contains laboratory tests for powders and planar surfaces. Characterizations of zirconia powder, such as IEP (isoelectric point) were investigated after ALP immobilization. In addition, ALP functionalized planar surface and its behaviour was studied in vitro in simulated body conditions with and without presence of osteoblast-like cells.

Main results of these studies supported that high mineralization accomplished by ALP functionalized zirconia in compare with non-functionalized zirconia in in vitro. In summary, the results of this research indicate the successful immobilization and surface modification of zirconia.

In addition, zirconia powder silanized by APTES, as the initial step of surface modification, showed acceptable stability in room temperature during 28 days.

In case of comparison between physical adsorption and covalent bonding methods, it can be observed that physical adsorption represents higher enzyme attachment. However, these attachments do not show suitable stability. In all the experiments regarding modification of the particle surface, Zirconia powder illustrates higher potential for protein (ALP) immobilization compare to Alumina.

PREFACE

My special thanks to Prof. Minna Kellomäki, head of Biomaterials major in Biomedical Engineering department in Tampere University of Technology, for her great advice to pursue my field of interest.

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Finally, I wish to thank my family, my lovely parents and sister for their support and love, which gave me strength in all the stages of my work in Germany. I also thank my boyfriend for his useful advices and support, which helped me a lot in completing my thesis.

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Aminopropyltriethoxysilane	APTES
beta-tricalcium phosphate	b-TCP
Bovine Serum Albumin	BSA
Cell Culture Medium	CCM
Coefficient of Thermal Expansion	CTE
Dulbecco Modified Eagle Medium	DMEM
Energy-dispersive X-ray spectroscopy	EDX
Extracellular Matrix	ECM
Fetal Bovine Serum	FBS
Human Bone-Derived Cells	HBDCs
Human Osteoblast cells	HOB
Isoelectric Point	IEP
Mesenchymal Stem Cells	MSC
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p-nitrophenol	pNP
P-nitro-phenylphosphate	pNPP
poly(ethylene glycol)	PEG
poly(methyl methacrylate	PMMA
primary Human Osteoblasts	pHOB
Scanning Electron Microscopy	SEM
Stimulated Body Fluid	SBF
Tetragonal Zirconia Polycrystal	TZP
Water-soluble tetrazolium salt	WST
Zirconia	Z
Zirconia+Silan	ZS
Zirconia+Silan+NHS+EDC	ZSNE
Zirconia+Silan+NHS+EDC+ALP	ZSNEA
β-Glycerol phosphate disodium salt pentahydrate	β-GP

1. INTRODUCTION

Zirconia is one of the important ceramic materials used in biomedical and bioengineering science. Recent advances in modern technology and science indicate that it plays a fundamental role in orthopedic and dental applications according to its unique physical and mechanical properties. High strength, toughness and good biocompatibility are the main properties, which make Zirconia unique among the other kind of materials. (Piconi, et al., 1999; Volpato, et al., 2011; Pilathadka, et al., 2007)

Nevertheless, the bioinert surface properties of Zirconia make a huge challenge for the scientists to make a surface with a suitable attachment to the surrounding biological environment. In the recent years, there have been many investigations on surface functionalization. This technique tries to emphasis on the analysis of surface properties while develops and optimizes them in order to obtain acceptable materials overall performance and improve the characteristics. (Treccani, et al., 2013) These studies have demonstrated the successful functionalized surfaces with improved properties. (Verne, et al., 2010). Surface functionalization and modification technique provide the bright future for improving the surface behavior while remaining the bulk properties of different materials such as Zirconia.

The main goal of this thesis is to modify the surface of Zirconia and create the bioactive properties by functionalizing Alkaline Phosphatase enzyme on the surface. This enzyme, which is a basic protein in biomineralization process, is able to make an ideal material in bone tissue engineering application. Alumina surface also have been modified in order to compare the results with Zirconia and get the better understanding of the modification.

This thesis reviews the basics of each functionalization steps, as long as every chemical, which have been used in this surface modification. Two different methods have been used in order to compare the results and study the characteristics of the materials powder: Adsorption and covalent bonding. In addition, two different in vitro investigations are defined to provide the performance of immobilized enzyme on Zirconia in biological condition. The results have been presented and discussed.

2. LITERATURE REVIEW

2.1. Biomaterials

Biomaterials are referred to as biocompatible materials, which can be inserted into the human body in biological environment in different forms in order to treat malfunctions of tissue and organs. They also perform their function in replacement surgery. Biomaterials with different functions are employed to fulfill the basic goal of improvement of human life. They also can be seen in history where the great civilizations used gold and seashell as the replacements for human lost tooth (Bandyopadhyay, et al., 2013; B D, et al., 2012). Biomaterials can be classified same as industrial materials based on bonding and structure; metals, ceramics, polymers and composite (figure 1) and as their compatibility and stability in human body. (Bandyopadhyay, et al., 2013)

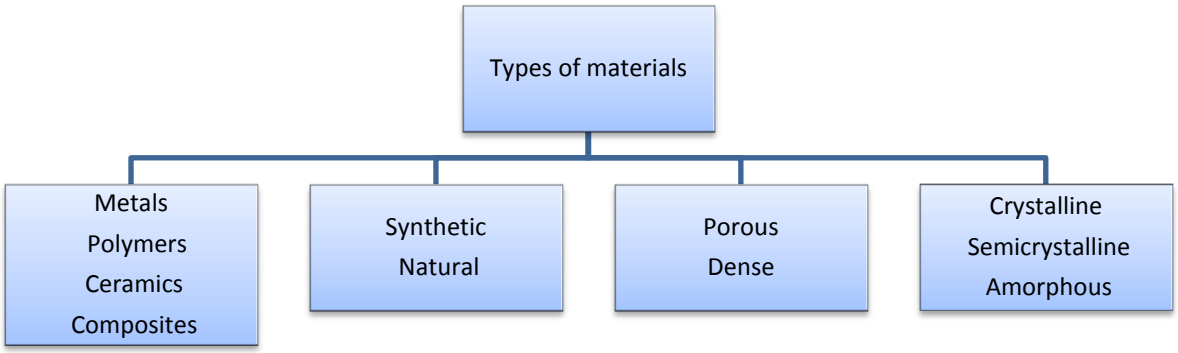

Figure 1. Different types of materials (Bandyopadhyay, et al., 2013)

Biocompatible biomaterials (even) as the delivery system or implant have positive biological host response, which cause no harm or toxic effect while performing dedicated functions or replacement of one tissue or organ. (Hisbergues, et al., 2009; Park J, 2008)

These materials may perform function with or without attachment to the host, which are considered as biostable or bioactive respectively. The other groups of biocompatible materials are those with bio-absorbable behavior, which can be absorbed by the host after performing their function. (Hisbergues, et al., 2009; Park J, 2008)

Biomaterials exhibit different and various properties, both in bulk and surface, such as; chemical, physical, mechanical and biological properties, which all depend on application of the materials, Figure2 (Bandyopadhyay, et al., 2013). In general, one

biomaterial as an implant must show good stability and mechanical properties like high strength and toughness.

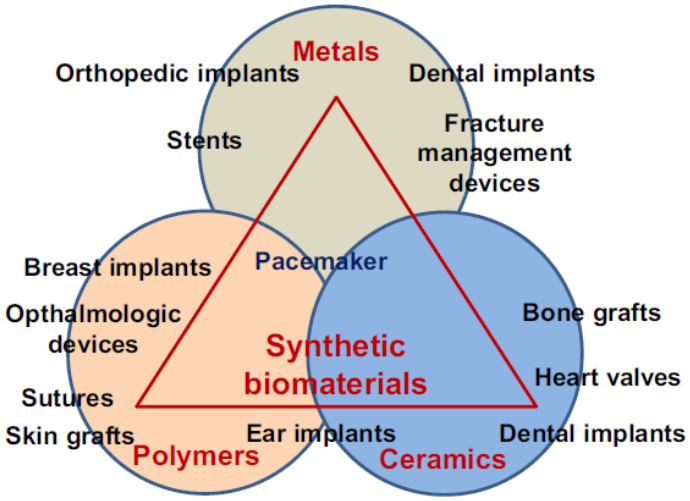

Figure 2. Biomaterials applications (Bandyopadhyay, et al., 2013)

Biomaterials are used in variety of applications such as, stents, heart valves, joint implants, tissue engineering, dental surgeries, drug delivery and bone fracture fixation. (Bandyopadhyay, et al., 2013)

2.1.1. Zirconia

Zirconia (zircon oxide) is one of the ceramic materials with outstanding mechanical properties used in applications in which high strength and toughness is required. The name comes from the two Persian words, Zar (gold) and Gun (color) (Hisbergues, et al., 2009). Unique properties of Zirconia make it useful in different applications in various industrial applications, such as, extrusion dies, valves, port liners for combustion engines, low thermal shock resistant refractory linings, blades, magnetic tapes, cigarette filters, fuel cells, oxygen sensors and so on (Piconi, et al., 1999).

This crystalline form of zircon can be found in monoclinic (M), cubic (C), and tetragonal (T), figure3. Zirconia is usually mixed with other metal oxides like MgO, CaO, and Y_2O_3 to increase its stability, prevent expansion and crack formation, which results of change in crystals forms. (Manicone, et al., 2007; Pilathadka, et al., 2007). Adding these oxides to the ceramic system makes partially stabilized Zirconia (PSZ), which consists of cubic crystals as major phase and monoclinic and tetragonal Zirconia as the minor phase.

ZrO_2-Y_2O_3 system also can be formed in tetragonal phase (TZP) which is the most studied type of Zirconia (Piconi, et al., 1999; Hisbergues, et al., 2009) as well as in our study. The diagram phase can be seen in figure 4.

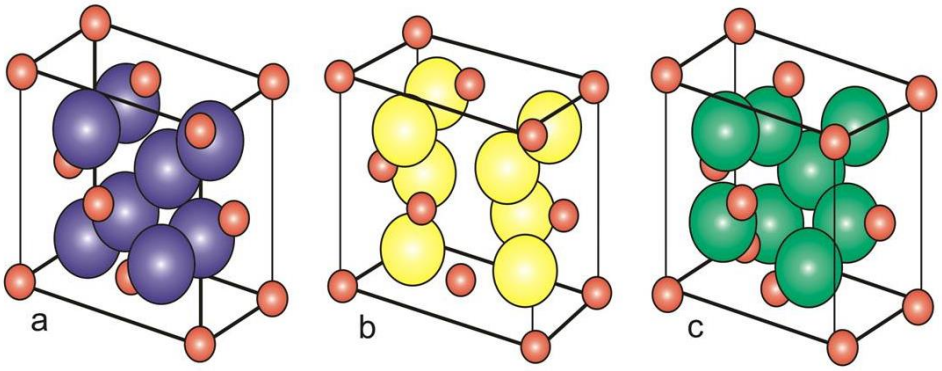

Figure 3. Crystal structure of Zirconia (a) monoclic , (b) tetragonal and (c) cubic (Volpato, et al., 2011)

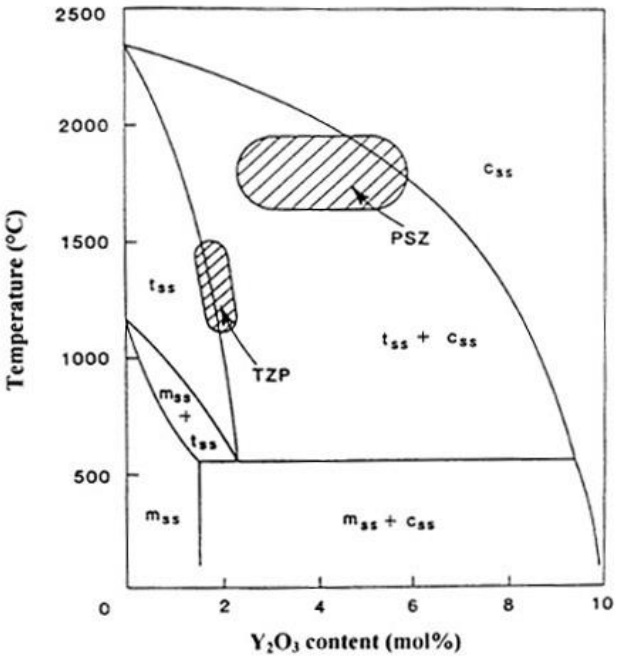

Figure 4. Phase diagram of Zirconia (Piconi, et al., 1999)

Main properties of zirconia such as high strength and toughness, chemical and dimensional stability and good biocompatibility make it one of the best implants materials. (Piconi, et al., 1999) In addition, in mechanical point of view, it shows suitable characteristics. These properties are similar to stainless steels with high-tension resistance (900-1200 Mpa) and excellent compression resistance almost 2000 Mpa. (Hisbergues, et

al., 2009; Park J, 2008) Modulus of elasticity of Zirconia is similar to steel and coefficient of thermal expansion (CTE) is close to iron (Piconi, et al., 1999). Table 1 compares the properties of Alumina and two kinds of Zirconia.

Table 1. Properties of ceramics used in biomedical application (Piconi, et al., 1999)

Property	Units	Alumina	Mg-PSZ	TZP
Chemical composition		99.9% Al_2O_3	ZrO_2	ZrO_2
		+ MgO	+8 /10mol %MgO	+3mol% Y_2O_3
Density	g cm^{-3}	≥3.97	5.74-6	>6
Porosity	%	<0.1	-	<0.1
Bending strength	MPa	>500	450-700	900-1200
Compression strength	MPa	4100	2000	2000
Young modulus	GPa	380	200	2110
Fracture toughness	MPa m^{-1}	4	7-15	7-10
Thermal expansion coeff.	K^{-1}	8×10^{-6}	$7\text{-}10 \times 10^{-6}$	11×10^{-6}
Thermal conductivity	W mK^{-1}	30	2	2
Hardness	HV 0.1	2200	1200	1200

Recent advances in modern technology and science indicate that Zirconia plays a fundamental role in orthopedic and dental applications due to its unique physical and mechanical properties. (Piconi, et al., 1999) Its first medical application was in 1969 in orthopedic implant, hip head replacement to substitute titanium and Alumina, the former candidates for hip surgery, and solve brittleness and failure problems. Nowadays, it is mostly used in tissue engineering and dental implants for its biocompatibility, nontoxicity, osseoconductivity, decrease in plaque accumulation, and having suitable interaction with soft tissues. (Piconi, et al., 1999)

Recent researches in dental field focus on metal-free prosthetic restorations. It has been found that they can improve aesthetical aspects of implants. Natural look of soft tissue in contact with fixed partial dentures is one of the important points, which should be considered. Metal-free restorations have this ability to maintain soft tissue color similar to the natural one. Among them, zirconia plays an important role in creating a structure, resistant to chewing stresses on both anterior molars teeth. It has a color, which helps harmonizing with natural teeth, along with opaque characteristics. Particularly, all ceramic restorations on posterior teeth caused worst 5-year survival percentage (84.4%). It is also reported that Zr-ceramic with Alumina oxide has the highest long-term strength. Zirconia

implants are able to tolerate fatigue, and stresses for anterior teeth implant replacement. Another advantage is radiopacity of zirconia, which is so beneficial in monitoring marginal adaptation in radiographic evaluation. In addition, in vivo investigations show that zirconia amassed fewer bacteria than metallic implants. (Manicone, et al., 2007)

In most cases, computer-aided design/manufacturing (CAD/CAM) system is used to create a zirconia core for a prosthetic restoration, fitting framework and zirconia implant abutments (figure5). (Manicone, et al., 2007)

Despite these advantages, Core–veneer interface is maybe one of the weakest points due to the chipping or cracking of the ceramic. Factors such as differences in thermal expansion coefficients between core and ceramic, firing shrinkage of ceramic, flaws on veneering and poor wetting by veneering on core can be the cause of these cracking. In general, additional evaluations are needed in order to investigate zirconia performance in long time period, especially, relationship between ageing of ZrO2 frameworks and long term loading. (Manicone, et al., 2007)

There are not many reports concerning surface treatment of zirconia implants in dental application but it is suggested that surface modification can magnificently improve their function. (Manicone, et al., 2007)

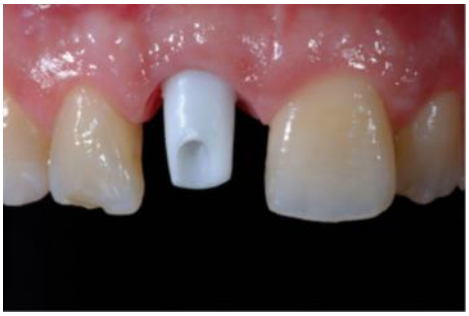

Figure 5. Zirconia implant abutment (Manicone, et al., 2007)

On the other hand, Zirconia is one of the bioinert materials, which means, it can be in contact with biological tissue without any host negative reaction such as immune response or inflammation. As long as one bioinert material is in vicinity of tissue, it does not have any connection or attachment to surrounding environment. It basically, performs its application during the diagnostic period. This specification stands in contrast to the bioactive materials, which have chemical reaction and create bonding with surrounding tissue. The goal is to give this property to the Zirconia-based materials to show both high strength and bioactivity at the same time in one bulk system. (Piconi, et al., 1999; Park J, 2008)

2.2. Enzyme and their classification

According to Oxford dictionary of biochemistry and molecular biology, enzyme is defined as "any naturally occurring or synthetic macromolecule substance composed wholly or largely of proteins that catalysis one or more biochemical reactions at relatively low temperature". (Smith A.D, 1997) Enzymes can be classified into different functional groups performing comparable chemical reactions, and each of them, under their own type, specifically catalysis a single type of reaction. (Alberts, et al., 2008) The eight most basic groups of enzymes are briefly explained in table 2.

Table 2.Enzyme classification and their functions (Alberts, et al., 2008; Berg, et al., 2007)

Enzyme class	Function
Oxidoreductases	Catalyze reactions in which one molecule is oxidized while the other is reduced.
Transferases	Enzymes Remove groups (not including H) from substrates and transfer them to acceptor molecules (not including water)
Hydrolases	Enzymes that catalyze a hydrolytic cleavage reaction
Lyases	Add or remove of groups to form double bonds (not by hydrolysis)
Isomerases	Catalyze the rearrangement of bonds within a single molecule
Ligases	Ligate two substrates at the expense of ATP hydrolysis
Polymerases	catalyze polymerization reactions such as the synthesis of DNA and RNA
Proteases	Break down proteins by hydrolyzing bonds between amino acids

The Alkaline Phosphatase, which has been used to promote mineralization in this study, can be classified in to hydrolases group. It is able to remove phosphate groups from molecules, such as nucleotides, proteins, and alkaloids. (Kogan, et al., 2002)

2.2.1. Alkaline Phosphatase

In 1912, high amount of alkaline phosphatase (ALP) was discovered in intestinal mucosa by Grosser & Husler and von Euler. They also reported the presence of this metalloenzyme in variety of tissues. This was the initial step in studying the properties of the enzyme. In 1962, the researchers determined the stoichiometric amount of zinc in E. coli, which is essential for initial phosphate bonding (Coleman J E, 1992).

In the structure, it is shown as homodimer with two similar subunit presenting two Zn and one Mg ions per dimer, which are responsible for catalysis properties of the enzyme by polarizing the molecules, figure 6 (Coleman J E, 1992; Colln S , 2007). The active places are known to be present around these three metal ions. (Coleman J E, 1992). Generally, ALP activity is based on pH and buffer constitution, and as it is clear, the suitable and efficient pH is in alkaline range. One ALP is able to hydrolyze phosphomonoesters and phosphodiesters in the presence of many R-groups indicating different molecules. (Colln S , 2007)

Zinc metalloenzyme alkaline phosphatase helps producing inorganic phosphate. It helps catalyzing the hydrolysis of organic phosphate esters in alkaline medium (at an optimum pH of 9.0). (Kunitz M, 1960) The protein ALP can be attached to plasma membrane of the cell by glycophosphatidylinositol anchor, and increases the mineralization of osteoblasts and at the same time diminishes the extracellular pyrophosphate, which acts as bone formation inhibitor. In fact, the ratio of inorganic phosphate and pyrophosphate has the direct relation with the hydroxyapatite formation and mineralization. The inorganic phosphates or P_i can be produced by the reaction of serine phosphates at the active site and high pH water as well as the phosphorylating process with the basic role of organic alcohol. TNAP (osteoblast protein) decreases the inhibitors (PP_i) and regulate P_i/PP_i ratio. Normally, ALP is classified in the initial groups of participating genes in calcification process, figure 7. (Golub, et al., 2007; Somerman, et al., 2009). These properties and functions make ALP a beneficial enzyme in hydroxyapatite formation for bone tissue regeneration and treatment.

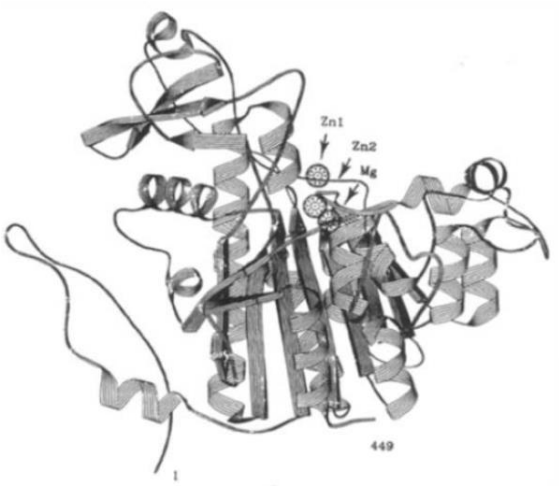

Figure 6. E. coli alkaline phosphatase. The three metal ions as spheres, Znl , Zn2, and Mg (Coleman J E, 1992)

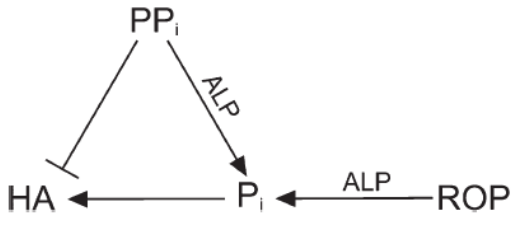

Figure 7. ALP enzyme function: hydrolysis of organic phosphatemonoesters (ROP) and pyrophosphate (PP_i) increase the inorganic phosphate (P_i) for hydroxyapatite formation. (de Jonge, et al., 2009)

Consequently, ALP is considered as an important enzyme in bone regeneration applications in biomedical science. The goal of several studies is based on grafting this enzyme on different substrate such as Zirconia with high strength, which makes it ideal for bone treatment. This can be possible by different methods such as immobilization.

2.3. Surface modification

Surface modification controls the reactions and properties of the surface while maintaining the bulk properties. It has a direct effect on adsorption of the proteins and enzymes. Furthermore, the cell interactions and positive host response can be obtained with this process. The recent investigations show the importance of the grafting biomolecules, which have a direct influence on cell and biomaterials interactions. Materials treated with this type of process can be used in different applications such as, biomedical implants, biosensors and bioreactors. (Treccani, et al., 2013; Rahmany, et al., 2013)

This technique can be classified into different methods such as mechanical (surface roughening, patterning and porosity) and chemical modification (coating and immobilization) (Oshida Y , 2007). Surface roughness is maybe the primary solution for improving stability and activity and cell attachment. Although, increase in roughness seems to improve the bone and implant connection and osteoblast cell attachment (Kim, 2013), there are many studies demonstrate the controversial aspect of this investigations. (Mueller, et al., 2003; Setzer, et al., 2009)

The reports are based on different methods, which improve the functions of enzymes and cells. An investigation conducted by H. Fischer, et al on modification of

Alumina which is done by adding sodium hydroxide to Alumina in 100°C for 24h to achieve Aluminum hydroxide group, reports promoted bioactivity. (Fischer, et al., 2005) The other factor, which has been studied for increasing the activity, is hydrophilic property of the surface. This property can be obtained by 48-hour UV light treatment using a 15W bactericidal lamp. By decreasing the contact angle, the hydrophilicity increases. Capacity for attachments spread and prolifiration of osteoblast cells can be increased by this UV light treatment of zirconia. Also, alkaline phosphatas and mineralization is doubled in this process.(Att, et al., 2009) Cold plasma treatment and high-energy oxygen ion irradiation also demonstrate enhance in osteoconductivity, bioactivity of ceramics and nucleation and growth of the apatite in Baohui Su, and E.K.Girija, et al studies. (Hui, et al., 2007; Parthibana, et al., 2008)

The two basic methods used in this science, are discussed separately below. Coating is one of the techniques, which is widely investigated on different materials such as glasses, reported by Lise T. de Jonge et al. (de Jonge, et al., 2009) and immobilization, which is mainly used in order to eliminate coating weakness.

2.3.1. Coating

There are many different researches which have studied functionalization of different materials by grafting proteins such as ALP.

One of these important methods is coating which has been employed by Lise T. de Jonge et al. They have been shown the successfully electrosprayed ALP-containing coatings on titanium surfaces and reported the high bioactivity and mineralization using simulated body fluid and cell culture medium. (de Jonge, et al., 2009) Electrosprayed alkaline phosphatase/calcium phosphate composite surface tested in 1ml Cell Culture Medium which was contained alpha minimal essential medium with 10 vol.% fetal calf serum, 50 µg/ml ascorbic acid , 10^{-8} M dexamethasone, 50 µg/ ml Gentamycin and 10 mM sodium β-glycerophosphate. The prepared samples were immersed in medium and studied in four day points. (de Jonge, et al., 2009) This quantities also used in this project along with Antibiotic-antimycotic (1%) and 1.5 mM $CaCl_2$ in order to create hydroxyapatite on the ceramic in the cell culture medium. The day points increased to seven days to consider the changes over time. The successful nucleation and growth of CaP and its morphology can be observed by scanning electron microscopy (SEM) in this study.

A very recent study, improved the same strategy by coating the ALP-containing polydopamine and improvement of cell morphology, mineralization and differentiation studied in supplemented cell culture medium (Nijhuis, et al., 2013). These studies are based on coating the active enzyme on the surface with the purpose of promoting mineralization. While there are, other researches preferred to coat the hydroxyapatite directly. The results show high ALP and osteocalcin expression. (Kim, 2013)

It is reported that, CaP have been tried to be coated on Ti implants by plasma-spraying technique. Although high deposition rate and the ability to coat large areas are two basic specifications of this technique, some features raise severe concerns, such as weak adhesion, undesired phases formation, delamination and poor control on thickness and surface morphology. Magnetron sputtering, electrophoretic deposition, hot isostatic pressing, sol–gel deposition, pulsed laser deposition, ion beam dynamic mixing deposition, electrospray deposition, biomimetic deposition, and electrolytic deposition are some other coating methods, which have tried to overcome the drawbacks of plasma-spraying technique. (de Jonge, et al., 2008) It should be mentioned that, these methods have also been used on Zirconia during recent years. Physical vapor deposition (PVD), for example, used by C. Hübsch et al. in order to coat transparent monolayers of titanium oxide and titanium oxide–alumina–titanium oxide multilayers on 3Y-TZP discs. While the results shows the satisfactory coating, it still suffers from the danger of aforementioned problems (Hübsch, et al., 2014) Apart from the method, it is believed that by creating a composite coating as organic and inorganic system, CaP, grow factors and surrounding tissues positive response would increase. (de Jonge, et al., 2008)

2.3.2. Immobilization

One of the Surface modification methods is immobilization. This term is referred to physical and chemical localization and attachment of enzyme in specific region of support materials to achieve the highest performance to show suitable stability and activity in desired time. The main goal of using this process is to achieve the enzymes which can be re-used over repeated times, which results in low cost, with longer lives, less degradation and the excellent properties i.e. activity and stability. (Guisan J.M, 2006; Spahn, et al., 2008; Tischer, et al., 2000)

Immobilization of enzymes can be performed by different techniques and methods as figure 8 depicts, in order to decrease the drawbacks of coating method, such as weak binding to the surface. To achieve this goal chemical reaction alters the surface behavior and makes it suitable to act as a support for different biomolecules. This can be done by binding and creating different active groups on the substrate. (Oshida Y , 2007)

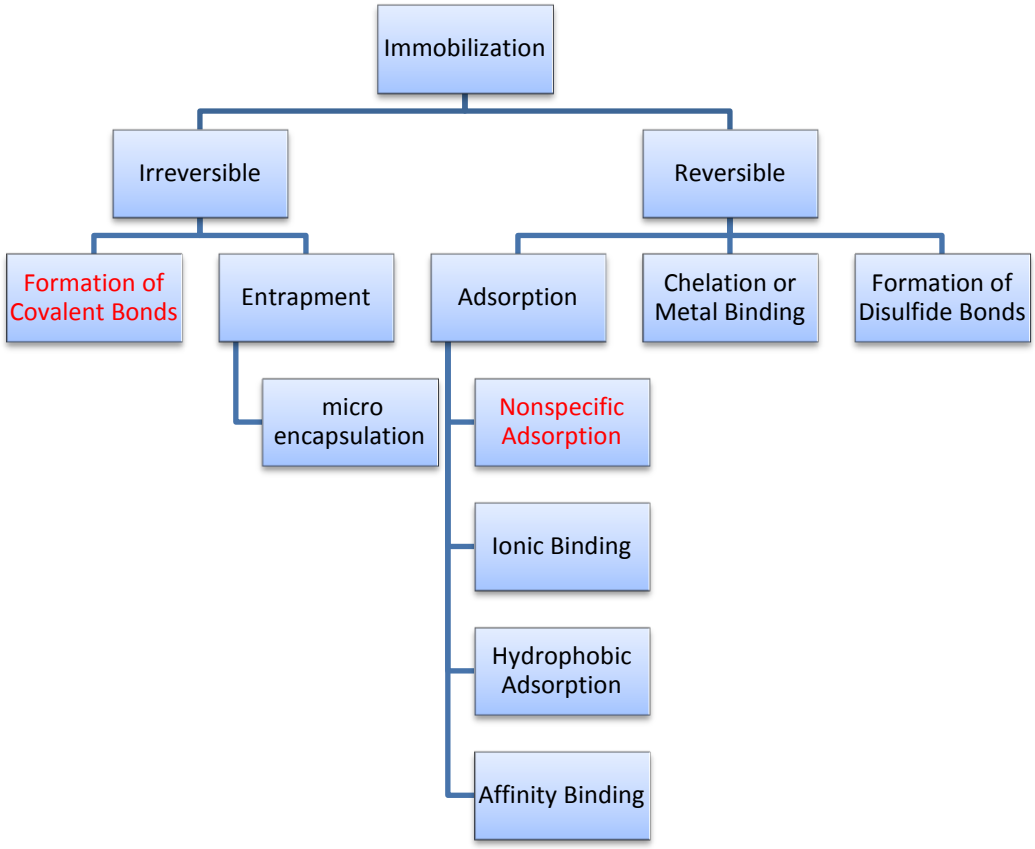

Figure 8. Immobilization techniques (Guisan J.M, 2006)

In present study, adsorption and covalent bonding are applied (shown in red in above chart). These methods can be classified in chemical functionalization group as they perform their function by adding functional group on the surface. (Guisan J.M, 2006)

The major factor, which influences immobilization, is the support or matrix that hosts the enzymes or cells. These materials should be biocompatible without any negative reaction in contact with enzymes, be resistant to any microbes and insoluble in solvent. They should present the proper surface properties, to accept hydroxyl or any active groups, and surface charges. The different types of materials can be classified in different groups. Natural minerals (bentonite and silica) and processed materials (glass, metal and ceramic) are inorganic while polymers such as polysaccharides and polystyrene are organic materials (Guisan J.M, 2006; Spahn, et al., 2008).

One of the basic properties of substrate is its morphology, which can be presented as different forms such as particle, planar surfaces, porous scaffold or membrane, nanofibres, nanotubes and glass capillaries. (Treccani, et al., 2013)

These different morphologies are associated with the final applications of the materials. For instance, planar substrates, discs or cylinders form of the materials mostly

are chosen for the dental applications. On the other hand, particles with high surface area to volume ratio, biocompatibility, stability and also easy handling, can be used for drug and gene delivery, tumor therapy or even medical imaging. (Treccani, et al., 2013)

Adsorption

The process in which the substances accumulate on the surface of the absorbent in physical (weak bonding) or chemical (Electrostatic bonding) manner is called adsorption with exothermic characteristic. (Dr. T.Ahmed)

In physical adsorption, enzyme is attached to surface by weak van der waals and hydrogen bonding. Although, the activity can be high with this simple technique, the stability is low. This is because different environmental factors such as pH, strength of bonds, temperature and solvent polarity, due to its non-covalent nature, are affecting this process, figure 10 (a). (Guisan J.M, 2006; Rusmini, et al., 2007) The strength of one adhesion between material and biomolecules can also present the mechanical strength of that system. This fact makes researchers work on the factors increasing the strength of the bonds. Electrostatic interaction is one of the factors influencing this process in hydrophilic surfaces. According to this, zeta potential measurement can be used to identify Isoelectric point (IEP), the point which the colloidal system is in its lowest state of stability. (Mueller, et al., 2012; Malvern, 2004)

M. Ghiaci et al. investigated the ALP functionalized on Na-bentonite and modified bentonite by van der Waals interaction in which ceramic properties such as surface area and pore size effect, along with activity of the enzyme, storage and thermal stability are studied. The result shows the acceptable enzyme adsorption on bentonite substrate. (Ghiaci, et al., 2009)

Mueller et al. (2009) studied two models of side-on and end-on adsorption orientation for bovine serum albumin (BSA) and lysozyme on hydroxyapatite and beta-tricalcium phosphate (b-TCP) particles equilibrated in 16hr based on biomolecules dimensions. Figure 9 has been prepared by combination of materials, which have been given in Mueller study, in order to depict these models. (Mueller, et al., 2012)

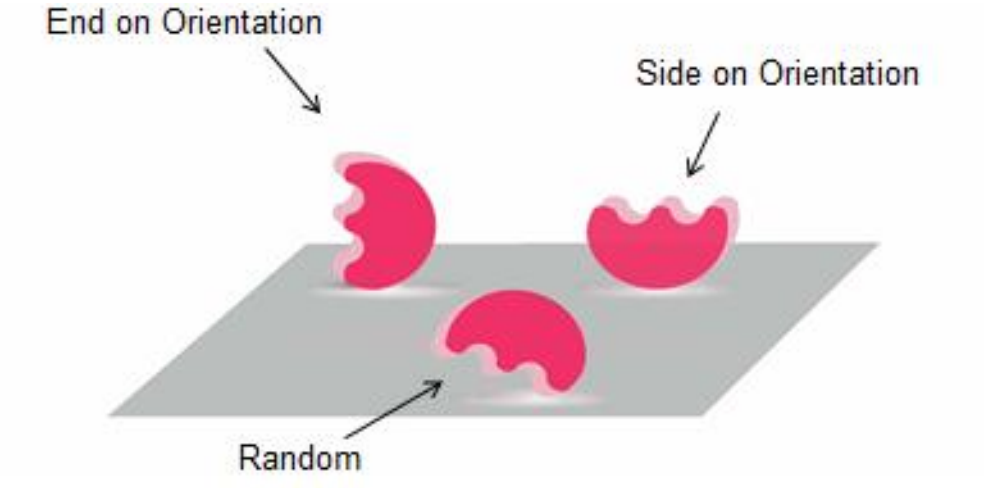

Figure 9. Three basic orientation models of enzyme attachment (adsorption) on Ceramic fine powder or planar surface

In this project, the same models are applied to calculate the amount of enzyme required for covering the whole zirconia particles. The end on orientation model chose between the three models depicted in figure 9. This model presents the most effective orientation on the particles with higher amount of immobilized enzymes.

Although adsorption is considered as simple and low cost method in grafting biomolecules on the substrate without changing the biomolecules nature, there are some disadvantages regarding the weak bonding. Due to the weak and unstable hydrogen and van der waals boding, this method can only be used in short time experiments. As aforementioned, it can be due to environmental factors, hydrophobic or hydrophilic behavior of the substrate or mobility of the biomolecules. (Górecka, et al., 2011; Guisan J.M, 2006; Rusmini, et al., 2007). The other drawback is based on surface microbe and undesired ion attraction on the surface. Besides, adsorption method cannot guarantee the attraction and grafting of any biomolecules to any substrate due to the surface charge properties. (Meder, et al., 2012)

Covalent bonding

Covalent bonding is one of the significant methods in immobilization due to its irreversible characteristics. This property allows the matrices to be used continuously in laboratory conditions without reducing stability. Contrary to adsorption method, this technique can be used by either adding functional groups or modifying the surface to produce them, figure10 (b) (Guisan J.M, 2006; Rusmini, et al., 2007). The immobilized enzymes made by this method cannot interfere in any intermolecular process such as aggregation or autolysis or any unwanted interaction. Many studies indicate that covalent

bonding strength of enzymes and matrix can be improved by increasing potential linkers and reducing inhibitors based on hydrophilicity or hydrophobicity factors. (Guisan J.M, 2006) However, it is reported that this method may decreases the enzyme movement and consequently decrease the enzyme activity. (Spahn, et al., 2008)

There are also studies discussing the surface modification of different materials such as metals, glasses, ceramics, glass ceramics by covalent bonding (Verne, et al., 2010), in order to achieve positive biological response. For example, Ti-6Al-4V alloy have been investigated by S. Ferraris, et al. to create the bioactive surface with ALP grafting by organic chloride (TC). The results show effective ALP functionalization (Ferraris, et al., 2011). Additionally, based on Zhou, et al. research, immobilization of β-glucosidase on magnetic nanoparticles demonstrates high storage stability and promotes enzyme properties. Both experiments led to satisfactory results. (Zhou, et al., 2013) Microporous nanofibrous fibrin scaffold also have been modified by linkers (EDC and NHS; which will be discussed later in this chapter) in order to immobilize ALP on the surface. The successfully grafted enzyme remained nontoxic and promoted osteoblast-like cell proliferation and differentiation in vitro and bone formation in vivo. (Somerman, et al., 2009).

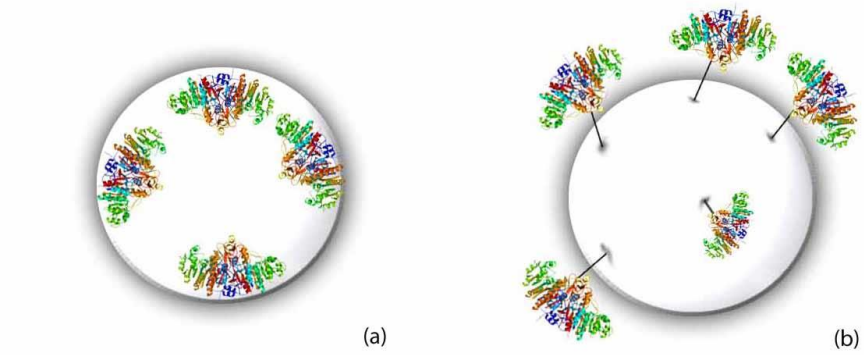

Figure 10. (a) Adsorption (b) Covalent bonding with linkers

Figure 10 have been made by revision of the figure presented in Yücel et al. studies in order to compare two method of immobilization process. (Yücel, et al., 12)

The first step of immobilization by this technique is silanization in order to create active groups on the surface such as amino groups.

a. Silanization

Silane, poly (ethylene glycol) (PEG) and Organophosphorous compounds are the most studied coupling agent, which can be used in the first step of surface modification. These materials are easy to use, cheap and stable in laboratory environment. Silanization process can be successfully practiced on different supports such as colloidal and porous particles, nanoporous membranes and planar surfaces. (Treccani, et al., 2013)

Reaction of Si and OH on the substrate surface leads to create a stable and strong bonding of Si-OH and the products such as water or alcohol that can be eliminated from the system. Silane layer can also be created in other organic solvents, which has some disadvantages compared to aqueous solvent such as formation of the thick and loose silane layer on the surface of the substrate. (Treccani, et al., 2013) Many studies suggest silane as one of the basic materials used for surface modification in order to reach biomineralization on biostable surfaces such as silicon oxide. (GK Toworfe;RJ Composto ;IM Shapiro;P Ducheyne ., 2006)

There are many types of silane, which can be classified by their active groups, Such as vinyl, epoxy, methacryloxy and amino silanes (Andriot, et al., 2007). 3-Aminopropyltriethoxysilane (APTES), as an organisilan agent (amino silanes), is utilized for creating amine groups on the surface of the host materials, which is considered as silinazation, the first step of immobilization process. This is the most frequently used technique on different substrates such as stainless steels, titanium, titanium alloys, magnesium and most of the ceramics such as SiO_2 , TiO_2 and Si_3N_4 (Landoulsi, et al., 2011; Kroll, et al., 2012). APTES contains NH_2 active groups, which easily react with functional groups such as OH groups and biomolecules. (Chai, et al., 2012) In addition, it should be mentioned that this functional group have different functions such as improving adhesion in glass–resin composites system, attachment of negatively charged species (DNA and NPs), controlling biomolecular adhesion and enzyme stability and cell adhesion and growth. (Treccani, et al., 2013)

Silicon oxide has studied by silane functionalization in order to create calcium phosphate precipitation by using simulated physiological fluid (SPF), Toworfe, et al. (GK Toworfe;RJ Composto ;IM Shapiro;P Ducheyne ., 2006) Taylor and Verné, et al. also used APTES to graft ALP on glass surfaces. In both researches, ALP successfully grafted and attached on silanized surface of the glass and preserved its activity after immobilization. (Taylor, et al., 2005)

After creating the desired active groups on the surface, there should be some linkers to graft the biomolecules on the surface. There are many kinds of linkers, which is discussed in the next topic.

b. Linkers

Cross-linking agents and linkers are mostly used to anchor the proteins and biomolecules to solid surfaces with various functional groups. They consist of at least two functional groups, which are able to create covalent bonds with other reactive groups such as amines, carboxyls, sulfhydryls, carbohydrates and carboxylic acids. (Wolfson, 2013)

In general, cross-linking agents can be divided to two groups: homobifunctional (with two identical reactive ends) and heterobifunctional agents (two different reactive ends). (Wolfson, 2013)

There are many kinds of heterobifunctional crosslinkers. Some of them consist of carbodiimides, which link between carboxyl groups (-COOH) and primary amines ($-NH_2$). While the others can be used to link proteins with amine and sulfhydryl groups, there are some others with one photoreactive end used under UV exposure. (Wolfson, 2013)

Due to the high reactions of the linkers, they are mostly applied under controlled buffer and pH. The objective is to obtain low ratio of cross linkers to proteins with high reactive bonds. There are some factors, which must be considered in choosing linkers to achieve this goal including reagent solubility, reactive groups and homobifunctional or heterobifunctional nature, obtaining photoreactive or thermoreactive groups in the structure and spacer arm length of the linkers. (Wolfson, 2013)

- N-Hydroxysuccinimide-Esters (NHS-Esters)

Water insoluble, membrane permeable N-hydroxysuccinimide (NHS) is one of the frequently used cross linkers with amine groups. By adding a charged sulfonate (SO3-) to N-hydroxysuccinimide ring, properties in solubility and permeability are changed which are suitable for crosslinking to the proteins. The reaction of NHS with amine groups and with nucleophilic groups of the proteins results amide bonds, and releases NHS ring. (Hermanson G.T , 2008; Rusmini, et al., 2007)

- 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)

Water-soluble EDC is one of the most popular linkers, which reacts with carboxylates and amines in biomolecules. EDC and NHS both can be utilized in planar surface and particles. This makes them applicable through a wide range. (Hermanson G.T , 2008)

As a chemistry reaction of EDC, the N-substituted carbodiimides link with carboxylic acids and make o-acylisourea intermediates. This is the active part, which then can bond with e.g. amine groups and create amide bond. By activating carboxylic acid or

formation of ester, an intramonolayer anhydride can be created. (Treccani, et al., 2013) In the other case, with the assistance of the sulfhydryl groups, thiol ester linkages can be made which are not as stable as amine linkage. (Hermanson G.T , 2008)

Studies indicated that the most optimum pH range for EDC-mediated amide bond formation is 4.5- 7.5. Using MES [2-(N-morpholino) ethane sulfonic acid] buffer 0.1 M can be the best for these linkers. It should be noted that, the salts in the mediums should not react with carbodiimide of EDC. (Hermanson G.T , 2008)

Using NHS and EDC simultaneously in one functionalizing system promotes bonding strength and increases reaction rate. (Hermanson G.T , 2008)

Functionalization stability

Functionalization stability of the modified surface in different lab conditions like changes in temperature, pH or prolonged time is always one of the basic interests in this field. (Treccani, et al., 2013) The basic goal in surface modification is to create the functionalized surface with high stability and activity so it would be applicable in industrial scale.

During recent years, many studies have been conducted to improve stability and activity as two basic factors in surface modification. For example, it is reported that the activity may be related to the phase and the temperature of calcination of materials and pH range. The a-amylase immobilized on Alumina calcinated in higher temperature present higher activity in higher pH range. (Reshmi, et al., 2006)

Different processes are also introduced to increase stability such as increasing the covalent strength by incubating the immobilized enzyme in reactive support or obtaining thermally stable materials in incubation time or increasing the temperature to enhance stability (Guisan, et al., 2000; Mateo, et al., 2007). Furthermore, there are studies indicating successful creation of covalent linking by vacuum (Simons, et al., 2002; Taylor, et al., 2005; Verne, et al., 2012). Simons et al. reports that zero-length cross-linking can be achieved by carboxyl groups of lyophilized protein and amino groups to form an amide bond under vacuum in order to take out the water products. This technique along with heating to facilitate taking up the unwanted product may create the strong bonding. (Simons, et al., 2002) There are many studies illustrates the importance of linkers such as EDC and NHS in increasing the stability of the enzyme on different surfaces. (Somerman, et al., 2009; Rusmini, et al., 2007; Kroll, et al., 2012; Li, et al., 2010)

It should be mentioned that all of these factors and processes depend on the enzyme and modification type and they can be specific to the certain biomolecules, support, buffer, pH or temperature.

2.4. In vitro investigation

In vitro experiments are the initial steps in considering biomaterials operation and function. These examinations are great help to predict the materials performance in living body and biological environment. (Lee, et al., 2011) Cells or any living component seed on the support surface and the biocompatibility or toxicity of the material can be investigated. In addition, cells grow and differentiation, proliferation, adhesion along with activity can be studied. These investigations can be analyzed by different microscopes such as fluorescence and SEM. The other specification, which can be measured, is bone mineralization obtaining by red alizarin the histological staining. (Treccani, et al., 2013)

Different studies indicate that stimulated body fluid (SBF), with same ion concentration as blood plasma; can be the first choice in testing materials in vitro. In 2006, Kokubo et al. present SBF as the solution needed to stimulated body conditions for studying bone-bonding ability. (Lee, et al., 2011) Although there are so many advantages in using SBF in vitro, there are some properties, which make other options be considered.

The perfect medium should share the similarities in ions concentrations with blood plasma. Cell culture medium (CCM), for instance, is used in acellular studies as it contains the essential materials, which are needed in cell culture studies. The inorganic salts, vitamins, amino acids are the main components in the medium, which make it suitable for cell experiments. (Lee, et al., 2011)

Besides, the buffers and mediums should carefully be selected based on the enzymes used in the test. As ALP enzyme main function is to break down the organic materials into inorganic components in order to start mineralization, the cell culture medium can be the best choice despite of its low amount of Ca^{2+} compare to SBF. This is due to the supplementation of the cell culture medium by β-Glycerol phosphate disodium salt pentahydrate (β-GP), the major source of organic substances. This fact stands in contrast to SBF, which contains only inorganic composition. (de Jonge, et al., 2009) It is also reported that SBF ion concentration cannot form calcium phosphate crystal core and for achieving this goal, the Ca and P ion concentration should be increased. (Hui, et al., 2007)

The other advantage of cell culture medium is the presence of serum. CCM contains fetal bovine serum (FBS) which is the source of growth factors, hormones, and attachment factors. In brief, the FBS provide food and supplements for the cells while maintain the pH. (Meenakshi A , 2013). Table3 demonstrates the comparison between Human blood plasma, SBF and Dulbecco modified Eagle medium (DMEM). The advantages of DMEM can be clearly observed in supplemented substances part such as Amino acids and vitamins.

Table 3. Ion concentrations (in mM) of human blood plasma, SBFs and DMEM (Lee, et al., 2011)

	Blood plasma		SBF			DMEM
	Total	Dissociated	Corrected	Revised	Ionized	
Na^+	142	142	142	142	142	155.3
K^+	5	5	5	5	5	5.3
Mg^{2+}	1.5	1	1.5	1.5	1	0.8
Ca^{2+}	2.5	1.3	2.5	2.5	1.6	1.8
Cl^-	103	103	147.8	103	103	119.3
HCO^{3-}	27	27	4.2	27	27	44
HPO_4^{2-}	1	1	1	1	1	0.9
SO_4^{2-}	0.5	0.5	0.5	0.5	0.5	0.8
pH	7.2-7.4	7.2-7.4	7.4	7.4	7.4	7.4
Amino acids	+	+	-	-	-	+
vitamins	+	+	-	-	-	+

In vitro investigation can be classified as two different tests, which have been discussed in the next topics.

2.4.1. Acellular investigation

Acellular test is referred to the test, which deals with no cells and permits the functionalized biomaterials to be in biological and simulated body conditions in order to consider immobilized enzyme performance. In this study, it can be observed whether the enzyme is dependent on cell presence or it can have its basic function, mineralization, in stimulated body conditions.

Cell culture medium (CCM) is utilized by many scientists in order to study the mineralization process of different materials such as collagen sponges (Andre-Frei, et al., 2000) in biological conditions. CCM can be supplemented by organic materials to review the Ca and P formations on the surface. This medium can be used either with or without cells. (Lee, et al., 2011; de Jonge, et al., 2009; Meenakshi A , 2013)

The materials, which are used in order to supplement the medium, are fetal calf serum and antibiotics. In many studies, depending on the aim of the project, $CaCl_2$ is also used in order to evaluate the mineralization and Ca formation. (Andre-Frei, et al., 2000)

2.4.2. Investigation of Cell behavior in vitro

In contrast to the acllular experiment, cell test deals with seeding cells, such as human osteoblast cells (HOB), to the samples in order to consider enzyme function in vitro. This process can be carried out in vitro or in vivo. Cell shape and morphology along with adhesion of the cells can also be studied by fluorescence microscopy. Calcium creation can be analyzed and its amount can be measured using Ca assay.

Different kinds of cells can be used based on the aim of the experiment to study various parameters in tissue engineering point of view. In fact, the type of the cells is the important factor in in vitro, which presented the proliferation rate and other specifications. Different responses can be obtained even with the same cell type. The number of passaging and cell seeding environment along with laboratory conditions such as; buffer, pH, temperature, CO2, humidity also affect the cell response. (Treccani, et al., 2013)

As aforementioned, the type of the cells is the basic factor in in vitro investigation with cells. There are different kinds of cells, which have been studied. For example, the mesenchymal stem cells (MSC) can be used in order to study the construction of MSC and surface modified titanium and Zirconia implants. In this experiment, the aspirated cells from bone marrow cultured in vitro in a medium containing serum and antibiotics, which resulted the bone formation in vivo. (Zhou, et al., 2010)

There are reports working with human osteoblast-like cells to carry out research on the cells behavior in contact with immobilized substrates. Human MG-63 osteoblastic cells is the other type of cells, cultured in serum free medium in order to investigate the cell adhesion and spreading on the titanium materials. (Nebe, et al., 2007) The other material, which can be used in vicinity of primary human osteoblasts (pHOB), is bone cement of COOL, the composition of bioceramics and chemically modified PMMA (poly(methyl methacrylate)). (Mitzner, et al., 2009) Human bone-derived cells (HBDCs) are also studied on modified Alumina and hydroxyapatite surface cultured in a medium containing fetal calf serum, L-glutamine, HEPES buffer, penicillin, streptomycin and L-ascorbic acid phosphate in 1998. (Zreiqat, et al., 1999)

Cell adhesion

Cell adhesion is one of the basic topics in in vitro or in vivo investigation. This process can be dealt with not only adhesion of the cells but also the cell functions such as signaling between cells and ECM (extracellular matrix). There are many kinds of adhesion

biomolecules families such as cadherins, immunoglobulin superfamily, selectins and integrins. (Marshall, et al., 2004)

The cell-biomaterials adhesion is an integrin-mediated process [19], which leads to promote cell differentiation and mineralization. (Reyes, et al., 2007) Integrins, as a basic cell adhesion mediator, can play the basic role in cell-cell and cell-ECM adhesion. (Marshall, et al., 2004) In addition, integrin receptors, which consist of non-covalent heterodimers of α/β transmembrane, have an influence on process such as; cell growth and embryonic development and tissue maintenance. (Rahmany, et al., 2013)

The immobilized proteins such as collagen type I function as ligand for integrin, which improves the cell attachment. Other component including actin cytoskeleton and vinculin, paxillin, and focal adhesion kinase also involve in this process. (Rebl, et al., 2010)

Vinculin is known as intracellular adapter protein, which is involved in intracellular signaling and creation of focal adhesion component. (Rebl, et al., 2010) The schematic of some kinases proteins is shown in figure 11 which are involved in a focal contact as linkers between the integrin receptors and actin cytoskeleton. They are responsible in growth, morphology, movement (cell migration and ECM formation), and differentiation process of cells. These contacts lead to adhesion of the biomaterials and cells. It is possible to say that increase in vinculin mobility may promote cell spreading and immigration. (Lotfi, et al., 2013; Rebl, et al., 2010)

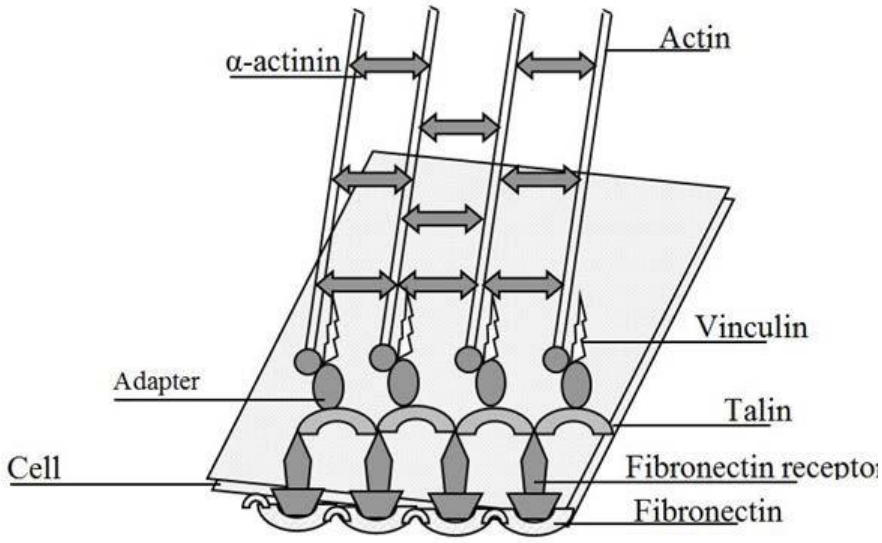

Figure 11. Adhesion proteins in focal contacts (Lotfi, et al., 2013)

The forces, which are responsible for cell-cell adhesion is called cohesion contact, which is weaker than the forces needed for cell-biomaterials adhesion. These forces and

their differences, depend on both cell and biomaterials type. The other parameter, which has an influence on cell adhesion, is surface free energy, which can control the surface wettability and cell behavior on the surface. This factor is dependent in electrical charge and chemical composition of the surface (physical and chemical properties). (Lotfi, et al., 2013; Bacakova, et al., 2004)

All of these parameters and factors indicate the specific properties of the surface, which makes one surface suitable for the integrin of cell to bind with the substrate. (Lotfi, et al., 2013)

Cell viability

Cell viability is a test, which is carried out in order to investigate the presence of viable cells. Water-soluble tetrazolium salt WST-1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium] assay is one of the important tests considering cells viability through reduction of tetrazolium salt by dehydrogenases and formation of formazan, figure12. (Tominaga, et al., 1999; Bernardo, et al., 2012)

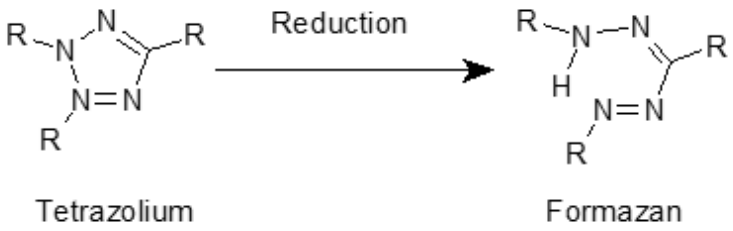

Figure 12. The reaction of WST-1 assay (Kregiel D , 2012)

Water solubility overcomes the disadvantages of the water insoluble assays that may damage the cells and show inaccuracy. (Tominaga, et al., 1999; Bernardo, et al., 2012)

Cell mineralization

Inorganic mineral formation by living component can be defined as biomineralization. (Epple, et al., 2002) In one physiological biomineralization, calcium cations and phosphate anions precipitated and create apatite by osteoblasts cells. [12]

Two important minerals, which have been widely studied and have a basic role in skeleton formation, are calcium carbonate and calcium phosphate. The process of mineralization can be expressed in basic stages including controlling mineralization space, ionic precipitations, nucleation and growth the crystals along with number and orientations.

In most of the cases, these factors are directly related to organic macromolecules. (Meldrum FC , 2003)

This mineralization can be assessed by alizarin red staining (1,2-dihydroxyanthraquinone) in which alizarin changes into red alizarin complex in chelation process by the effect of calcium. (Carson, et al., 2009) The structure is presented in figure 13.

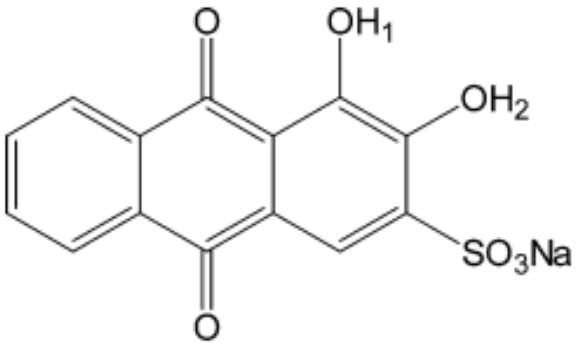

Figure 13. Chemical structure of Alizarin Red Staining (Córdobaa, et al., 2011)

This investigation method is widely used for determining the mineralization in different stages and times, to study the first step of bone formation on different materials or even in anchoring the surfaces in the desired systems or applications such as dental pulp biomineralization. (Du, et al., 2013; Berendsen, et al., 2007)

3. EXPERIMENTAL PART

3.1. Materials and methods

The material examined in this experiment was Zriconia TZ-3YS-E(3% mol yttria stabilized tetragonal Zirconia with Fine sintered grain size) (TOSOH,OH,USA). This type of Zirconia need lower sintering temperature of 1350ºC (TOSOH, 2013). The powder properties are provided in table 4.

Table 4.Powder characteristics of TOSOH-Zirconia TZ-3YS-E (TOSOH, 2013)

Powder Characteristics	TZ-3YS-E
Y_2O_3 (mol %)	3
Specific Surface Area (m^2/g)	7±2
Appearance	Granule
Density (g/cm3)	6.05
Bending Strength R.T. (MPa)	1,200
Hardness (Hv10)	1,250

Zirconia samples were fabricated by hydraulic dry pressing process and sintered in 1500 °C for 2 hours with the final size of 13.97mm diameter. (Figure14)

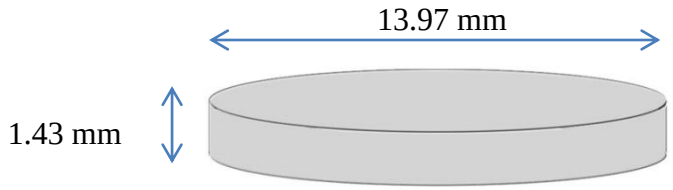

Figure 14. Zirconia planar substrate dimensions

The enzyme immobilzed on the Zirconia sutface was Alkalin phosphate enzyme(USBiological phosphatase alkaline chicken intestine, ME, USA) with dimension reported as 5nm×8nm×5nm. (US Biological life science, 2013) This immobalization carried out by covalant bonding methods, created by APTES, 3-aminopropyltriethoxysilane

(Sigma-Aldrich Co., MO, USA) in order to create amino group on the surface. In addition, linkers such as 1-ethyl-3-[3-dimethylaminopropyl] carboiimide hydrochloride, EDC, (Sigma-Aldrich Co., MO, USA), and N-Hydroxysuccinimide-Esters, NHS (Sigma-Aldrich Co. MO, USA), are used to graft enzyme on the Zirconia planar surface as well as zirconia particles. The buffer used in this experiment was, 2-(N-morpholino)ethanesulfonic acid, MES (Sigma-Aldrich Co., MO, USA).

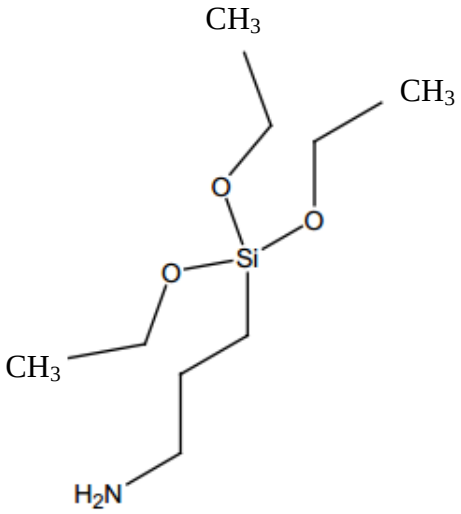

Figure 15.Structure of APTES (Oscar H, 2003)

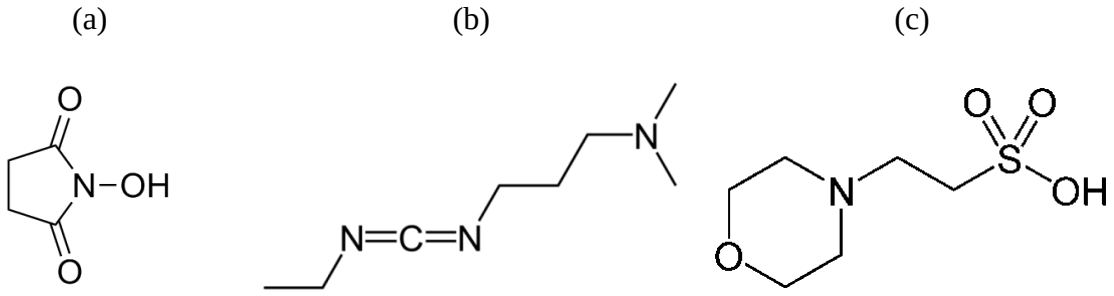

Figure 16. NHS (a), EDC (b) and MES buffer (c) main structure

Grafting of the enzyme on the ceramics considered as three basic models; Side on, End on and random (figure 9).

Molecular weight and dimensional size of enzyme and specific surface area of the powder were used to calculate the amount of enzyme which is required to create one ALP monolayer covering whole ceramics particle.

The ALP amount for 0 to 2 monolayer on the particles has been calculated which can be seen in table 5-8. It should be mentioned that End on orientioan model has been considered in all of the experiments.

Table 5. Characterization of Zirconia powder

Data	
Specific surface area	$6.9\ \frac{m^2}{g}$
Density	$5.98\ \frac{g}{cm^3}$
N	6.0221415×10^{23}

Table 6. Characterization of ALP enzyme

Data	
ALP dimensions	5nm× 8nm× 5nm.
ALP $_{MW}$	$53000\ \frac{g}{mol}$
Form	lyophilized powder

Table 7. The amount of ALP calculated for covering zirconia particles

Powder (A)g	**Specific surface area (B) $\frac{m^2}{g}$**				
1	**6.9**		**(C) g Quantities ALP**	**(D) mol ALP (1 monolayer)**	**(E) g ALP (1 monolayer)**
5.98	**41.26**	**Side**	**(B)** $\times \frac{10^{18}}{40\ m^2} = 1.0315 \times 10^{18}$	$\frac{(C)}{N} = 0.1712 \times 10^{-5}$	**(D)**$\times 53000\ \frac{g}{mol} = 0.0907784$
		End	**(B)** $\times \frac{10^{18}}{25m^2} = 1.65048 \times 10^{18}$	$\frac{(C)}{N} = 0.274 \times 10^{-5}$	**(D)**$\times 53000\ \frac{g}{mol} = 0.14522$
2.99	**20.63**	**Side**	**(B)** $\times \frac{10^{18}}{40m^2} = 0.51577 \times 10^{18}$	$\frac{(C)}{N} = 0.0856 \times 10^{-5}$	**(D)**$\times 53000\ \frac{g}{mol} = 0.045389$
		End	**(B)** $\times \frac{10^{18}}{25m^2} = 0.82524 \times 10^{18}$	$\frac{(C)}{N} = 0.137 \times 10^{-5}$	**(D)**$\times 53000\ \frac{g}{mol} = 0.07261$

Table 8. ALP amount for 1%vol Zirconia for different monolayers

	1	**0.25**	**0.5**	**0.75**	**1.5**	**2**
Side 5.98g powder	0.09077 g	0.02269 g	0.045389g	0.06808g	0.13616g	0.18155g
End 5.98g powder	**0.14522 g**	**0.036305 g**	**0.07261g**	**0.10891g**	**0.21783g**	**0.29044g**
Side 2.99g powder	0.045389 g	0.011345g	0.02269g	0.034035g	0.06807g	0.09046g
End 2.99g powder	**0.07261 g**	**0.01815g**	**0.0363058g**	**0.0544g**	**0.1089g**	**0.14522g**

3.1.1. Enzyme immobilization on Zirconia particles: Adsorption

The 1 vol. % Zirconia (and Alumina) suspensions were prepared and sonicated by ultrasound horn (Sonifier 450, Branson, CT, USA). Other Zirconia (and Alumina) suspensions also composed in the same method and autoclaved (SYSTEC 2540, Wettenberg, Germany) in order to produce hydroxyl group on the surface and to sterile the system. They have centrifuged (Thermo Fisher Scientific Inc. MA, USA) for 10 minutes, 4000 rmp, re-prepared and sonicated again for 10 minutes.

Suspensions have been immobilized directly by ALP enzyme solution without any other significant process in order to create 0, 0.5 and 1 monolayer of enzyme on the ceramics particles. The calculation of needed enzyme can be found in table 8. They have been incubated 16hr on the shaker at room temperature. Finally, the samples has been examined with Zeta Potential device, DT 1200 (Dispersion Technology, NY, USA) and titrated with 1 M HCl and 1 M KOH to measure the zeta potential and isoelectric point (IEP).

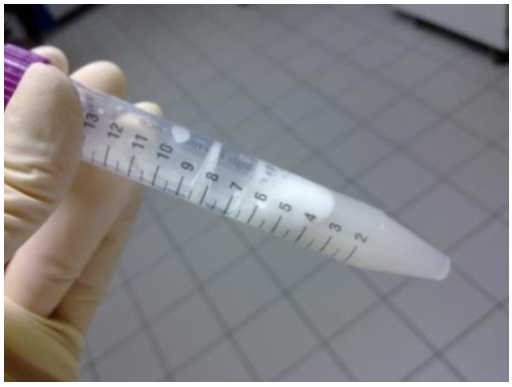

Figure 17. ALP solution prepared for adding to 1vol% Zirconia for measuring zeta potential

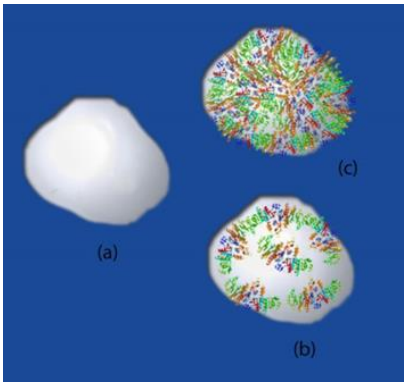

Figure 18. (a). Zirconia particle (b). Partially covered with ALP, (c). completely covered with one monolayer of ALP

3.1.2. Stability of silanized powder

2 vol.% of APTES was added to every sonicated Zirconia suspension and all of the samples were stored on the shaker for 1hr in room temperature and 1hr in 70°C. The extra and non-bonded APTES were removed from the suspensions by washing the reacted powder. The zeta potential of the samples were measured at different time points (day 1, 4, 7, 10, 14, 21, 28) to inquiry any IEP changes during these 28 days. Silanization of planar

surfaces is the same as above, except that samples after rinsing were dried overnight in oven at 70°C.

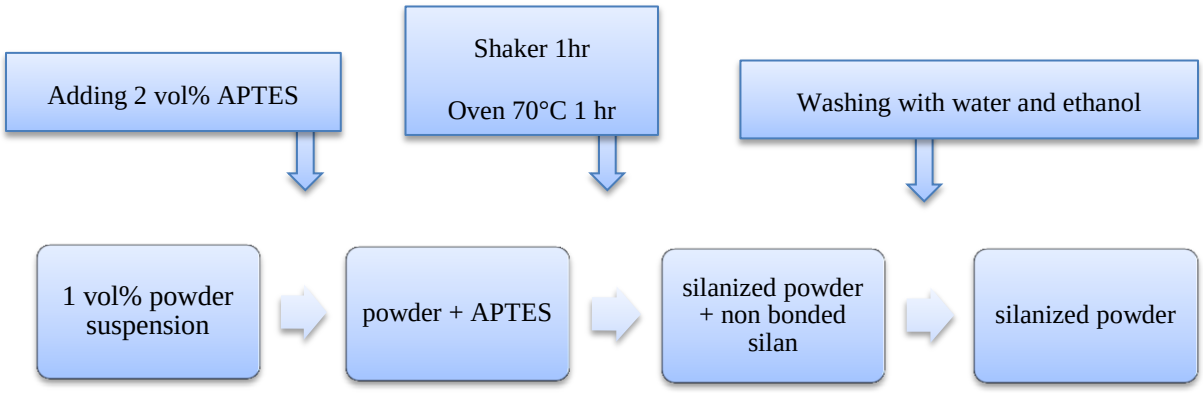

Figure 19. Schematic of silanization process

3.1.3. Enzyme immobilization on Zirconia: covalent bonding

Two types of samples immobilized as follows:

1) Ceramic powder

1 vol% Zirconia suspensions was treated by APTES (2 vol%) and stored on shaker for 1 hour and heated at 70°C for another 1 hour. Afterwards, unbounded APTES removed from the system by washing. The silanized suspension was centrifuged and added to 0.1M MES buffer (SIGMA, MO, USA) with pH 6 includes, 0.6mg/mL of N-hydroxysuccinimide (NHS), 10 ml (0,0.5,1,1.5 monolayer) ALP enzyme and 10mg/mL 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and placed on shaker at 4°C for 2 hours. Subsequently, the suspensions were washed 10 times with double deionezed water and one time with ethanol. (figure 20)

2) The planar surface

Sintered Zirconia Samples were silanized by 2 vol% of APTES in 24 well plates with the same procedure as mentioned for Zirconia powder and dried overnight (8hours at 70°C). Afterwards they were functionalized with 0.1 M MES at pH 6, 0.6mg/ml NHS, 20mg/ml ALP solution and 10mg/ml EDC respectively in 1 ml solution for 2 hours at 4°C. In the last step, they were rinsed for 10 times in order to remove the unbounded linkers or enzymes (Figure 21).

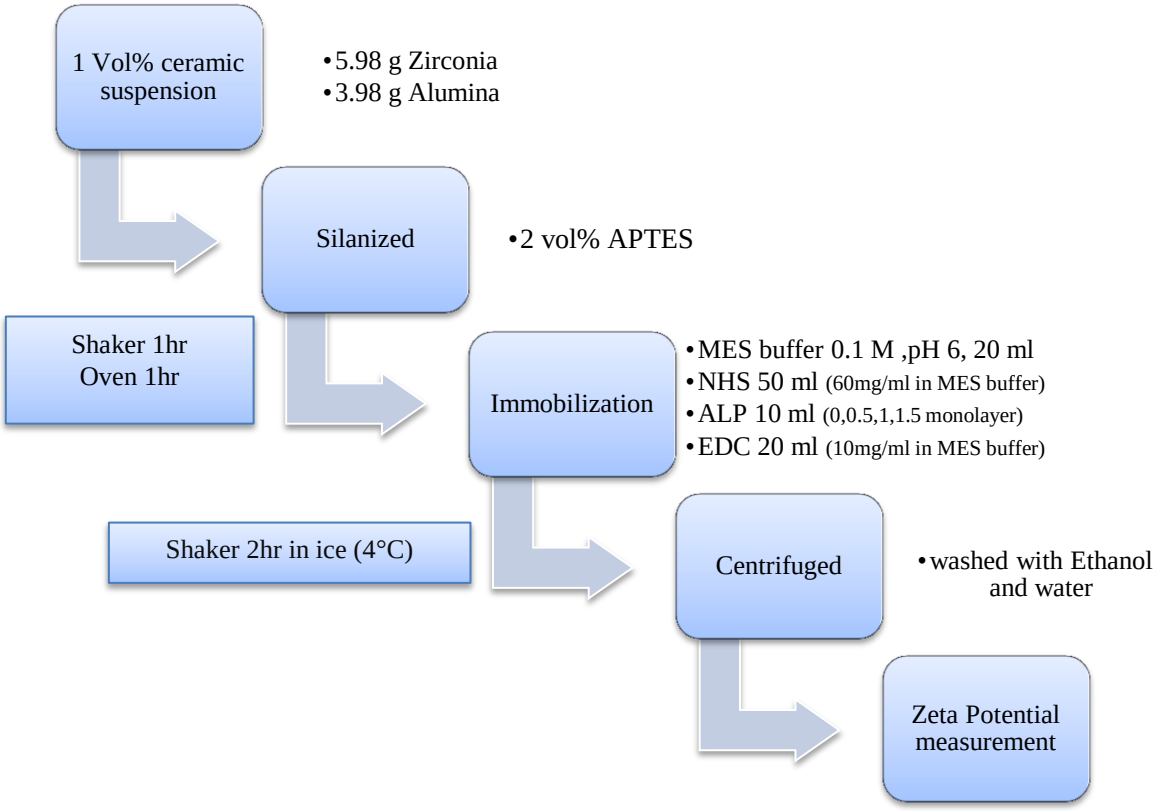

Figure 20. The steps of immobilization process for Zirconia powders

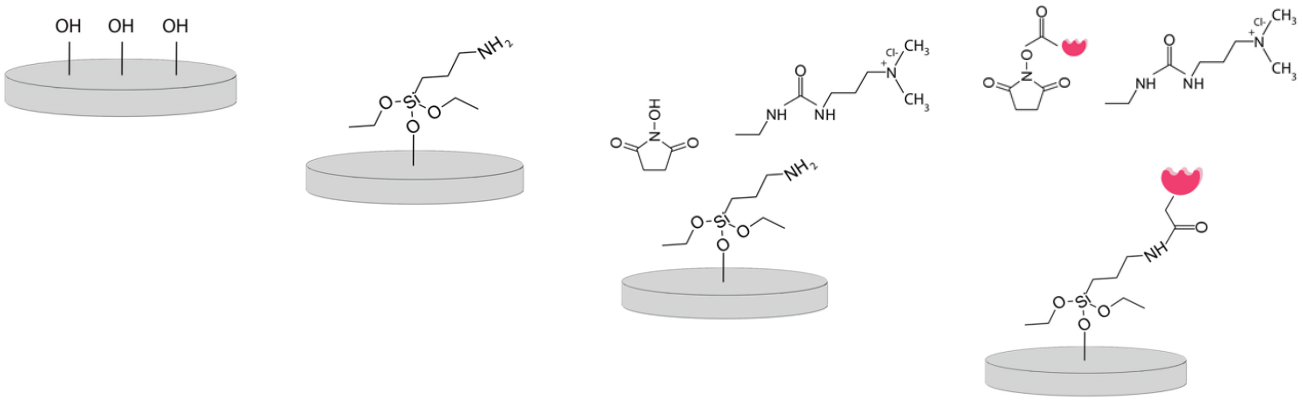

Figure 21. Immobilization steps for planar surfaces; (a) without modification. Autoclaved samples with OH groups on the surface (b) silanization. with APTES to produce amino active groups (c) adding NHS and EDC as the linker (d) functionalization of ALP

Table 9. Concentration of buffer, linkers and ALP in immobilization solution per sample

MES buffer	**0.1 M (pH=6)**	**300 µl**
NHS	0.6mg/ml in MES buffer	500 µl
ALP	20mg/ml	50 µl
EDC	10 mg/ml in MES buffer	200 µl
Total		1 ml

Enzymatic activity

In order to measure the enzymatic activity of ALP after Functionalization on planar surface Zirconia, a chemical assay, which is called pNPP assay, was used.

In this assay, P-nitro-phenylphosphate (pNPP) (SIGMA, MO, USA), as a substrate, reacts with enzyme, in hydrolysis reaction process and the yellow p-nitrophenol is produced which indicates the activity of alkaline phosphate, (the principle of this reaction is in equation 1). The reaction can be stopped by 1M NaOH after desired (2, 10 and 30 minutes) time. p-nitrophenol light absorbance was measured in 405nm. (Snyder, et al., 1972; Bosque, et al., 2002; Jan, et al., 2000)

$$\mathbf{pNPP + H_2O \xrightarrow{ALP} p-nitrophenol + Phosphate}$$ **(Equation 1) (Snyder, et al., 1972; Bosque, et al., 2002; Jan, et al., 2000)**

This assay was carried out by two different buffers, 2-Amino-2-Methyl-1-Propanol (AMP from ALDRICH CHEMISTRY, MO, USA) at pH 10 and Glycin buffer at pH 10. The planar surface Zirconia samples functionalized and 500µl ALP regeant solution (made by 1.0 mg/mL pNPP tablet) added to 10 replicates and the standards contains different range of enzyme concentrations, (from 0.1 to 8 mU/mL). The reaction was stopped by 1 M NaOH after 2, 10 and 30 minutes. The product was pipetted into the 96 well plates and the light absorbance measured in 405nm by plate reader, (HIDEX Co., Turku, Finland). The standard curve plotted demonstrating activity as concentrations and the activity of the samples calculated using the standard curve.

It should be mentioned that the activity stability of the adsorbed and covalent bonded enzyme on the Alumina during 24 hours have already been investigated. The results have been compared, which can be found in results and discussion section.

3.1.4. In vitro investigation

Acellular in-vitro investigation

In total, four types of Zirconia (Autoclaved zirconia, Silanized zirconia, Functionalized without enzyme and Functionalized with enzyme, with 5 replicates of each) were used in this experiment (which is called briefly CCM test) for seven different time points. The cell culture medium, required for this investigation method, is supplemented with ß-glycerol phosphate (ß-GP) and serum protein (table 10).

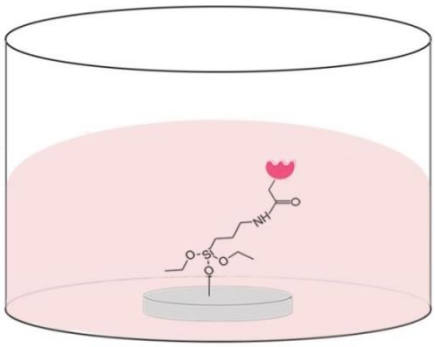

Figure 22. Immersion of ALP immobilized Zirconia in cell culture medium without cells (acellular investigation: CCM test)

Table 10. Concentration of integrant for cell culture medium in CCM test

CCM test materials	
White DMEM	493 ml
αA/αm (antibiotic)	1%
Ascorbic acid	50 µg/mL
Gentamycin	50 µg/mL
Dexamethasone	10^{-8} M
β-Glycerol phosphate disodium salt pentahydrate(β-GP)	10 mM
$CaCl_2$	1.5 mM

Samples were immersed in 7mL of the prepared medium in 6well plates and stored in incubator (Labotect GmbH Co., Göttingen, Germany) in 37°C, 9.5% CO_2 and 90%RH, hereafter they were removed from CCM, washed carefully then dried in 70°C oven (for 2hours) after 1 , 4 , 7 , 10 , 14 , 21 and 28 days. The method of washing is very important at this step, it should be in appropriate manner, to avoid washing out the crystals, and instead the undesired medium should be totally removed from the surface to avoid negative effect on Scanning Electron Microscopy (SEM) image. The soaked immersing medium was refreshed every 2 days to substitute the old ones.

The surface morphology of dried samples were observed by scanning electron microscopy, SUPRA 40 (Zeiss, Jena ,Germany) without gold coating and Energy-dispersive X-ray spectroscopy (EDX) was used for the elemental analysis or chemical characterization of the sample. Furthermore, the amount of calcium deposited on zirconia substrate was determined using a Calcium assay.

Samples were incubated in 0.6 N HCl (Sigma-Aldrich Co., MO, USA), at 37°C for 24 hours in order to quantify deposited calcium on the surface (Somerman, et al., 2009). Calcium assay carried out by Fluitest CA CPC assay with standard Ca concentration from 0- 2.5 mM (analyticon Co., Lichtenfels, Germany) using Spectrophotometer Multiskan GO (Thermo scientific, MA, USA). The absorbance was measured at 578 nm and the standard curve plotted by the software and calcium concentration obtained.

In-vitro investigation with human osteoblast cells

The Human Osteoblast cells (HOB) were provided by Provitro GmbH Co., Berlin Germany.

Cells were cultured in medium, containing DMEM (life technologies Co., USA), (10 %) Fetal Calf Serum (FCS) (Sigma-Aldrich Co., MO, USA) and (1%) antibiotics-antimycotic (life technologies Co., USA). Afterwards, they were incubated (Labotect GmbH Co., Göttingen ,Germany) at 37°C, 9.5% CO_2 atmosphere and 93% humidity. Four-time Passaged cells were trypsinized, counted with cell counter Scepter (EMD Millipore

Co.,Life science Biotech, MA, USA) and 10^4 cell/cm^2 seeded on 4 types zirconia planar surfaces (Autoclaved zirconia, Silanized zirconia, Functionalized without enzyme and Functionalized with enzyme) in 24 well-plates. The cells early differentiation can be characterized by proteins such as alkaline phosphate and Collagen type I. These are the cells markers, which illustrates the cell viability, which has been studied in this project. (Treccani, et al., 2013)

The cell culture medium was refreshed every two days. The following analyses were carried out in seven different time points: Day 1, 4, 7, 10, 14, 21 and 28.

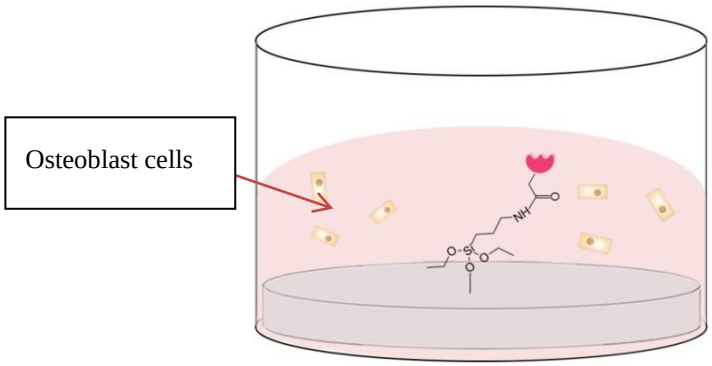

Figure 23. Cells are seeding on ALP functionalized zirconia

a. Cell attachment and morphology

In order to investigate the cell morphology, attachment and dispersion on the substrate, 3-fold fluorescence staining employed. Samples classified in two groups for investigating the secretion of collagen type I and Vincullin attachment to the surface with four replicates each for four types of samples as same as pervious experiments.

The samples were fixed by paraformaldehyde (PFA) followed by Triton X-100 permeabilization in 3 minutes. After blocking process by BSC, the cells were stained fluorescently for 40 minutes (in the dark) by first antibodies, the anti-vinculin (Sigma-Aldrich Co., MO, USA) and anti-collagen (Acris Antibodies Co., Herford, Germany) and 45 minutes by second antibodies, Phalloidin 448 (life technologies Co., CA, USA), AF 546(Alexa Fluor) (Invitrogen Co., CA, USA) and DAPI (Sigma–Aldrich Co., MO, USA). After washing the samples, they have been observed by fluorescence microscopy (Carl Zeiss AG Co., Jena, Germany) to monitor the cell dispersion and morphology (cell nuclei, cytoskeleton, Collagen type I and vinculin). .

Scanning electron microscopy SUPRA 40 (Zeiss, Jena, Germany) was applied to detected cell morphology. Cells were washed and fixed by OsO_4-Fixation solution, containing 0.2 M and 0.1 M cacodylic acid buffer (pH=7.3), Glutaraldehyde and

Osmiumtetroxide. They were dehydrated by different percentage range of ethanol and HMDS. SEM images were taken from prepared gold-coated samples.

b. Cell viability

Cell viability investigated by colorimetric WST-1 assay, which is based on formazan production (n=6 four types of samples). WST-1 assay was carried out by adding 100 μL WST-1(Roche Co., Basel, Switzerland) to each samples in 24-well plates. The TE buffer (invitrogen Co., CA, USA) used in this assay contained Tris and EDTA to solve the DNA while protecting it from degradation (Aitken A , 2012). Finally, 100 μL of the samples solution transfer in the 96 well plate and formazan production measured and read at 450 and 650 nm with Plate Reader (HIDEX Co.,Turku, Finland).

c. Cell differentiation

pNPP assay were used to evaluate the alkaline phosphate activity produced as marker of early cell differentiation. Four types of samples with 6 replicates in 24-well plates washed with Phosphate buffered saline (PBS) and trypsinized (Sigma-Aldrich Co., MO, USA). The cell lysate was stored in ice in order to be used in following assays. This assay is carried out in 0.1 mM Glycine buffer (pH 10), contained 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 60mM p-nitrophenyl phosphate. The standard curve is plotted by 0.0075 to 0.75 mM p-nitrophenol (pNP) concentration. The reaction between ALP and p-nitrophenylphosphate started after adding the reagent to the samples and stopped by 1M NaOH after 30 min incubation in 37°C. The absorbance of the yellow colour, produced in this reaction was measured at 405 nm with plate reader (HIDEX Co., Turku, Finland).

d. Biomineralization

Biomineralization of the cells was investigated by alizarin red staining on four types of samples with 6 replicate. Samples were washed after different culturing days and stained with 40 mM Alizarin red Staining pH (4.1- 4.3) at room temperature on shaker. The 150 μL of red alizarin products transferred into 96 well plate and measured at 405 nm with plate reader (HIDEX Co., Turku, Finland)

CaP morphology also studied by Scanning electron microscopy SUPRA 40 (Zeiss, Jena, Germany) and chemical characterization investigated by Energy-dispersive X-ray spectroscopy (EDX). The biominealizatioan is indicating the late differentiation of the cells.

3.2. Results and discussion

3.2.1. Functionalization of zirconia particle

Physical adsorption of ALP on Zirconia particles

Sonication of Zirconia suspensions creates homogenous ceramic samples with high temperature. As a result, all the sonicated suspensions placed into the freezer in order to reduce the temperature obtained by ultra-sound to avoid destruction of the proteins added in the next step. It is reported that ALP will be inactive at pH <5 and temperatures >40°C, as it is sensitive to temperature and pH. (Verne, et al., 2010)

The zeta potential measurement as a function of pH (2-12) for Zirconia (as purchased and autoclaved) before and after ALP adsorption, were presented in figures 24-25.

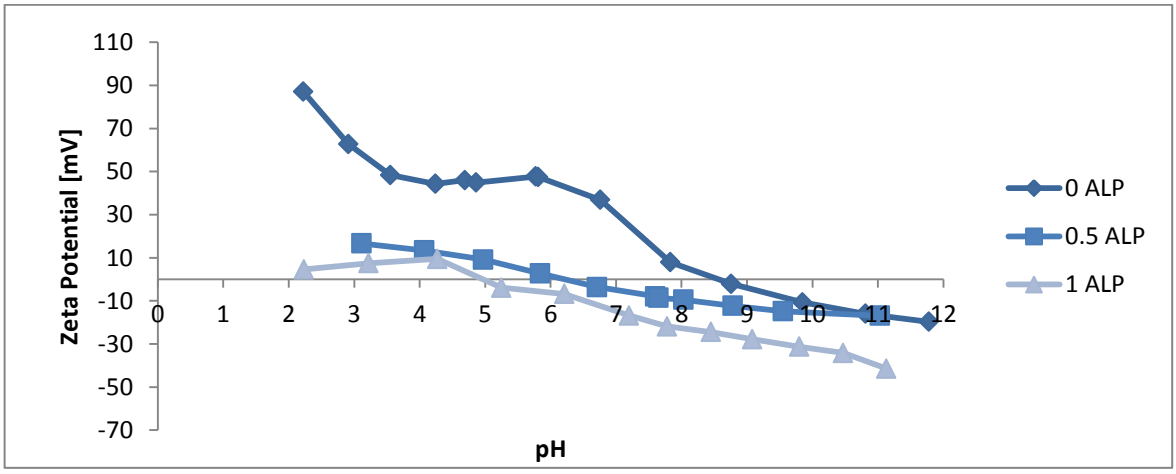

Figure 24. Zeta potential of as purchased zirconia and ALP adsorbed zirconia with different initial ALP concentration (0.5=1.37µM and 1monolayer=2.74µM) after 16 h of adsorption time [calculated in table8]

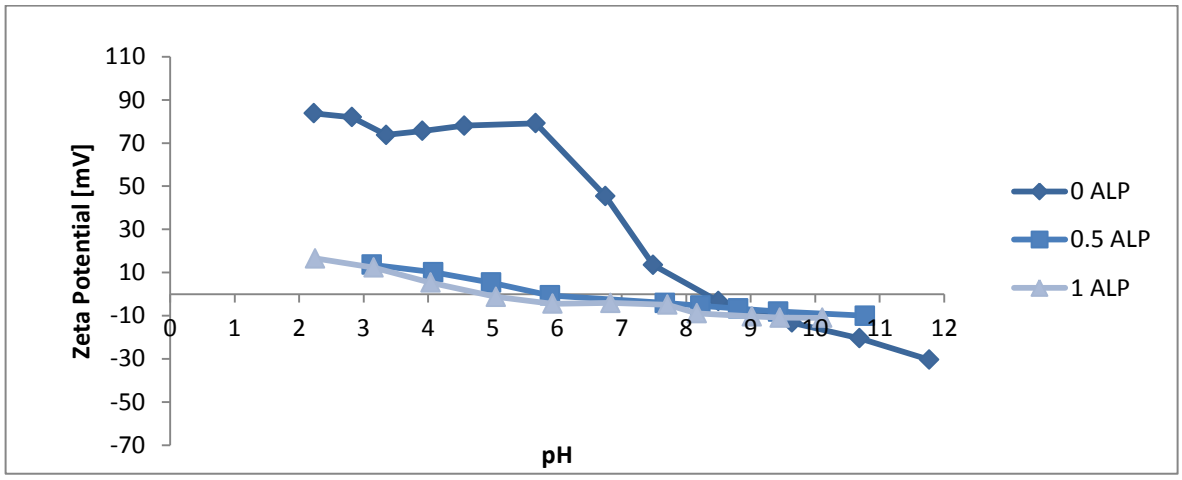

Figure 25. Zeta potential of autoclaved zirconia and ALP immobilized autoclaved zirconia with different initial ALP concentration (0.5 and 1 monolayer) after 16 h of adsorption time[calculated in table8]

Figures 24-25 indicate, the 0.5 and 1 monolayer of ALP on the zirconia particles show lower suspension stability than 0 ALP. This was concluded from the low zeta potential changes over pH range (Malvern, 2004) and it is due to the high concentration of enzyme adsorbed on the particles.

Figure 26 illustrates the IEP (Isoelectric point) of ALP adsorption on as purchased and autoclaved Zirconia particles, which is obtained from the figures 24-25. The same type of Alumina particles were also treated by the same amounts of ALP for comparison, which is depicted in figure 26.

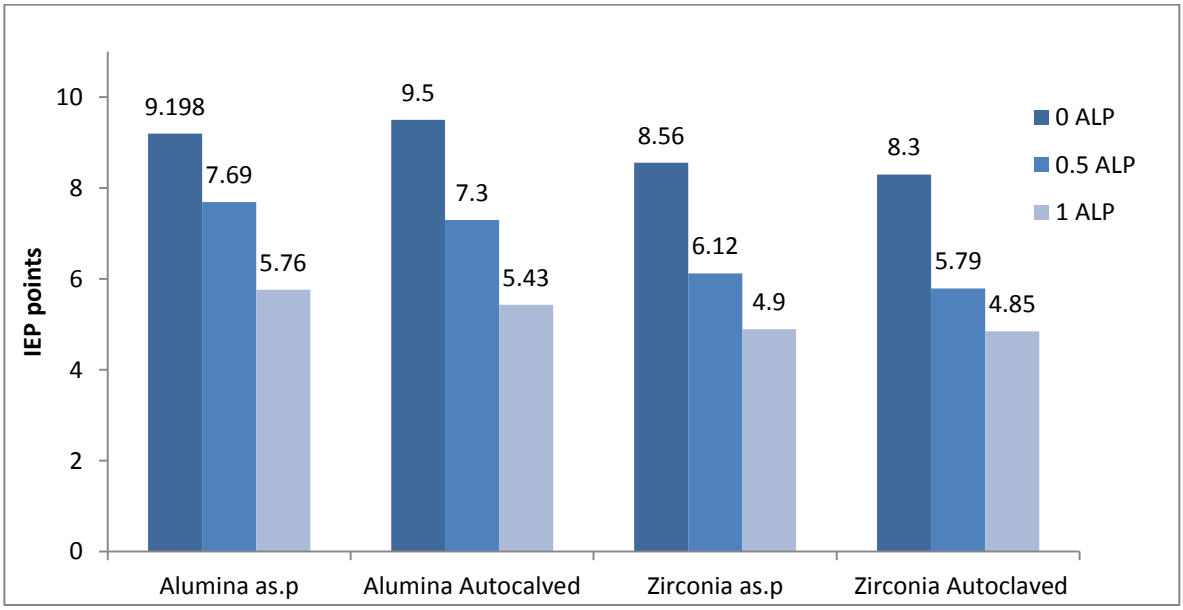

Figure 26. Isoelectric point of as purchased and autoclaved zirconia before and after ALP adsorption with different initial ALP concentration (0.5=1.37µM and 1 monolayer=2.74µm) after 16 h of incubation time [calculated in table8] compared with the same type of alumina

The figure indicates that, by increasing the amount of adsorbed ALP to one monolayer on zirconia and Alumina surfaces, the IEP decreases significantly from almost 9 to 5, through acidic pH, which can also be seen in figure24-25. This decrease is based on IEP of the enzyme, which is between 2-4 pH range (US Biological life science, 2013; Sigma-Adrich, 2013; Worthington biochemical Co, 2013). These results demonstrate the adsorption of ALP on Zirconia particles.

By comparing the IEP of autoclaved powder with as purchased powder after ALP adsorption, it can be concluded that the IEP of ALP adsorbed autoclaved powder with hydroxyl groups (IEP of 0.5ALP= 5.79, 1ALP= 4.85) is lower than ALP adsorbed as purchased powder without any pretreatment (IEP of 0.5ALP= 6.12, 1ALP= 4.9). This is based on better attachment of enzymes with hydroxyl group on the surface. This result can be achieved in Alumina IEP as well. As a whole, Zirconia powder shows higher potential in absorbing enzyme on its surface. This can be realized from the lower IEP points compared to Alumina. (Verne, et al., 2010)

a. ALP activity after adsorption on zirconia substrate

Stability of ALP activity grafted by adsorption and covalent bonding immobilization on Alumina has already investigated. As it is shown in figure 27, the ALP adsorbed on Alumina does not show any stability during 24 hours. Although the covalent bonded ALP activity is lower than the adsorbed one, it remains constant during the same period.

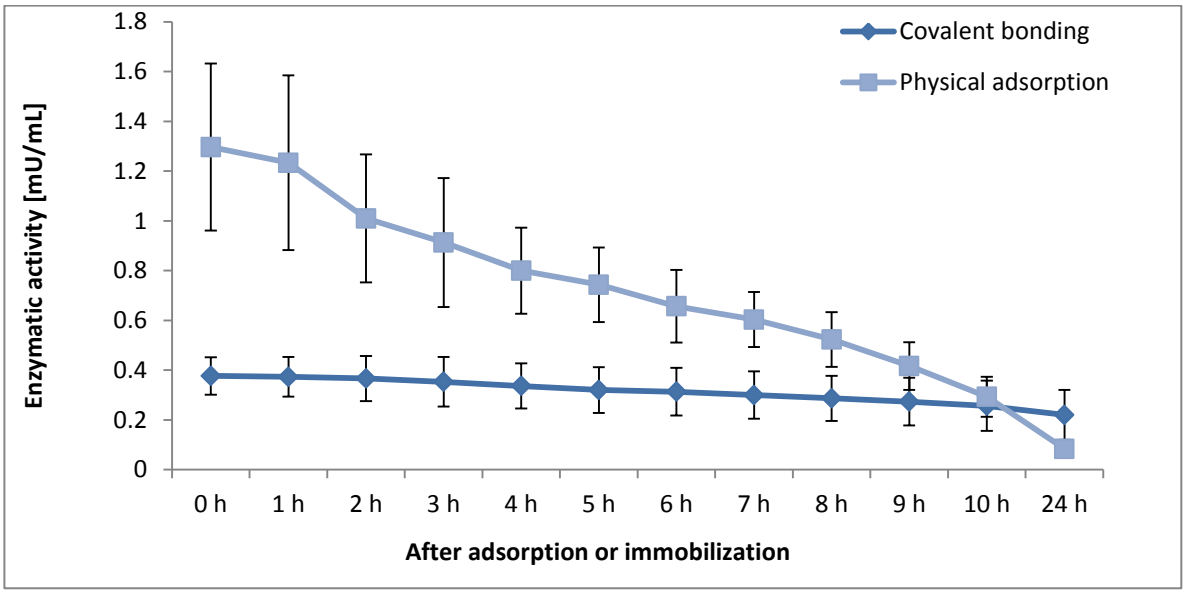

Figure 27. Stability of grafted enzyme activity on Alumina by adsorption and covalent bonding methods [Data by Advanced Ceramics Department]

It can be illustrated that the covalent bonding method is more appropriate compared to other method, due to higher stability.

Enzyme immobilization on zirconia: covalent bonding

a. Stability of silanized zirconia particles

In order to create active bonds on the surface of the powder samples, silanization is carried out on the powder surfaces. (3-aminopropyl)triethoxysilane (APTES) helps producing the active groups called amino group (NH_2-) on Zirconia particles which is considered as the first step of surface modification. High stability of this process is also important fact, which should have been considered. This test examined the stability of silanization during 28 days in seven different time points.

Zeta potential of these particles evaluated after 1, 4, 7, 10, 14, 21 and 28 days. The result is shown in the figures 28.

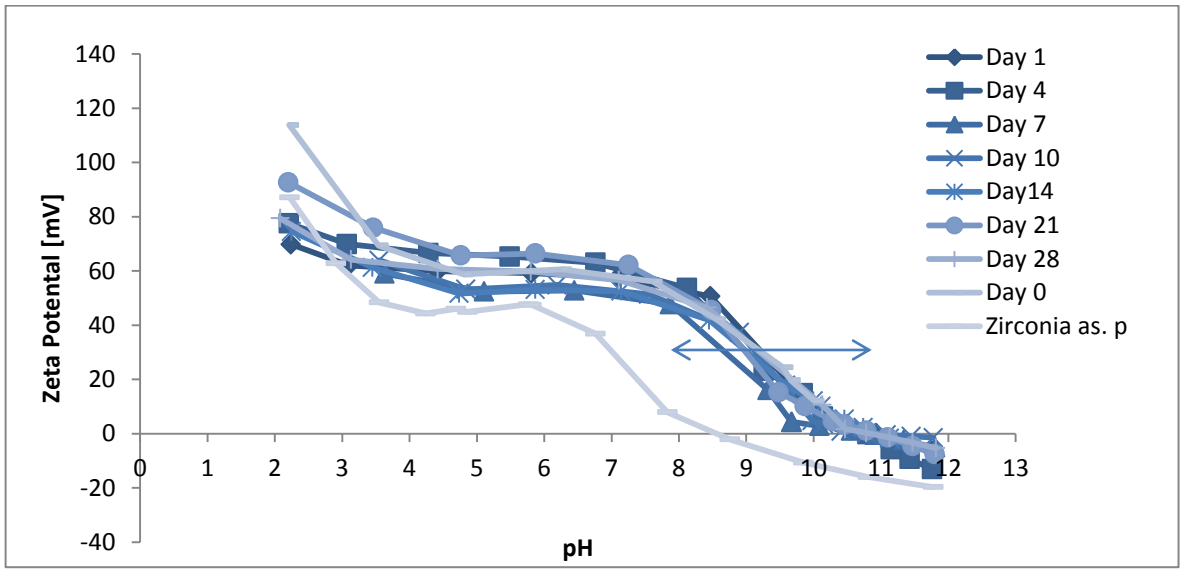

Figure 28. Zeta Potential of silanized particles after different storage time (1, 4, 7, 10, 14, 21 and 28 days, The arrow indicates the shifts in IEP of silanized zirconia powder compare to zirconia as purchased

Based on these results, silanized zirconia was stable during 28 days since the IEP after different storage time didn't change dramatically and it remained at 10.83 close to APTES IEP (IEP~11) (Meder, et al., 2012; Lee, et al., 2006)

b. Covalent bonding of ALP on zirconia particle

Immobilization is localizing enzymes for desired time, in order to perform a function continuously. There are many types of immobilizations such as adsorption or entrapment. In the following experiments, the covalent binding immobilization has applied; within this method, the strong covalent attachment between enzyme and solid particles or planar surface is created. The main goal of this step is to build a stable connection between enzyme and surface for long period in different conditions such as various pH and temperature with acceptable activity. Linkers mostly make these connections, like NHS (N-hydroxysuccinimide) and EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride).

Alkaline phosphatase was immobilized covalently on zirconia particles, with different initial ALP concentration, calculated for 0, 0.5, 1 and 1.5 monolayer of this enzyme based on end on orientation (table8). The zeta potential of these functionalized powder were measured and illustrated in figure 29.

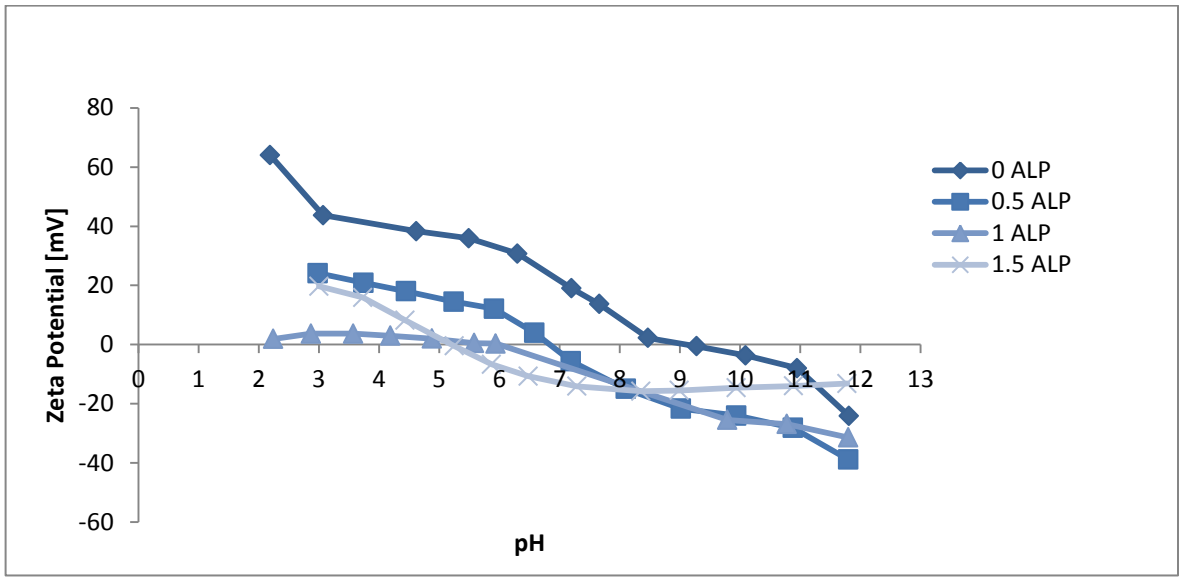

Figure 29. Zeta Potential of ALP functionalized zirconia with different initial ALP concentration (0.5 ,1 and 1.5 monolayer)[table8]

Figure 30 illustrates, as the calculated ALP monolayer on zirconia surface increases from 0 to 1.5 monolayer, the suspension loses its stability as a result of high enzyme concentration, which can be deduced in low changes of zeta potential over pH range (Malvern, 2004). The IEP shifted from 9 to 5 (close to IEP of ALP) declares the higher immobilized enzyme on zirconia surface. Due to this fact, the suspension starts to flocculate and the particles strongly agglomerates. Same as adsorption method, Zirconia powder demonstrates higher enzyme attachment compared to Alumina.

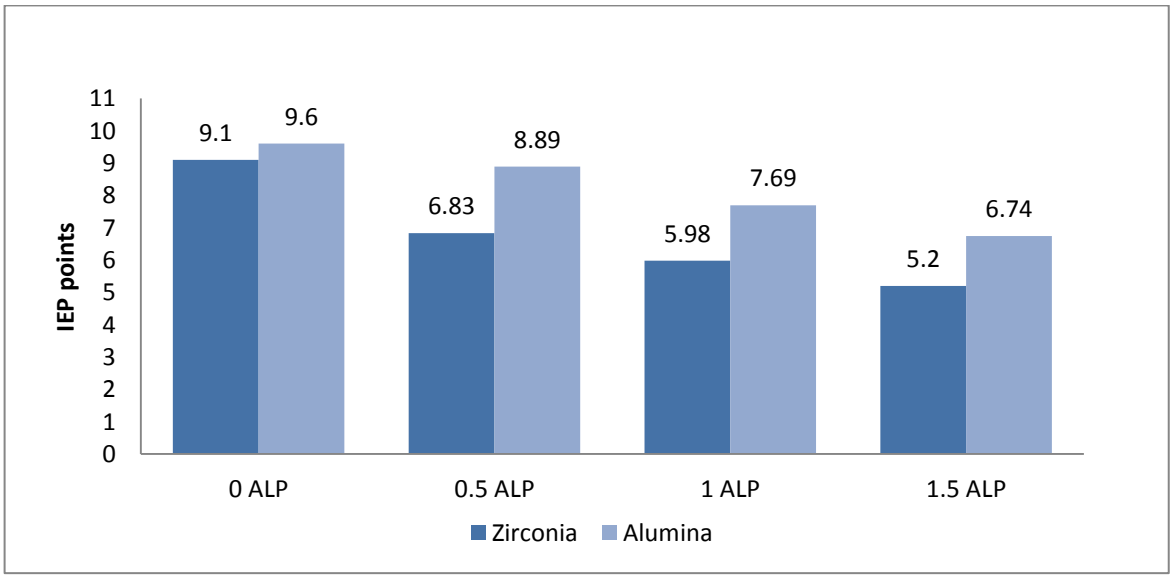

Figure 30. IEP comparison between functionalized Alumina and Zirconia

Figure 31 indicates the IEP comparison between physical adsorption and covalent bonding immobilization of ALP on Zirconia and Alumina particles. From the figure, it can be concluded that there is higher enzyme attachment in physical adsorption method compared to covalent bonding immobilization process due to lower IEP of the second approach.

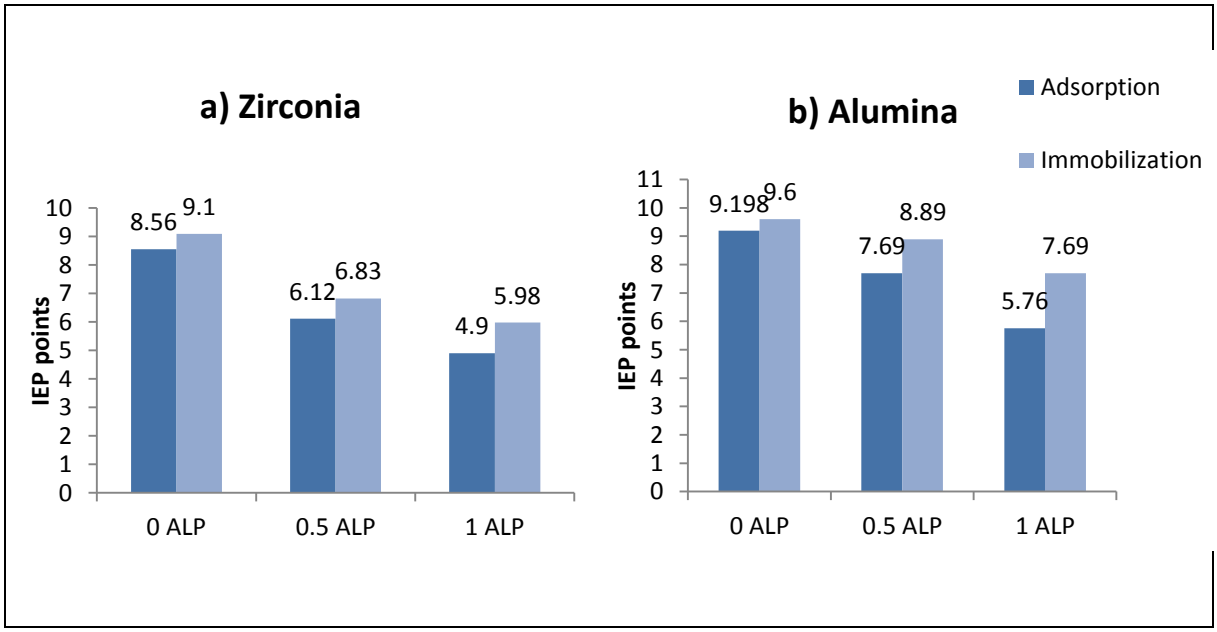

Figure 31. IEP of ALP functionalized a) zirconia and b) alumina with physical adsorption and covalent bonding methods

Although adsorption test shows higher enzyme attachment on both Alumina and Zirconia, the stability is crucially low due to weak hydrogen bonding and Van der Waal's forces. Besides physical adsorption depends on environmental factors, as behavior of the substrate or mobility of the biomolecules (Górecka, et al., 2011; Guisan J.M, 2006; Rusmini, et al., 2007; Meder, et al., 2012)

3.2.2. Functionalization of zirconia planner surface

Based on the results of zirconia particles, as the covalent bonding methods confirm higher stability of ALP activity after immobilization in contrast to physical adsorption, this method is selected to immobilized ALP on zirconia planner surface.

ALP enzymatic activity assay for planar surface

Enzymatic activity of ALP after functionalization on zirconia planar surface was studied by pNPP assay with two different buffers, Glycin and AMP in 2, 10 and 30

minutes. The aim of this experiment was to analyze and compare the two buffers and investigate the optimum reaction time for each one in both ceramics. The results are gathered in figure 32.

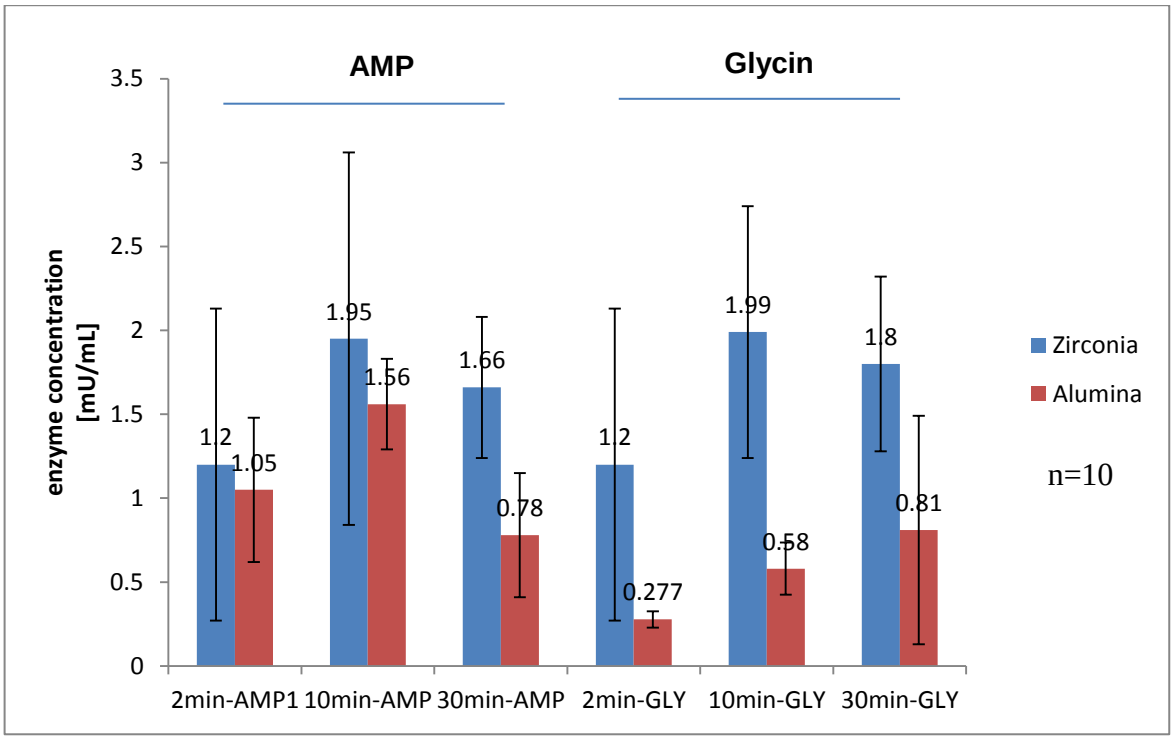

Figure 32. Enzymatic activity of functionalized Alumina and Zirconia by pNPP assay with AMP and Glycine buffer after 2, 10 and 30 minutes of incubation.

Zirconia samples in Glycine buffer in 10 min and AMP buffer in 30 min show higher enzyme activity compared to other sample (Alumina). Optimum reaction time for AMP and Glycine buffer is 30 min in zirconia samples

3.2.3. Optimized sample

According to the results presented in pervious sections, Covalent-bonding immobilization method selected in order to functionalize ALP on zirconia planner surfaces, to fabricate samples for experiments in biological environment (in vitro investigation with and without cells). The zirconia planar surface samples were prepared by hydraulic pressing and sintered at 1500°C. For our experiments, we classified using substrates in four types:

Group 1 includes the samples, which were autoclaved, in order to sterile and create the hydroxyl group on the samples surface. The samples of second group were silanized by 2 vol% of APTES (1hour at RT+ 1hour 70°C) to produce amino groups on them. Third

group of samples were functionalized by linker such as NHS and EDC without ALP at 4°C (2hour) while in group 4, ALP were functionalized on zirconia surface with the same linkers plus ALP at 4°C for 2 hours with covalent bonds. (Figure 33)

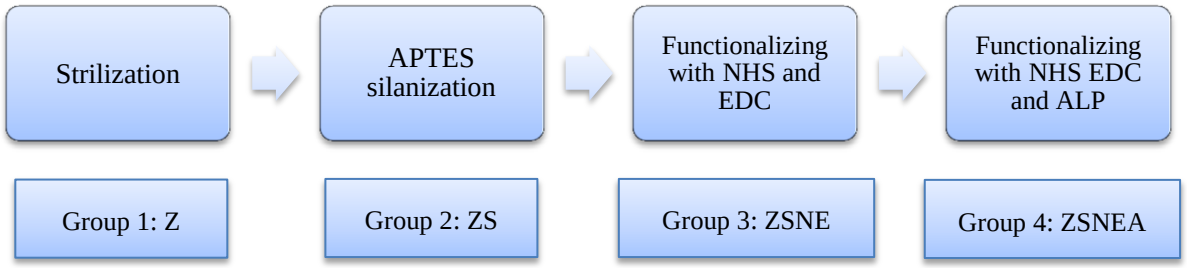

Figure 33. Classification of different types of zirconia planar surface, used in in vitro tests in different processes

3.2.4. In vitro investigation in acellular conditions (CCM)

Zirconia planar surface were immersed in cell culture medium (CCM) to examine the effect of enzymatic function after immobilization on Zirconia in acellular environment. These experimental substrates were soaked in CCM for 1, 4, 7, 10, 14, 21 and 28 days. The SEM micrographs of all samples are shown in figure 34 and 35.

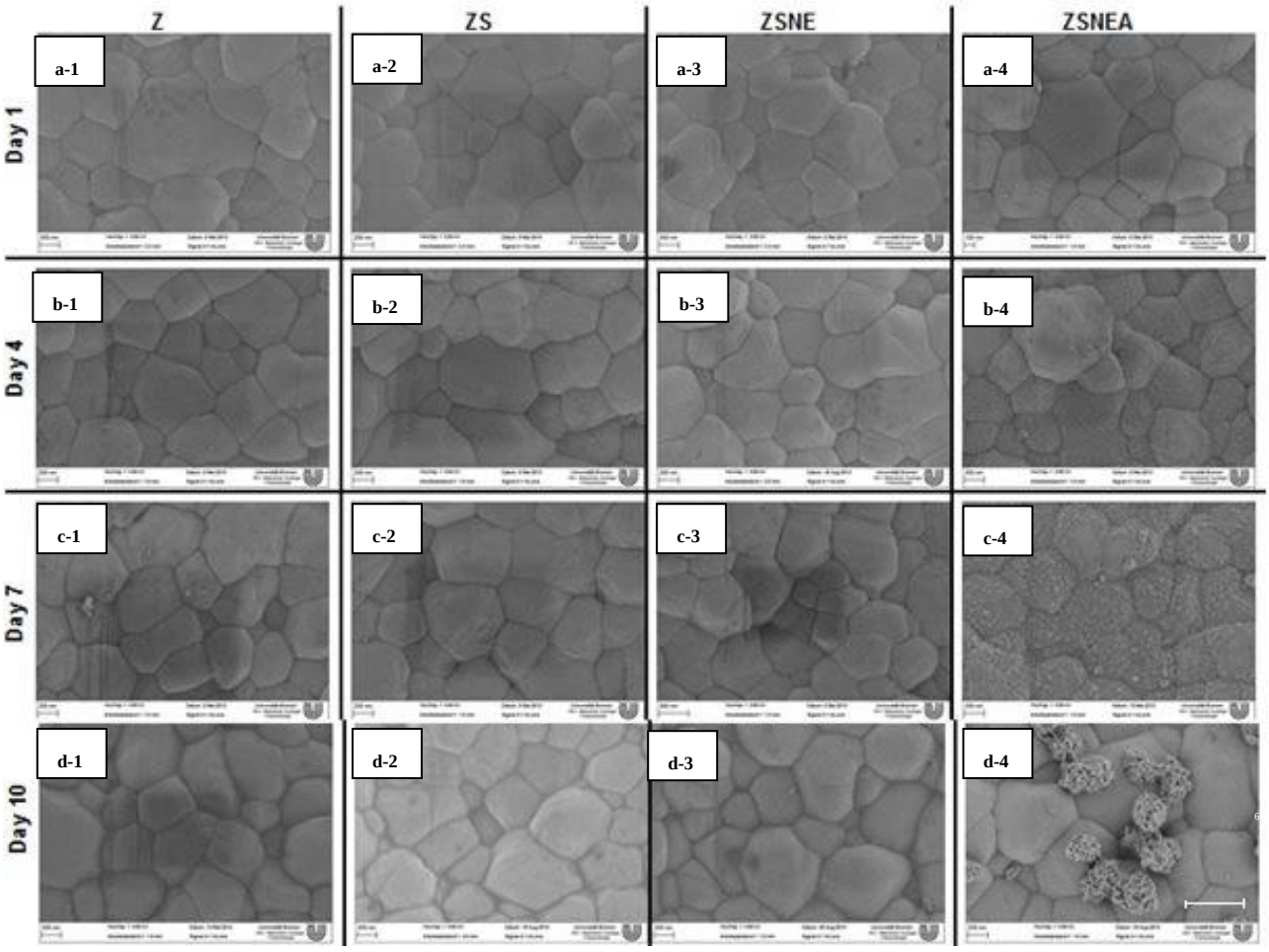

Figure 34. SEM images of a-1) Z, a-2) ZS, a-3) ZSNE, a-4) ZSNEA after 1 day immersion in CCM, e b-1) Z, b-2) ZS, b-3) ZSNE, b-4) ZSNEA after 4 day immersion in CCM, c-1) Z, c-2) ZS, c-3) ZSNE, c-4) ZSNEA after 7 day immersion in CCM, d-1) Z, d-2) ZS, d-3) ZSNE, d-4) ZSNEA after 10 day immersion in CCM

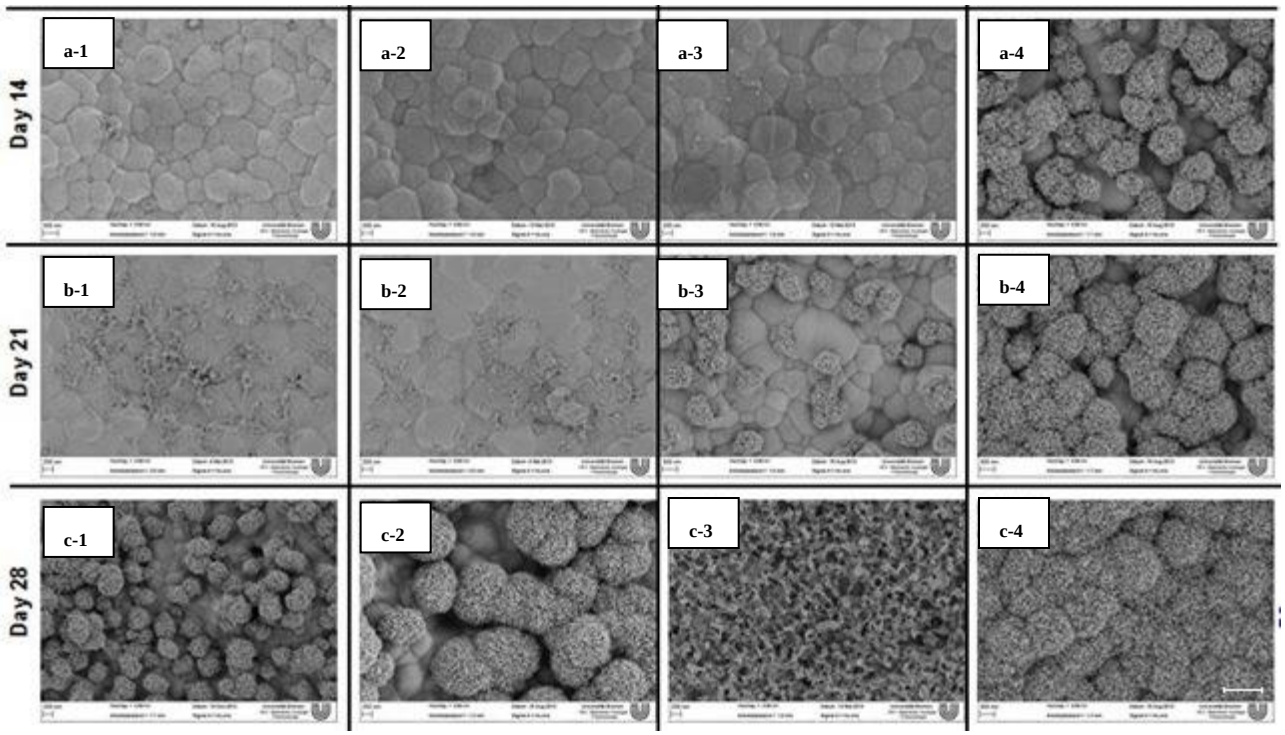

Figure 35. SEM images, a-1) Z, a-2) ZS, a-3) ZSNE, a-4) ZSNEA after 14 day immersion in CCM, b-1) Z, b-2) ZS, b-3) ZSNE, b-4) ZSNEA after 21 day immersion in CCM, c-1) Z, c-2) ZS, c-3) ZSNE, c-4) ZSNEA after 28 day immersion in CCM

SEM images exhibit that calcium phosphate started to deposit on ALP functionalized zirconia after 4 days of immersion (figure 36). Nevertheless, there is no crystal formation on the other substrates even after 10 days. These minerals cover the ALP functionalized zirconia's surface after 14 days of soaking in CCM, which resulted in the deposition of a homogeneous CaP layer. Data of EDX revealed CaP mineralization with the ratio of Ca to P in the range of 1.5 to 1.7 (data are not shown).

The main role of ALP in mineralization (simulation of biomineralization) is clearly observed on ZSNEA samples in this acellular investigation. In fact, ALP is able to assist in the attraction of CaP mineral on surfaces of functionalized sample in CCM by hydrolyzing organic phosphates of the β-GP.

Moreover, it should be realized that physiological testing solutions such as CCM is metastable solutions, which easily tend to give rise to apatite deposition upon slight fluctuations in ionic composition (and especially those of Ca^{2+} and PO^{3-}) (de Jonge, et al., 2009). This is the reason of CaP deposition on the other sample after 14 days of immersion.

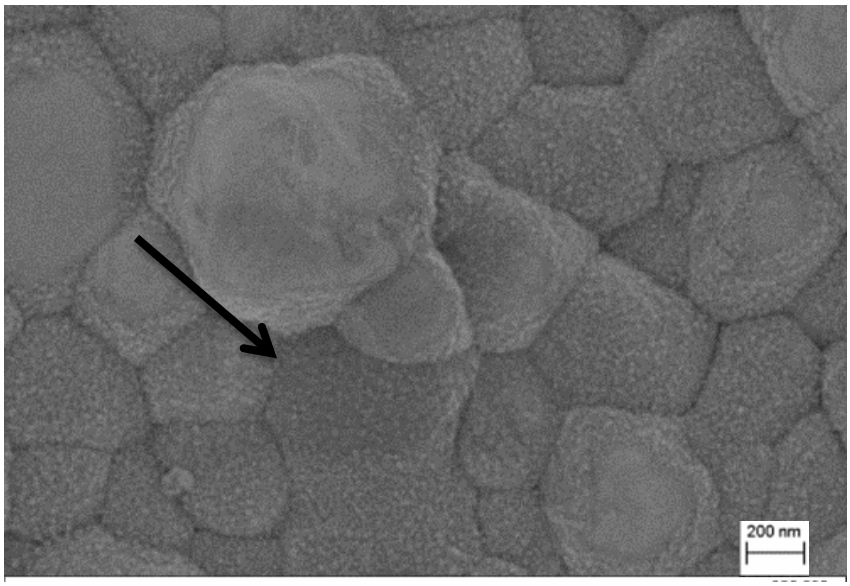

Figure 36. Crystals of hydroxyapatite started to grow on ZSNEA samples day4

The calcium content on deposited CaP was further investigated using the calcium assay. (Somerman, et al., 2009) Calcium content on ALP functionalized zirconia was significantly increased compared to non-functionalized zirconia when incubated them in CCM for 28 days (figure37). These results supported the observation of SEM images, that the amount of mineralization on ALP functionalized zirconia is remarkably more than non-functionalized zirconia.

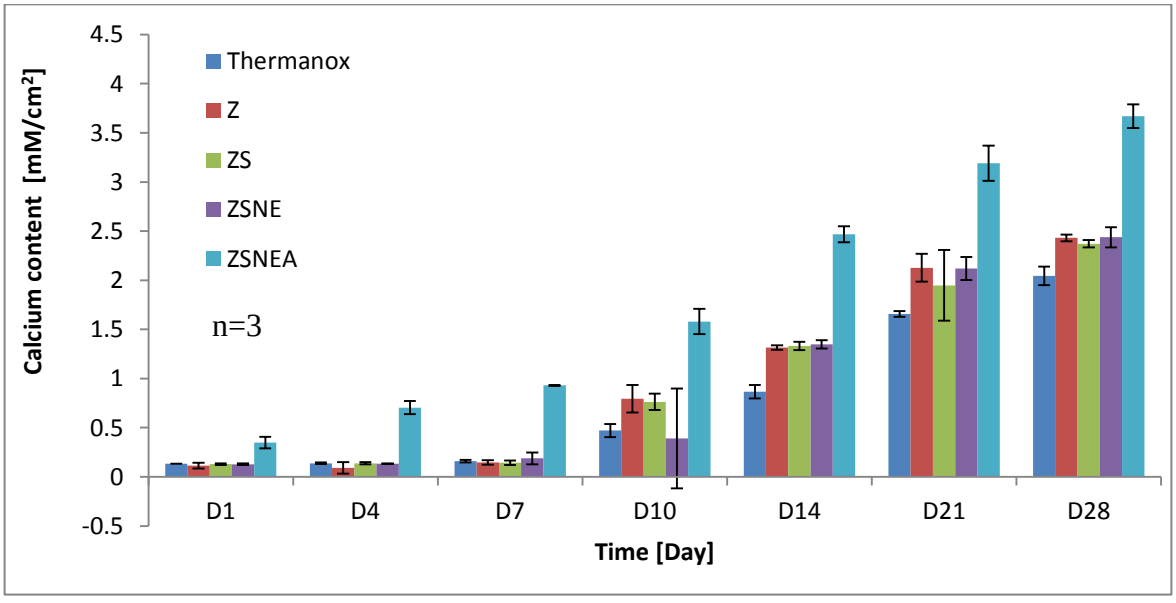

Figure 37. Calcium content of experimental substrate after soaking for 1, 4, 7, 10, 14, 21 and 28 days in CCM

3.2.5. Osteoblast cell culture experiments

This experiment is the process in which the cells are seeded on samples in simulated biological environment in vitro for desired time in order to study the cells reaction and behavior. This stage can be the first step for in vivo test and the results show if the cells differentiate on the functionalized samples and start mineralization by producing calcium phosphate.

Cell culture experiments with human osteoblast cells were carried out and the results of this test were presented in section below.

Cell morphology

SEM Fluorescence microscope (figure38) and SEM (figure 39) were employed to observe the morphology of the cells on different types of samples in every time points. No outstanding differences were observed in cell morphology in both kind of images (fluorescence microscope images (figure38) and SEM images (figure39)) between the experimental samples.

Osteoblast-like cells established a flat appearance and spread out over the all types of surfaces. Generally, after 7 days of culture nearly all of the surfaces of the experimental samples were covered completely with a confluent layer of osteoblast-like cells (figure 38). However, underneath the osteoblast-like cells, mineralized layers were visible on ALP functionalized zirconia (ZSNEA) after 7 days (figure 40). No mineral deposition was observed underneath the other substrates, which were spread out over the ALP free surfaces (Z, ZS and ZSNE). Although after 14 days of culture, a cellular multilayer had formed on all experimental substrates. Furthermore, fibrous extracellular matrix with mineralized globules was observed on all experimental substrates (figure 40).

This normal cells growth demonstrates the biocompatibility of linkers and enzymes and the method of immobilization.

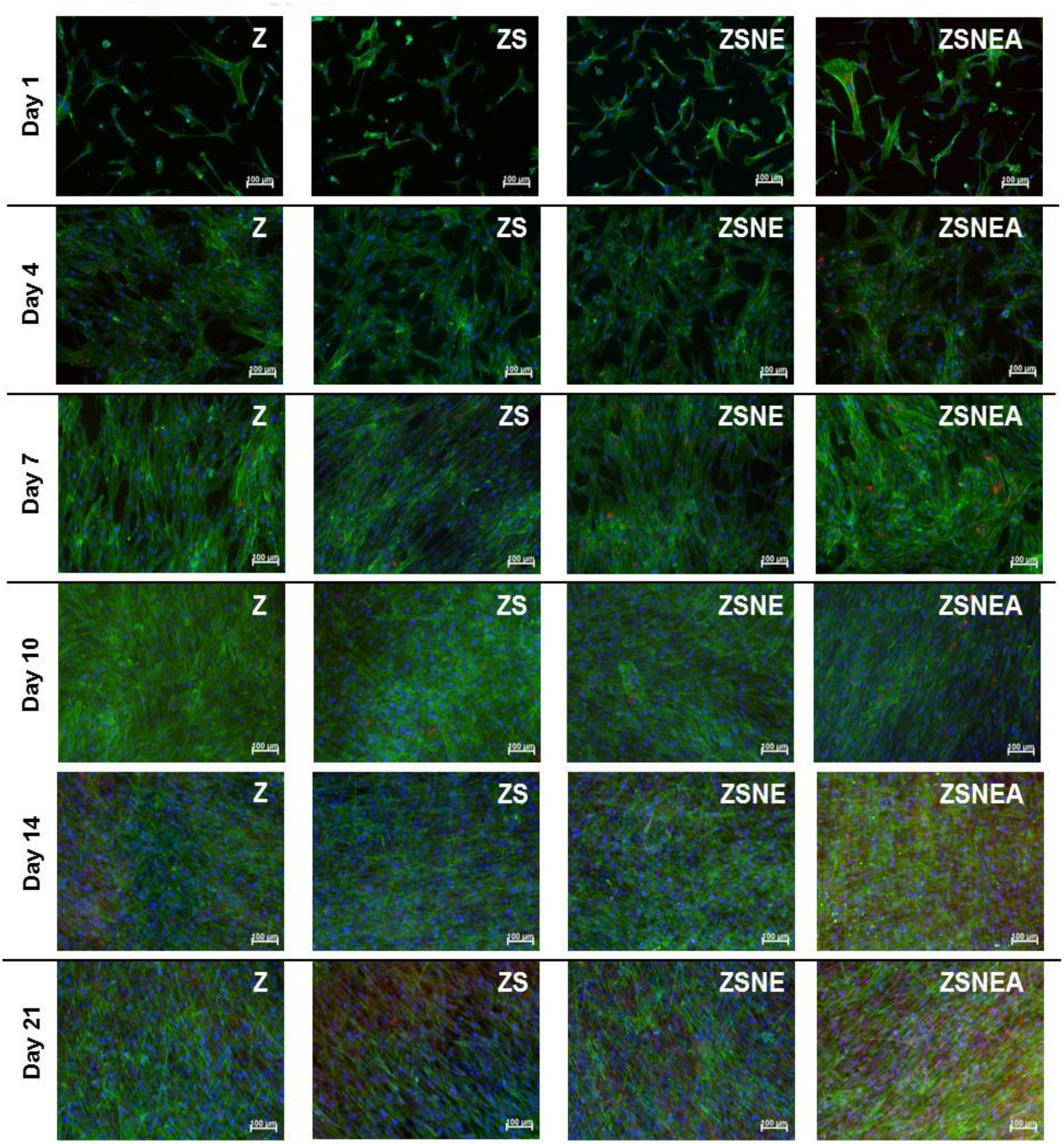

Figure 38. Fluorescence microscopy of HOBs grown on all types samples (Z, ZS, ZSNE, ZSNEA) after 1, 4, 7, 10, 14, 21 and 28 days of culturing (Blue: Nucleus, Green: Actin cytoskeleton, Red: Collagen type I)

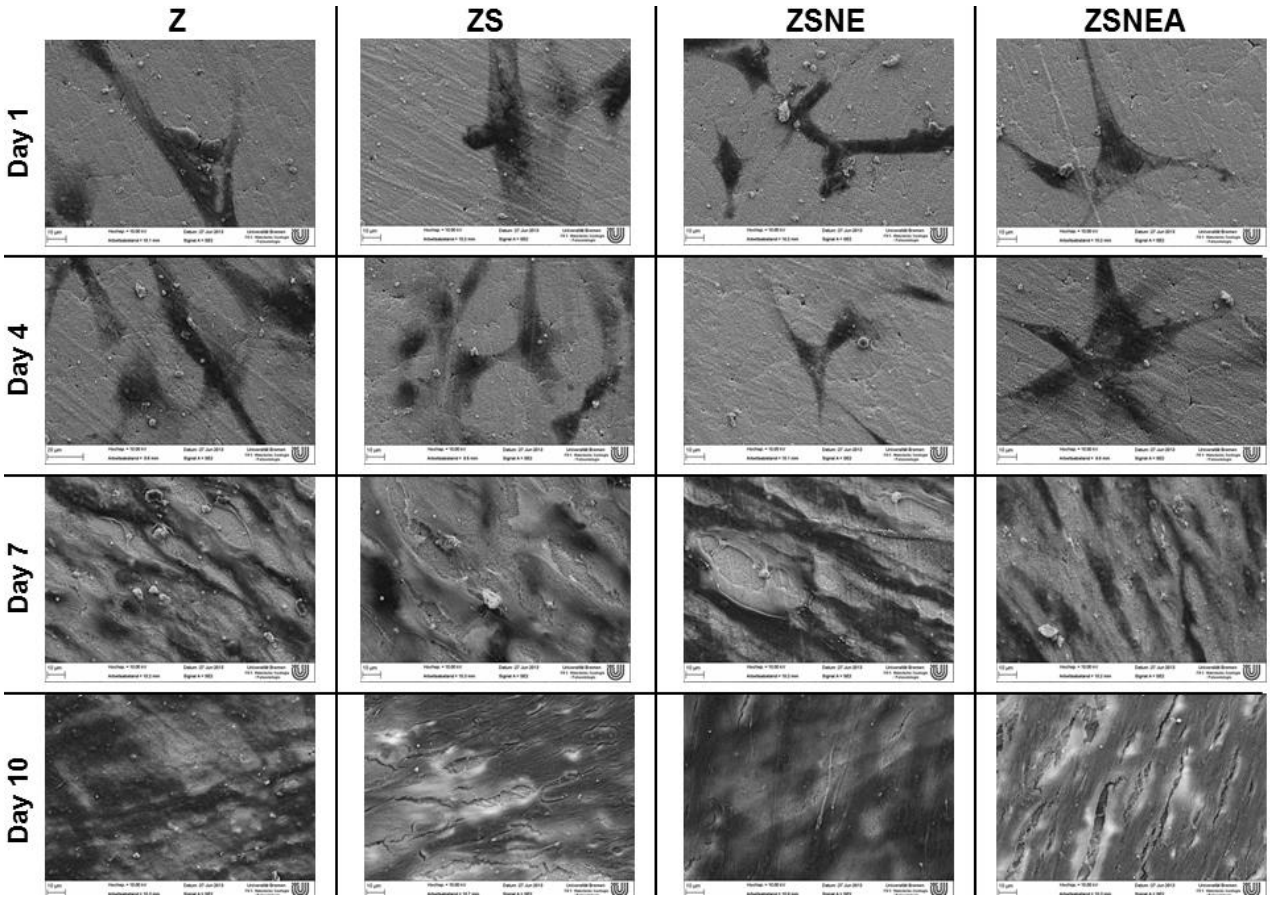

Figure 39. Cell morphology and cell attachment

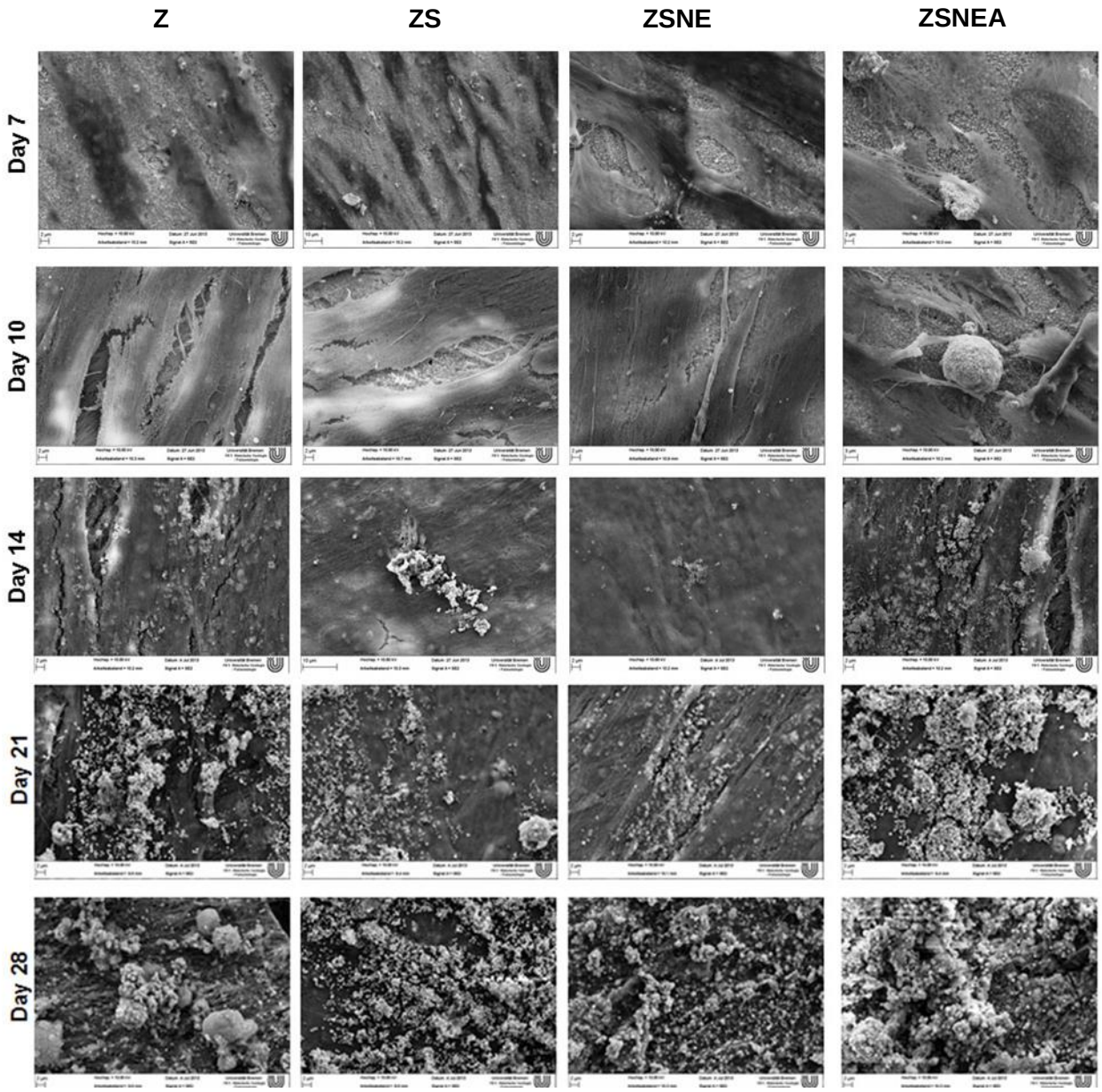

Figure 40. Biomineralization after cell culture in osteoinductive medium (with cells)

Cell viability and proliferation

Proliferation of human osteoblast-like cells on all types of samples are assessed by cell number, which is measured by DNA assay (Figure 41-a). The calculated cell numbers on experimental samples are nearly the same and there is no notable difference between them.

Cell viability was determined using colorimetric assay, WST-1, the results are plotted in figure 41-b. Indeed, there is no considerable difference between experimental samples, and they are almost in the same range, and after 10 days cells stopped proliferation due to the osteoinductive medium, used for feeding. These two data also can confirm non-toxicity of ALP functionalization process in this study.

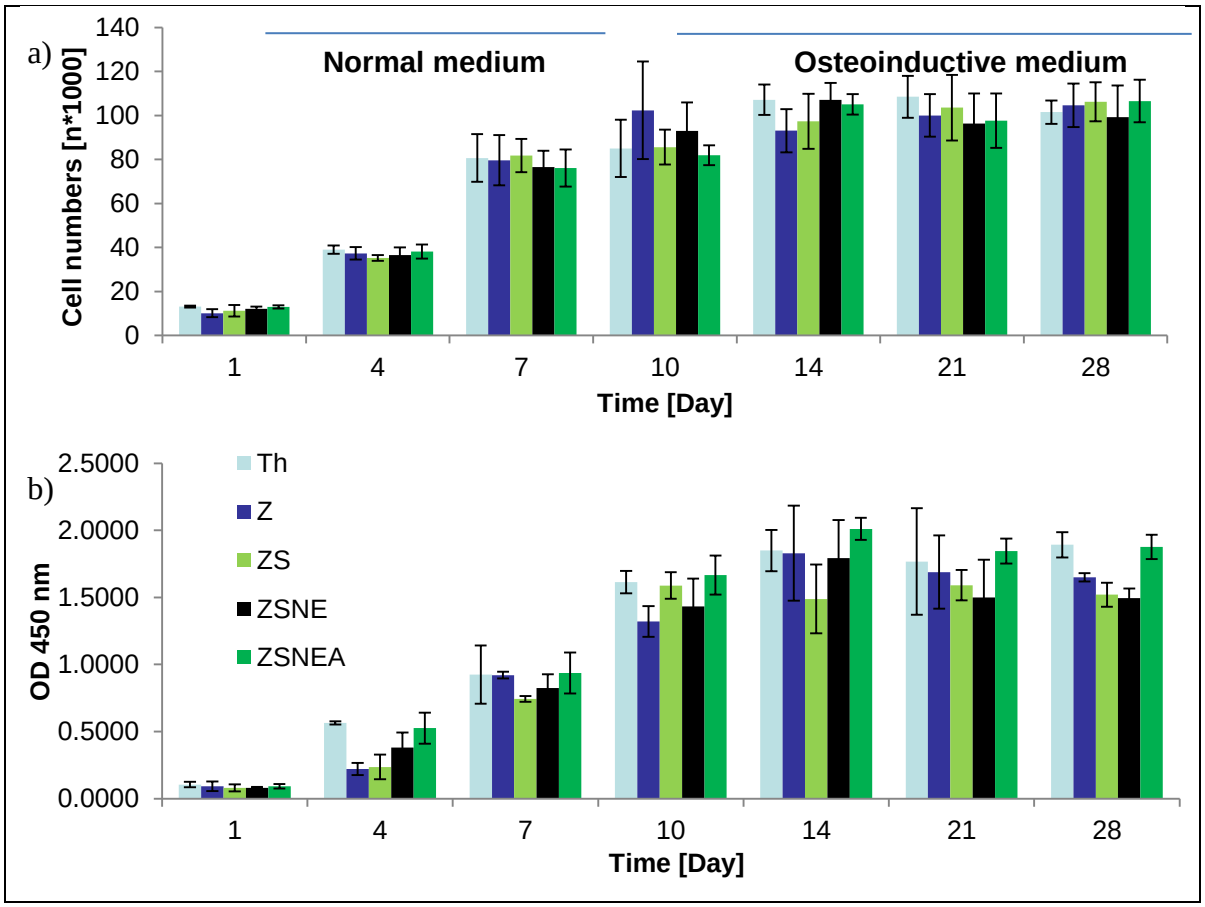

Figure 41. a) DNA assay indicating the HOBs number and b) WST-1 assay indicating the viability of the cells on all types samples (Z, ZS, ZSNE, ZSNEA) after 1, 4, 7, 10, 14, 21 and 28 days of culturing

Cell attachment

In order to have a preliminary overview about the effect of immobilized ALP on focal adhesion of cells on experimental surfaces, fluorescent microscope imaging was applied (figure 42).

Immunoreaction for vinculin was also found in the peripheral regions of cells on all four materials, vinculin-immunoreactive plaques in the cells on non-functionalized zirconia seemed to be fewer than those on ALP functionalized zirconia (figure 42).

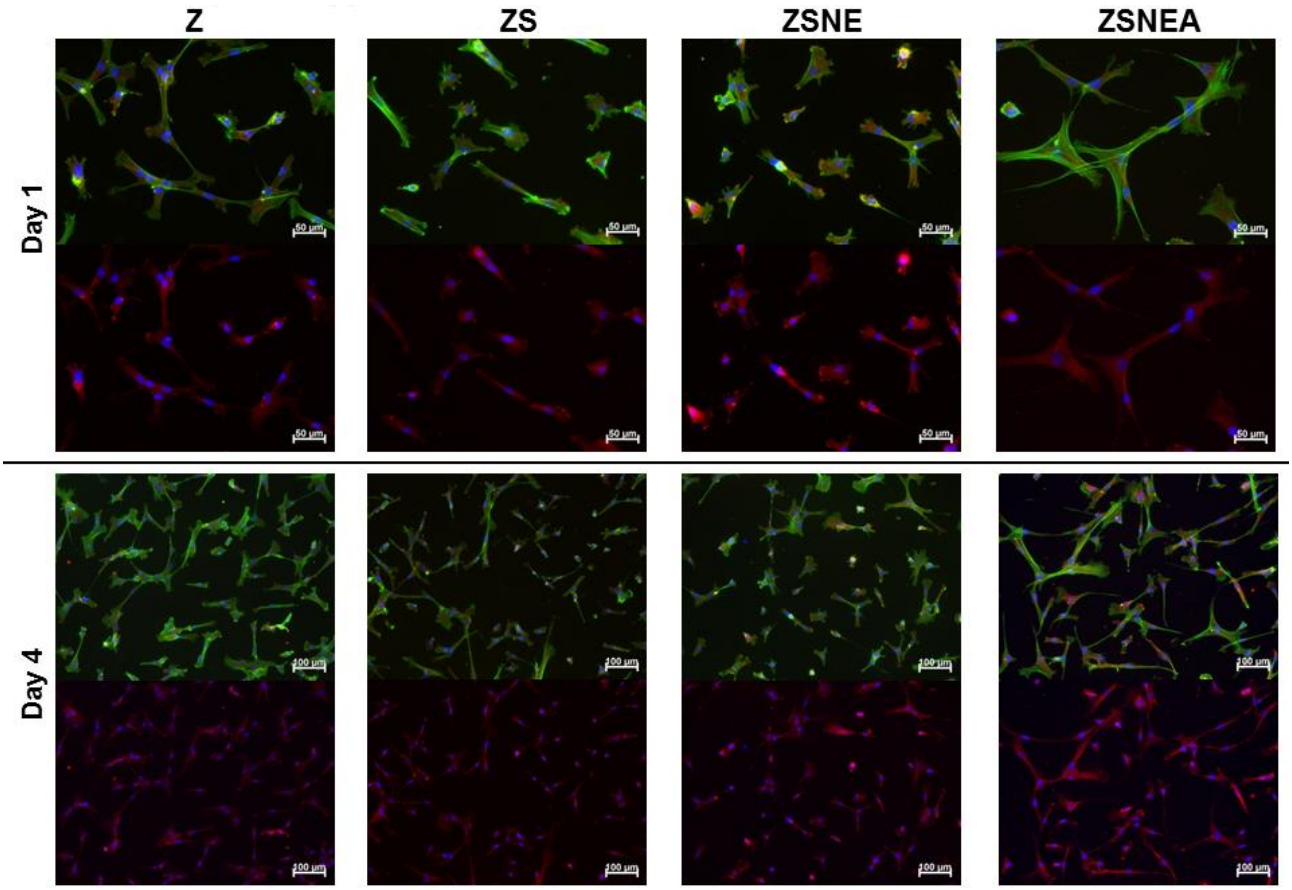

Figure 42. .Cell morphology and attachment (Blue: Nucleus, Green: Actin cytoskeleton, Red: vinculin)

Cell differentiation

Production of collagen type I and the activity of ALP are indicative for the early differentiation of osteoblast-like cells (Treccani, et al., 2013).

The result of the ALP activity measurements are depicted in figure 43 and the ALP activity on ALP functionalized zirconia is significantly higher than the other types of zirconia and the ALP activity increased till day 14 and afterward it seem to be constant untill day 28.

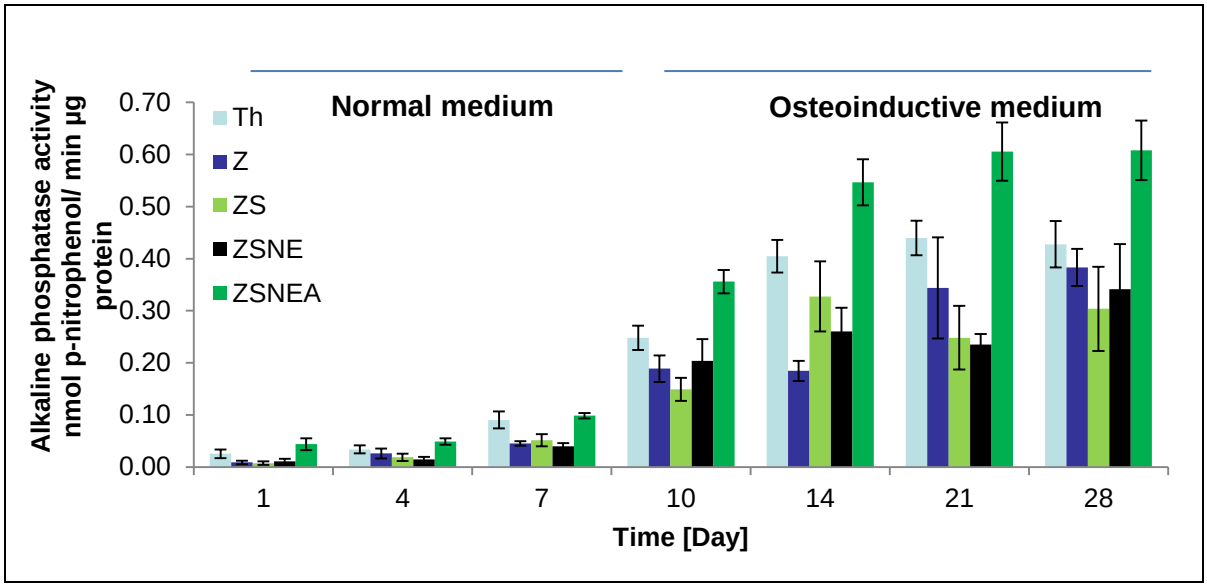

Figure 43. pNPP assay indicating the ALP activity of the cells on all types samples (Z, ZS, ZSNE, ZSNEA) after 1, 4, 7, 10, 14, 21 and 28 days of culturing

Collagen type I are stained and it can be detected on florescent micrographs in figure 44-46, the red color of images indicates the collagen type I and it seems on ALP functionalized zirconia the amount of the collagen type I is higher than the other types of samples.

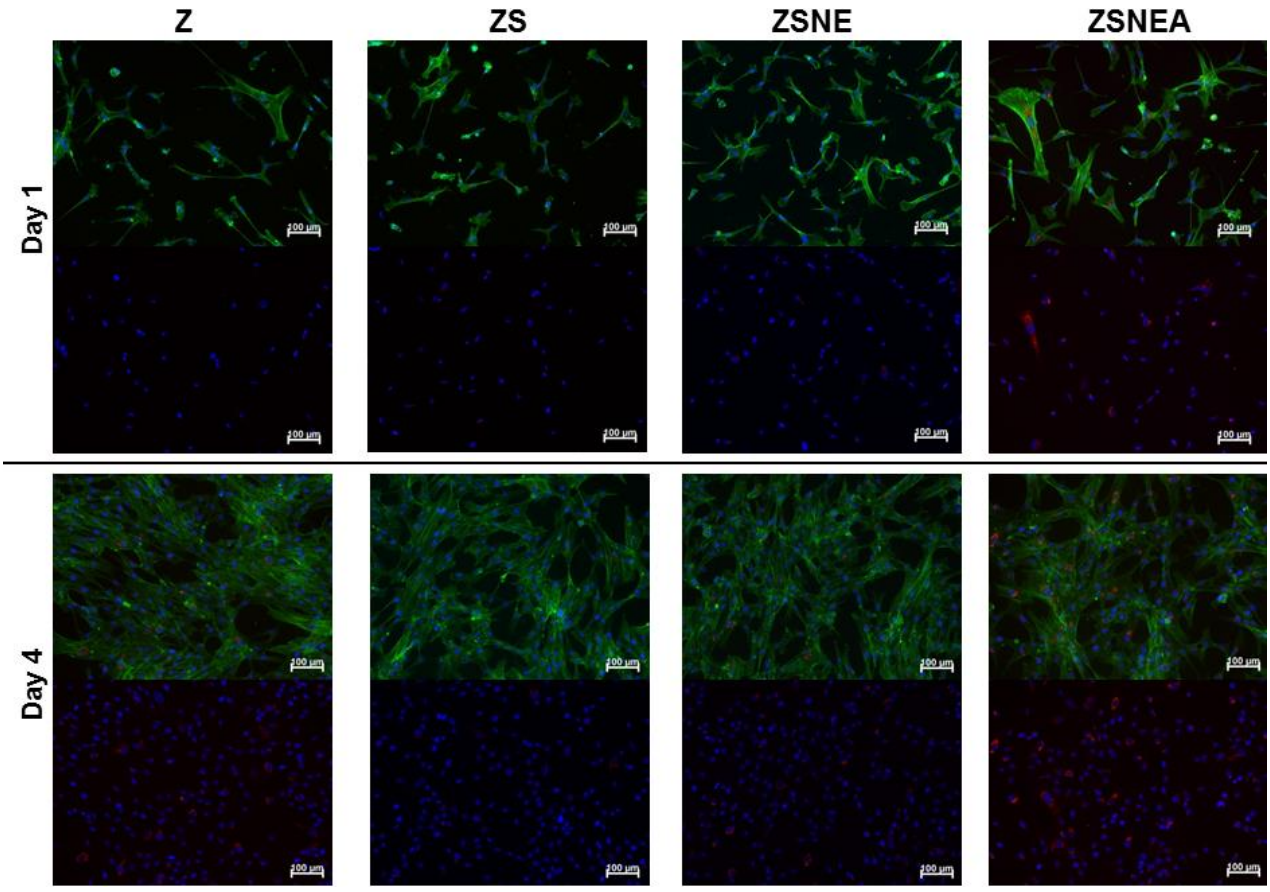

Figure 44. Confocal micrographs of experimental samples stained for collagen type I day 1-4(Red), cytoskeleton (Green) and nucleus (Blue) of cells

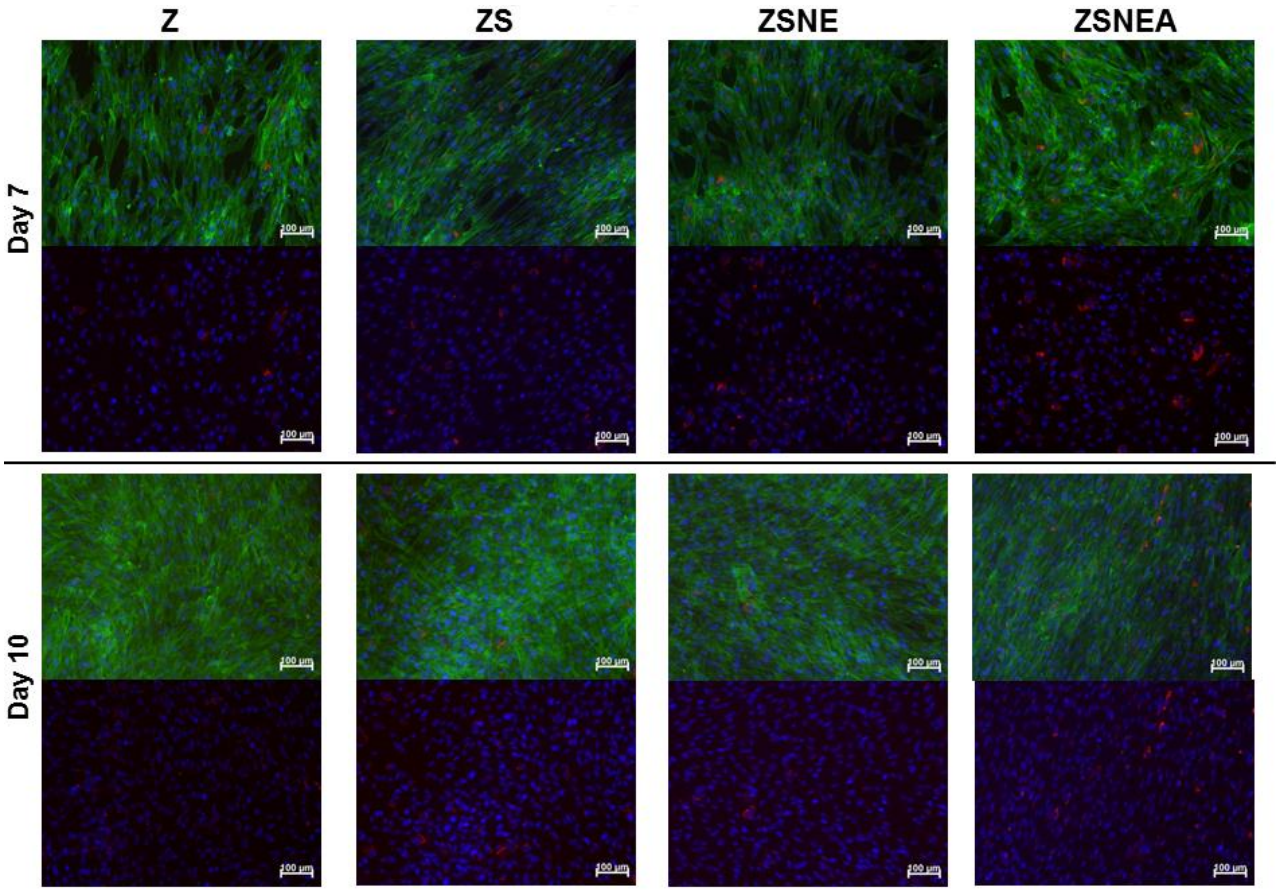

Figure 45. Confocal micrographs of experimental samples stained for collagen type I day 7-10 (Red), cytoskeleton (Green) and nucleus (Blue) of cells

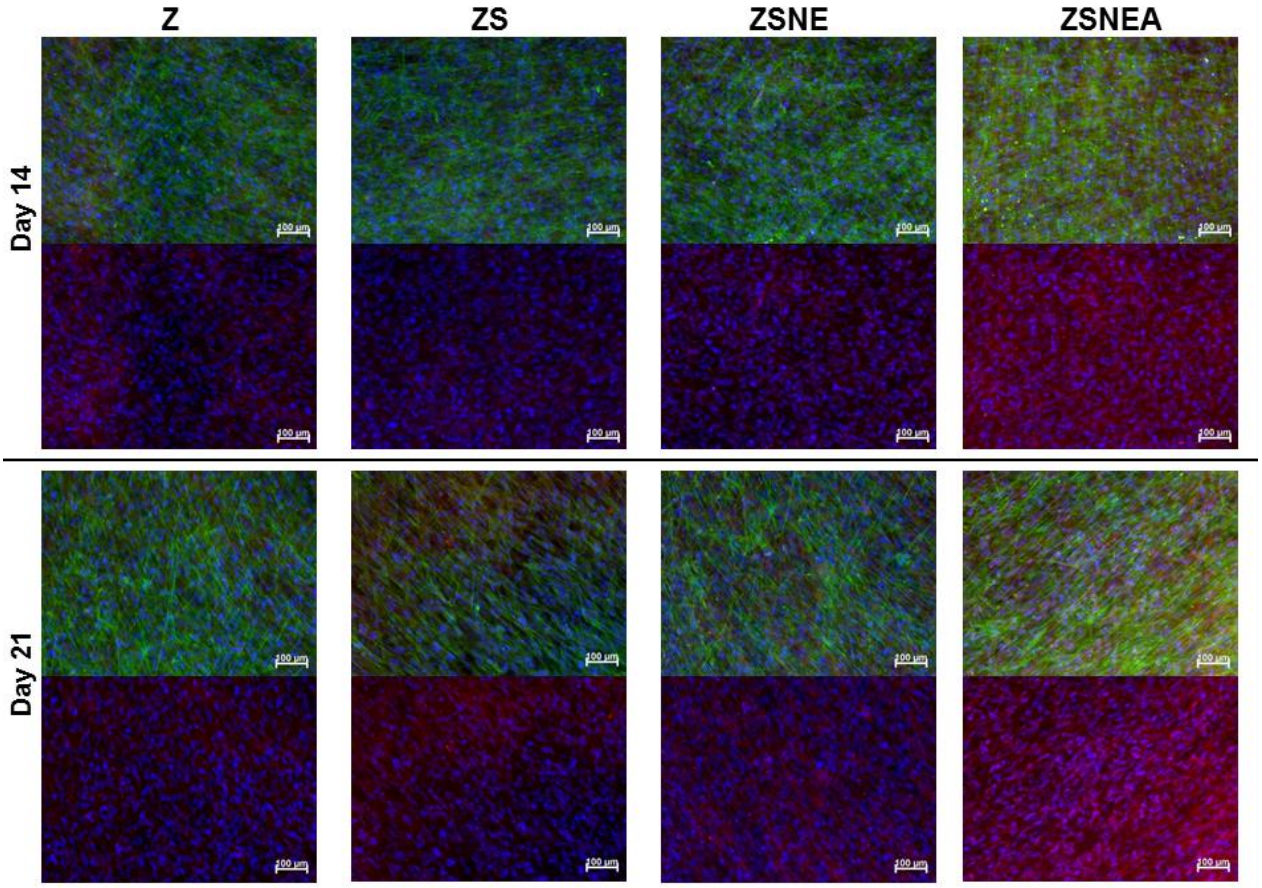

Figure 46. Confocal micrographs of experimental samples stained for collagen type I day 14-21(Red), cytoskeleton (Green) and nucleus (Blue) of cells

Cell mineralization

Calcium deposition or in the other words mineralization is the marker of late differentiation. In this research, the calcium deposition is visualized by SEM images and measured by calcium assay and Alizarin red staining methods.

SEM images of the experimental samples were illustrated in figure 40, as aforementioned the mineralized globules are observed on all types of samples after 14 days. Nevertheless, these mineralized globules are appeared sooner on ALP functionalized zirconia, and in summary, the amount of the CaP deposition, is also higher on ALP immobilized zirconia compared to the other sample types (figure 40). This visualization is quantified by alizarin red staining (figure 47). By alizarin red staining, calcium is detected from day 4 on all samples, even though, the ALP functionalized zirconia shows higher calcium phosphate content compared to rest of the samples.

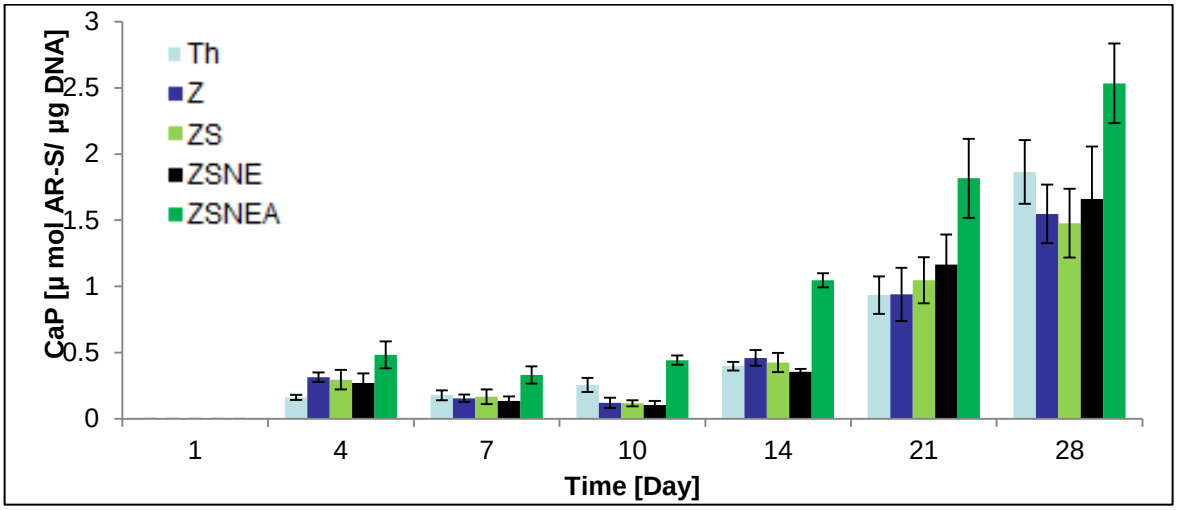

Figure 47. Alizarin assay indicating the biomineralization of the cells on all types samples (Z, ZS, ZSNE, ZSNEA) after 1, 4, 7, 10, 14, 21 and 28 days of culturing

Experimental samples are also scanned with scanning electron microscopy after cell detachment with trypsin and these micrographs are shown in figure 48. Chemical composition of theses mineralization was investigated using EDX, and the results affirmed, that this crystal part is CaP with the ratio of calcium to phosphor in the range of 1.54 to 1.76. The results are in coincident with the previous results and Calcium amount of these samples are calibrated with calcium assay (figure 50).

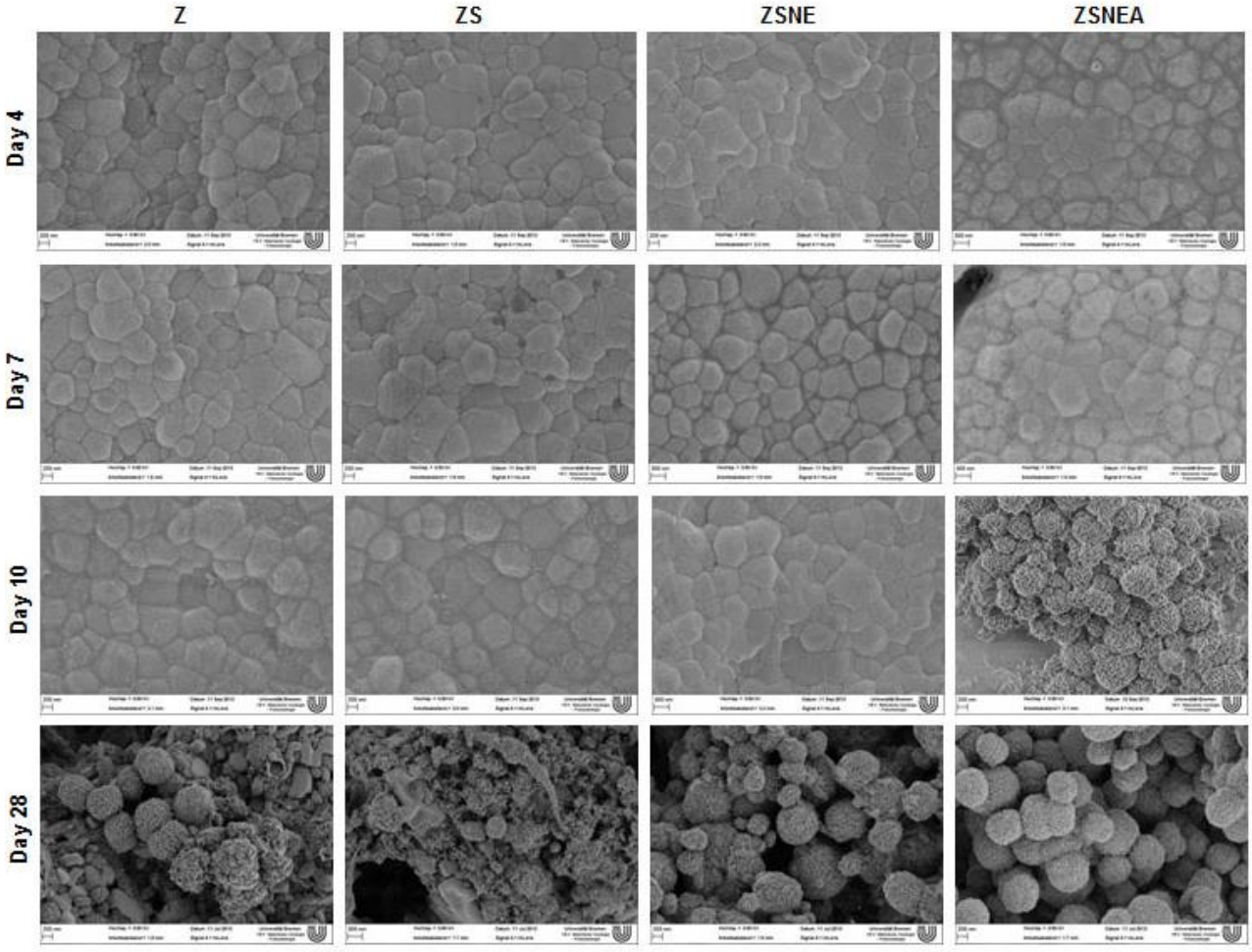

Figure 48. Calcium phosphate formation underneath the cells after cell removal (50×)

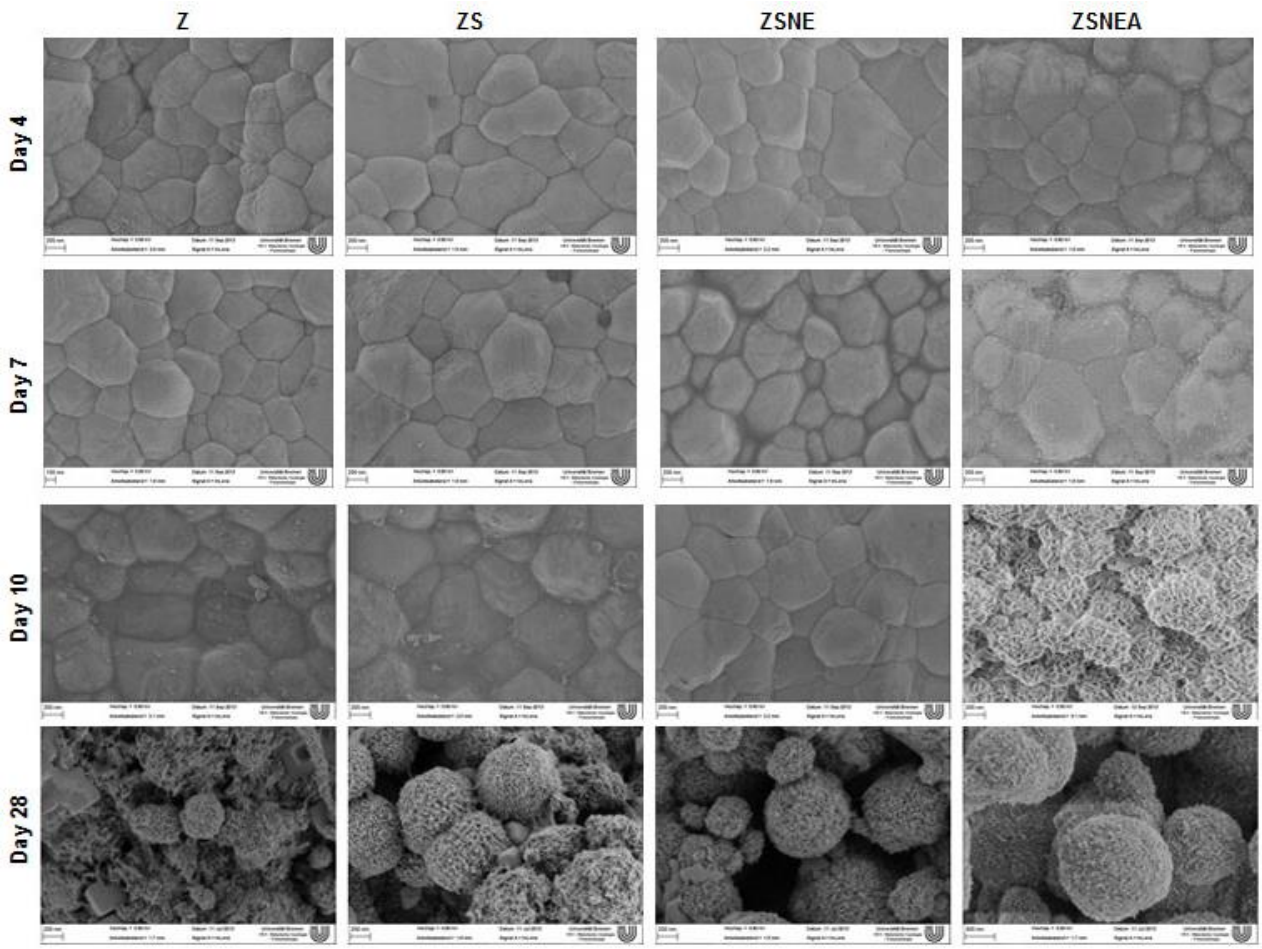

Figure 49. Calcium phosphate formation underneath the cells after cell removal (100×)

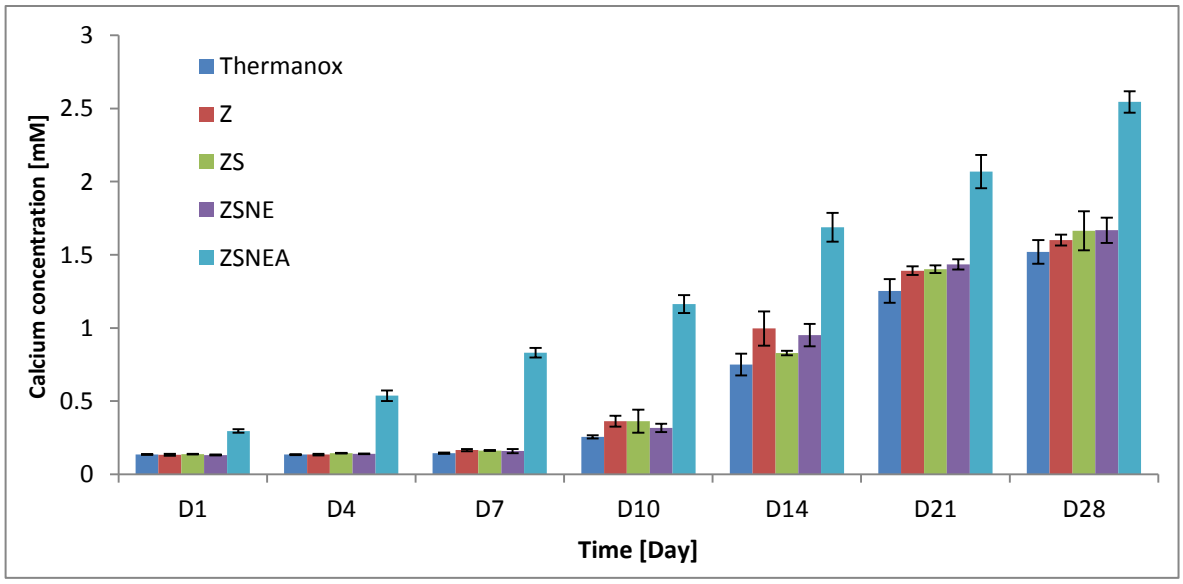

Figure 50. Calcium content of experimental substrate after 1, 4, 7, 10, 14, 21 and 28 days after cell removal

3.3. Conclusion

The present research tried to graft ALP enzyme on zirconia by adsorption and covalent bonding methods (using APTES as amino group source and EDC and NHS as linkers) to study the function of immobilized enzyme in cell culture medium with and without presence of human osteoblast cells.

In both adsorption and covalent bonding methods, Zirconia particles indicate higher potential in enzyme attachment on the surface compared to Alumina. Both autoclaved ceramic particles show better enzyme adsorption due to hydroxyl groups. Despite of higher enzyme activity and attachment in adsorption technique, the stability is low due to weak physical bonding. In contrast, covalent bonding method illustrates lower activity and attachment but constant stability during time. Silanized Zirconia particles exhibited suitable stability during 28 days aged in room temperature. Activity of the enzyme immobilized by covalent bonding method also investigated by two different buffers, in which 30 minutes show optimum reaction time. In summary, it is possible to conclude that the alkaline phosphatase is successfully grafted on Zirconia via covalent bonding method and it remains active after immobilization.

In vitro investigation confirmed functionalization process and the linkers; implicated in covalently bonding method is not toxic. In fact, zirconia is still biocompatible after this surface modification. This can be derived from the cell proliferation, morphology, and attachment on the surface of Zirconia. ALP functionalized zirconia presents the capability of biomineralization in vitro in acellular condition and investigation with osteoblast-like cells. In addition, Acceleration of cell metabolism was significantly observable on immobilized ALP Zirconia. The two stages of differentiation of the cells were successfully completed by production of ALP and collagen type I as the first, and biomineralization as the second stage.

This thesis illustrated the stability of silanized samples in room temperature in 28 days. For the future investigation, it is suggested to examine this stability in different storage conditions such as temperature and pH. This experiment will show the sensitivity of immobilized enzyme to different environments. Furthermore, the stability of the ALP activity after immobilization can be monitored during different storage time. By this investigation, the optimum storage time will be obtained which is vital information especially in industrial scale.

For the biological investigation, it is strongly recommended to study the initial cell adhesion on ALP functionalized and non-functionalized Zirconia by cell adhesion assays. These assays can demonstrate and approve the better initial cell adhesion on functionalized Zirconia. Finally, the in vitro investigations results in this thesis, strongly demands the future experiments in vivo analysis and research.

REFERENCES

Ahmed T CE6301:Theory of Water Treatment [Online] // http://www.scribd.com/. - 2013. - Jan 28, 2013. - http://www.scribd.com/doc/122569487/Adsorption.

Aitken A Natural History Museum [Online]. - 2012. - 10 11, 2012. - http://www.nhm.ac.uk/resources-rx/files/te-buffer_aug12-118648.pdf.

Alberts B [et al.] Molecular Biology of the Cell, fifth edition [Online]. - 2008. - 2013. - http://www.gopetsamerica.com/bio/enzyme_types.aspx.

Andre-Frei V [et al.] Acellular Mineral Deposition in Collagen-Based Biomaterials Incubated in Cell Culture Media [Journal] // Calcif Tissue Int. - 2000. - Vol. 66. - pp. 204–211.

Andriot M [et al.] Silicones in Industrial Applications [Book Section] // Inorganic Polymers / book auth. Jaeger R De and M Gleria. - [s.l.] : Nova Sciences, 2007.

Att W [et al.] Enhanced osteoblast function on ultraviolet light-treated zirconia [Journal] // Biomaterials. - Laboratory for Bone and Implant Sciences (LBIS), The Jane and Jerry Weintraub Center for Reconstructive Biotechnology, Division of Advanced Prosthodontics,Biomaterials and Hospital Dentistry, UCLA School of Dentistry : Elsevier, 2009. - 7 : Vol. 30. - pp. 1273–1280.

B D Ratner [et al.] A History of Biomaterials [Book Section] // Biomaterials Science, 3rd Edition. - [s.l.] : Elsevier, 2012.

Bacakova L [et al.] Cell Adhesion on Artificial Materials for Tissue Engineering [Journal] // Physiol Res. - 2004. - 1 : Vol. 53. - pp. S35-45.

Bandyopadhyay A and Bose S Characterization of Biomaterials [Book]. - [s.l.] : Elsevier, 2013. - pp. 1-9.

Berendsen A.D [et al.] Alkaline phosphatase-induced mineral deposition to anchor collagen fibrils to a solid surface [Journal] // Biomaterials. - 2007. - 24 : Vol. 28. - pp. 3530-6.

Berg J M, Tymoczko J L and Stryer L BIOCHEMISTRY. 6th ed [Book]. - New York : W. H. FREEMAN AND COMPANY, 2007.

Bernardo E [et al.] Porous wollastonite–hydroxyapatite bioceramics from a preceramic polymer micro-and- or nano-sized fillers [Journal] // Journal of the European Ceramic Society. - 2012. - 2 : Vol. 32. - pp. 399–408.

Bosque P J. [et al.] Prions in skeletal muscle [Journal]. - [s.l.] : Proc. Natl. Acad. Sci. USA, 2002. - Vol. 99. - pp. 3812-3817.

Carson F.L and Hladik C Histotechnology: A Self-Instructional Text 3rd Edition [Book]. - [s.l.] : American Society for Clinical Pathology, 2009. - http://library.med.utah.edu/WebPath/HISTHTML/MANUALS/ALIZARIN.PDF.

Chai Ch [et al.] Antibody immobilization on a nanoporous aluminum surface for immunosensor [Journal] // Applied Surface Science. - 2012. - Vol. 263. - pp. 195-20.

Coleman J E Structure and mechanism of alkaline phosphatase [Journal] // Annu Rev Biophys Biomol Struct. - 1992. - Vol. 21. - pp. 441-83.

Colln S Observation and Control of Immobilized [Report]. - [s.l.] : University of Bremen, Institute of Biophysics, 2007.

Córdobaa A, Magarioa I and Ferreira M L Experimental design and MM2–PM6 molecular modelling of hematin as a peroxidase-like catalyst in Alizarin Red S degradation [Journal] // Journal of Molecular Catalysis A: Chemical. - 2011. - Vol. 355. - pp. 44–60.

de Jonge L T. [et al.] Electrosprayed Enzyme Coatings as Bioinspired Alternatives to Bioceramic Coatings for Orthopedic and Oral Implants [Journal] // Advanced Functional Materials. - 2009. - 5 : Vol. 19. - pp. 755-762.

de Jonge L T. [et al.] In vitro responses to electrosprayed alkaline phosphatase/calcium phosphate composite coating [Journal] // Acta Biomater. - 2009. - 7 : Vol. 5. - pp. 2773-82.

de Jonge L T. [et al.] Organic–Inorganic Surface Modifications for Titanium Implant Surfaces [Journal]. - [s.l.] : Pharmaceutical Research, 2008. - 10 : Vol. 25.

Dr. T.Ahmed CE6301:Theory of Water Treatment [Online]. - http://www.scribd.com/doc/122569487/Adsorption.

Du R [et al.] Role of the Extracellular Signal-regulated Kinase 1/2 Pathway in Driving Tricalcium Silicate–induced Proliferation and Biomineralization of Human Dental Pulp Cells In Vitro [Journal] // Journal of Endodontics. - 2013. - 8 : Vol. 39. - pp. 1023–1029.

Epple M and Dorozhkin S.V Biological and Medical Significance of Calcium Phosphates [Journal] // Angewandte Chemie International Edition. - 2002. - 17 : Vol. 41. - pp. 3130-46.

Ferraris S [et al.] Surface modification of Ti-6Al-4 V alloy for biomineralization and specific biological response: part II, alkaline phosphatase grafting [Journal] // J Mater Sci Mater Med. - 2011. - 8 : Vol. 22. - pp. 1835-42.

Fischer H [et al.] Bioactivation of inert alumina ceramics by hydroxylation [Journal] // Biomaterials. - 2005. - 31 : Vol. 26. - pp. 6151-7.

Ghiaci M [et al.] Enzyme immobilization: Part 2 Immobilization of alkaline phosphatase on Na-bentonite and modified bentonite [Journal] // Applied Clay Science . - 2009. - 3-4 : Vol. 43. - pp. 308–316.

GK Toworfe;RJ Composto ;IM Shapiro;P Ducheyne . Nucleation and growth of calcium phosphate on aminecarboxyl- and hydroxyl-silane self-assembled monolayers [Journal] // Biomaterials. - 2006. - 4 : Vol. 27. - pp. 631-42.

Golub E E and Boesze-Battaglia K The role of alkaline phosphatase in mineralization [Journal] // Current Opinion in Orthopaedic. - [s.l.] : 01/2007; 18(5):444-448. DOI:10.1097/BCO.0b013e3282630851, 2007. - 5 : Vol. 18. - pp. 444-448.

Górecka E and Jastrzębska M Immobilization techniques and biopolymer carriers [Journal] // Biotechnology and Food Science. - 2011. - 1 : Vol. 75. - pp. 65-86.

Guisan J M [et al.] Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment [Journal] // Enzyme and Microbial Technology. - 2000. - 7 : Vol. 26. - pp. 509-515.

Guisan J.M Immobilization of enzymes and cells , second edition [Book]. - 2006.

Hermanson G.T Bioconjugate techniques (Second Edition) [Book]. - [s.l.] : Elsevier, 2008.

Hisbergues M, Vendeville S and Vendeville P Zirconia: Established Facts and Perspectives for a Biomaterial [Journal] // J Biomed Mater Res B Appl Biomater. - 2009. - 2 : Vol. 88. - pp. 519-29.

Hübsch C [et al.] Protection of yttria-stabilized zirconia for dental applications by oxidic PVD coating [Journal]. - [s.l.] : Acta Biomaterialia, 2014. - Vol. 14.

Hui S.B [et al.] Formation of Bone-like Apatite of Bioceramics Modified by Cold Plasma in SBF and Mechanism of Active Modification [Journal] // Key Engineering Materials. - 2007. - Vols. 280-283. - pp. 1575-1580.

Jan JT [et al.] Potential Dengue Virus-Triggered Apoptotic Pathway in Human Neuroblastoma Cells: Arachidonic Acid, Superoxide Anion, and Nf-Kappab Are Sequentially Involved [Journal]. - [s.l.] : JOURNAL OF VIROLOGY , 2000. - 18 : Vol. 74. - pp. 8680-8691.

Kim Ch-H The Effect of Surface Treatment of Titanium with Sand-Blasting/Acid-Etching or Hydroxyapatite-Coating and Application of Bone Morphogenetic Protein-2 on Attachment, Proliferation, and Differentiation of Stem Cells Derived from Buccal Fat Pad [Journal] // Tissue Engineering and Regenerative Medicine. - 2013. - 3 : Vol. 10. - pp. 115-121.

Kogan G [et al.] Effect of Carboxymethyl Chitin-Glucan on the Activity of Some Hydrolytic Enzymes in Maize Plants [Journal]. - [s.l.] : Chem. Pap, 2002. - 5 : Vol. 56. - pp. 326-329 .

Kregiel D Succinate Dehydrogenase of Saccharomyces cerevisiae – The Unique Enzyme of TCA Cycle –Current Knowledge and New Perspectives [Book Section] // Dehydrogenases / book auth. Canuto Rosa Angela. - [s.l.] : InTech, 2012.

Kroll S [et al.] Highly Efficient Enzyme-Functionalized Porous Zirconia Microtubes for Bacteria Filtration [Journal] // Environ. Sci. Technol. - 2012. - 16 : Vol. 46. - pp. 8739–8747.

Kunitz M Chicken Intestinal Alkaline Phosphatase [Journal]. - [s.l.] : J Gen Physiol., 1960. - 6 : Vol. 43.

Landoulsi J [et al.] Silanization with APTES for Controlling the Interactions Between Stainless Steel and Biocomponents Reality vs Expectation [Book Section] // Biomaterials - Physics and Chemistry. - [s.l.] : InTech, 2011.

Lee JT [et al.] Cell culture medium as an alternative to conventional simulated body fluid [Journal] // Acta Biomater. - 2011. - 6 : Vol. 7. - pp. 2615-22.

Lee K [et al.] Surface properties of submicrometer silica spheres modified with aminopropyltriethoxysilane and phenyltriethoxysilane [Journal] // Journal of Colloid and Interface Science . - 2006. - Vol. 304. - pp. 119–124.

Lee SY [et al.] Inorganic nanomaterial-based biocatalysts [Journal] // BMB reports. - 2011. - 2 : Vol. 44. - pp. 77-8.

Li D [et al.] Functionalization Strategies for Protease Immobilization on Magnetic Nanoparticles [Journal] // Advanced Functional Materials. - 2010. - 11 : Vol. 20. - pp. 1767–1777.

Lotfi M, Nejib M and Naceur M Cell Adhesion to Biomaterials: Concept of Biocompatibility [Book Section] // Advances in Biomaterials Science and Biomedical Applications. - [s.l.] : InTech, 2013.

Malvern Zeta Potential theory [Book Section] // Zetasizer Nano Series User Manual. - [s.l.] : Malvern Instruments Ltd, 2004.

Manicone P, Iommetti P and Raffaelli L An overview of zirconia ceramics: Basic properties [Journal] // journal of dentistry. - 2007. - Vol. 35. - pp. 819-826.

Marshall J. F and Davies D The Role of Integrin-Mediated Processes in the Biology of Metastasis [Book Section] // Cancer Metastasis, Molecular and Cellular Mechanisms and Clinical Intervention. - [s.l.] : Springer Netherlands, 2004.

Mateo C [et al.] Improvement of enzyme activity, stability and selectivity via immobilization techniques [Journal] // Enzyme and Microbial Technology. - 2007. - 6 : Vol. 40. - pp. 1451–1463.

Meder F [et al.] Protein adsorption on colloidal alumina particles functionalized with amino, carboxyl, sulfonate and phosphate groups [Journal] // Acta Biomaterialia. - 2012. - Vol. 8. - pp. 1221-1229.

Meenakshi A Cell Culture Media: A Review [Journal] // MATER METHODS. - 2013. - 3 : Vol. 175.

Meldrum FC Calcium carbonate in biomineralisation and biomimetic chemistry [Book Section] // International Materials Reviews, volume 48. - [s.l.] : Maney Publishing, 2003.

Mitzner E [et al.] Material Properties and In Vitro Biocompatibility of a Newly Developed Bone Cement [Journal] // Materials Research. - 2009. - 4 : Vol. 12. - pp. 447-454.

Mueller B, Rezwan K and Zacharias M Bovine Serum Albumin and Lysozyme Adsorption on Calcium Phosphate Particles [Journal] // Advanced Engineering Materials. - 2012. - 1-2 : Vol. 12. - pp. B53-B61.

Mueller W-D [et al.] Evaluation of the interface between bone and titanium surfaces being blasted by aluminium oxide or bioceramic particles [Journal] // Clin Oral Implants Re. - 2003. - 3 : Vol. 14. - pp. 349-56.

Nebe B [et al.] Improved initial osteoblast functions on amino-functionalized titanium surfaces [Journal] // Biomol Eng. - 2007. - 5 : Vol. 24. - pp. 447-54.

Nijhuis AWG [et al.] In vitro response to alkaline phosphatase coatings immobilized onto titanium implants using electrospray deposition or polydopamine-assisted deposition [Journal] // J Biomed Mater Res Part A. - 2013. - 4 : Vol. 102. - pp. 1102-9.

Oscar H SIDS Initial Assessment Report for SIAM 17 [Online]. - U.S. Environmental Protection Agency, 2003. - http://www.inchem.org/documents/sids/sids/919302.pdf.

Oshida Y Surface Modifications [Book Section] // Bioscience and Bioengineering of Titanium Materials. - [s.l.] : Elsevier, 2007.

Park J Bioceramics properties, characterizations and applications [Book]. - [s.l.] : Springer, 2008.

Parthibana S. P [et al.] High energy irradiation, a tool for enhacing the bioactivity of Hydroxyapatite [Journal] // Journal of the Ceramic Society of Japan. - 2008. - 1350 : Vol. 116. - pp. 320-324.

Piconi C and Maccauro G Zirconia as a ceramic biomaterial [Journal] // Biomaterials. - 1999. - Vol. 20. - pp. 1-25.

Pilathadka S, Vahalová D and TVosáhlo The Zirconia: a New Dental Ceramic Material. An Overview [Journal] // Prague Medical Report. - [s.l.] : Prague Med Rep., 2007. - 1 : Vol. 108. - pp. 5-12.

Rahmany MB and Dyke M V Biomimetic approaches to modulate cellular adhesion in biomaterials: A review [Journal] // Acta Biomater. - 2013. - 3 : Vol. 9. - pp. 5431-7.

Rebl H [et al.] Positively Charged Material Surfaces Generated by Plasma Polymerized Allylamine Enhance Vinculin Mobility in Vital Human Osteoblasts [Journal] // Advanced Engineering Materials. - 2010. - 8 : Vol. 12. - pp. B356–B364.

Reshmi R, Sanjay G and Sugunan S Enhanced activity and stability of a-amylase immobilized on alumina [Journal] // Catalysis Communications. - [s.l.] : Elsevier, 2006. - 7 : Vol. 7. - pp. 460–465.

Reyes C D [et al.] Biomolecular surface coating to enhance orthopaedic tissue healing and integration [Journal] // Biomaterials. - 2007. - 21 : Vol. 28. - pp. 3228-35.

Rusmini F, Zhong Zh and Feijen J Protein Immobilization Strategies for Protein Biochips [Journal] // Biomacromolecules. - 2007. - 6 : Vol. 8. - pp. 1775-1789.

Setzer B [et al.] The gene-expression and phenotypic response of hFOB 1.19 osteoblasts to surface-modified titanium and zirconia [Journal] // Biomaterials. - 2009. - 6 : Vol. 30. - pp. 979-90.

Sigma-Adrich http://www.sigmaaldrich.com [Online]. - Sigma-Aldrich Co., 2013. - http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase.html.

Simons B L [et al.] Covalent cross-linking of proteins without chemical reagents [Journal] // Protein Sci. - 2002. - 6 : Vol. 11. - pp. 1558-64.

Smith A.D Oxford dictionary of biochemistry and molecular biology [Book]. - [s.l.] : Oxford University press, 1997.

Snyder S.L, wilson I and bauer W [Journal]. - [s.l.] : Biochim. Biophys. Acta, 1972. - Vol. 258. - pp. 178-187. - http://www.piercenet.com/product/pnpp-substrates.

Somerman M J, Osathanon T and Giachelli CM Immobilization of alkaline phosphatase on microporous nanofibrous fibrin scaffolds for bone tissue engineering [Journal] // Biomaterials. - 2009. - 27 : Vol. 30. - pp. 4513–4521.

Spahn C and DMinteer Sh Enzyme Immobilization in Biotechnology [Journal] // Recent Patents on Engineering. - 2008. - Vol. 2. - pp. 195-200.

Taylor R H [et al.] Covalent protein immobilization on glass surfaces:Application to alkaline phosphatase [Journal] // Journal of Biotechnology. - 2005. - Vol. 118. - pp. 265–269.

Tischer W and Wedekind F Immobilized Enzymes: Methods and Applications [Book Section] // Biocatalysis: From Discovery to Application. - [s.l.] : Springer, 2000.

Tominaga H [et al.] A water-soluble tetrazolium salt useful for colorimetric cell viability assay [Journal] // Anal. Commun. - 1999. - Vol. 36. - pp. 47-50.

TOSOH http://www.tosoh.com [Online]. - TOSOH Corporation, 2013. - http://www.tosoh.com/our-products/advanced-materials/zirconia-powders.

Treccani L [et al.] Functionalized ceramics for biomedical, biotechnological and environmental applications [Journal] // Acta Biomater. - 2013. - 7 : Vol. 9. - pp. 7115-50.

US Biological life science usbio [Online]. - US Biological life science, 2013. - http://www.usbio.net/item/P4071-02.

Verne E [et al.] Alkaline phosphatase grafting on bioactive glasses and glass ceramics [Journal] // Acta Biomaterialia . - 2010. - Vol. 6. - pp. 229–240.

Verne E [et al.] Effects of sterilization and storage on the properties of ALP-grafted biomaterials for prosthetic and bone tissue engineering applications [Journal] // Biomed. Mater. 7 (2012) 054102 (13pp). - 2012. - Vol. 7. - p. 054102 (13pp).

Volpato C.Â M [et al.] Application of Zirconia in Dentistry: Biological, Mechanical and Optical Considerations [Book Section] // Advances in Ceramics - Electric and Magnetic Ceramics, Bioceramics, Ceramics and Environment. - [s.l.] : InTech, 2011.

Wolfson Double-Do Protein Cross-Linkers Handbook & Selection Guide [Online]. - 2013. - 2013. - http://www.docstoc.com/docs/155589378/Protein-Cross-linkers-handbook-and-selection-guide-The-Wolfson.

Worthington biochemical Co worthington-biochem [Online]. - worthington biochemical Co, 2013. - http://www.worthington-biochem.com/bap/default.html.

Zhou W [et al.] The performance of bone marrow mesenchymal stem cell – Implant complexes [Journal] // Biomaterials. - 2010. - 12 : Vol. 31. - pp. 3212-21.

Zhou Y [et al.] Immobilized β-glucosidase onto magnetic nanoparticles and evaluation of the enzymatic properties [Journal] // BioResources. - 2013. - 2 : Vol. 8. - pp. 2605-2619.

Zreiqat H, Evans P and Howlett CR Effect of surface chemical modification of bioceramic on phenotype of human bone-derived cells [Journal] // J Biomed Mater Res. - 1999. - 4 : Vol. 44. - pp. 389-96.

SUPPLEMENTARY DATA

The effect of acid and base on functionalized Zirconia

The stability and activity of immobilized Enzyme on sample's surface are the two basic factors, which should have been considered. In this experiment, surface area and new IEP of the silanized and immobilized powder analyzed to investigate the effect of acid and base. The acid and base had been added to samples during the previous zeta potential measurement.

Freeze-drying technique is applied to separate the solid powder from the suspension. The wet powders were in freeze-dryer for three days (72 hr), eventually their zeta potential were evaluated by zeta potential device (DT1200 Dispersion Technologies, NY,USA) to obtain new IEP. The specific surface area of these powders was measured by BET device (Bel Japan Inc, Osaka, Japan) using nitrogen adsorption technique. The results are presented in figure 51- 52.

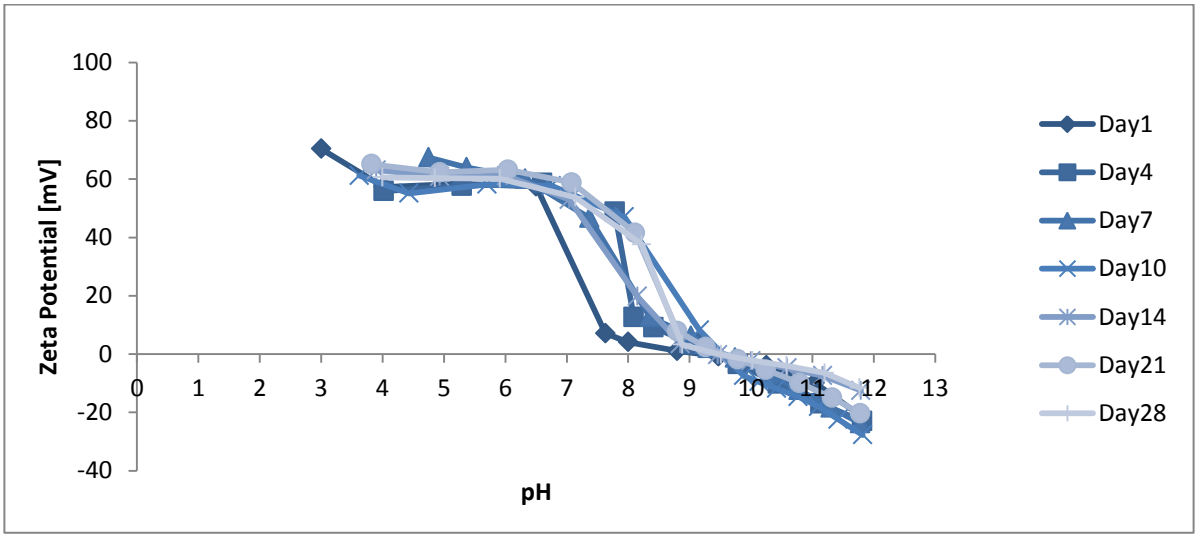

Figure 51. Zeta Potential of silanized particles after acid and base effect in seven time point

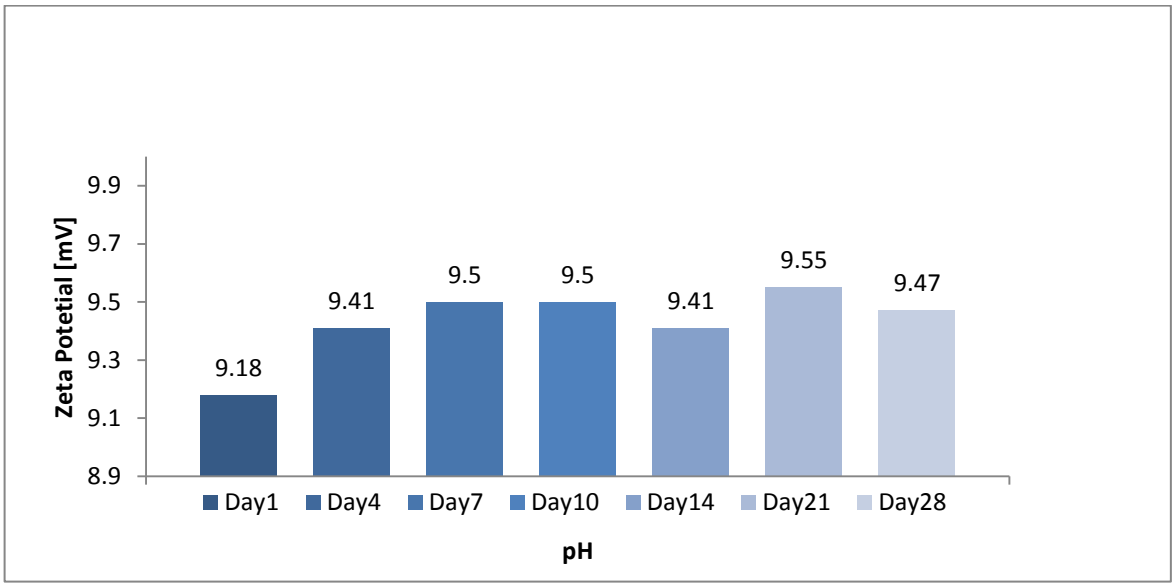

Figure 52. IEP point of silanized particles after acid and base effect in seven time point

The zeta potential curves of before and after acid and base treatments were compared. As it is clearly shown in figure 53, the IEP decreased after acid and base treatment, by one unite. It can be concluded that, the silanized zirconia is not stable in acidic and basic environment.

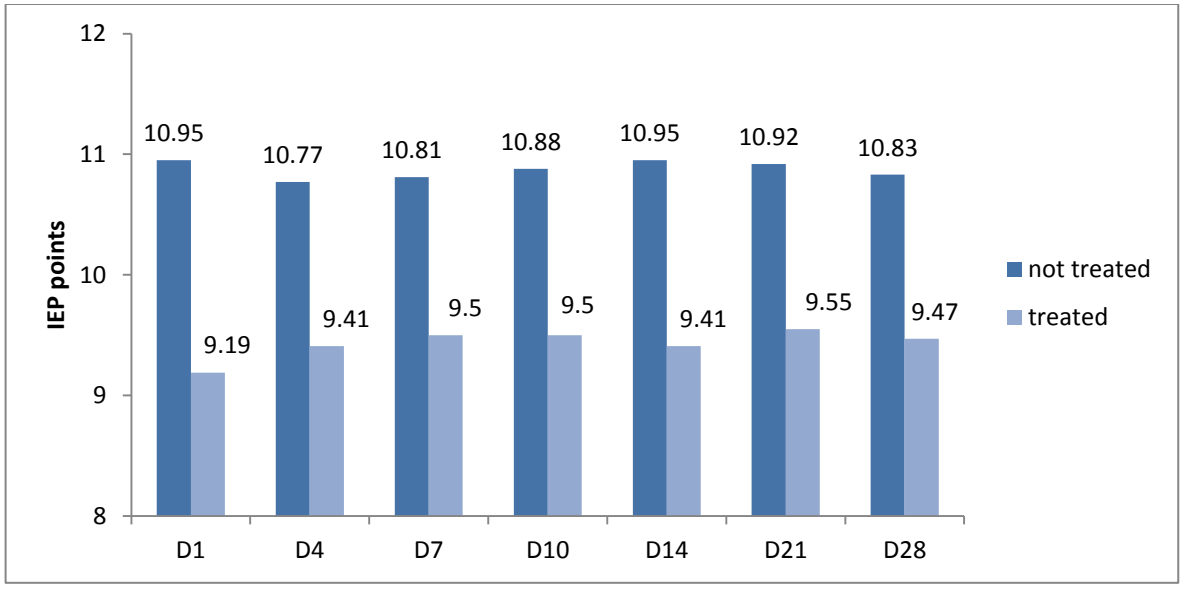

Figure 53. IEP points comparison between not treated and treated Zirconia with acid and base in seven day point

The zirconia powders which were used in functionalization by covalent bonding technique, also freeze-dried and new IEP investigated by new zeta potential to study the effect of acid and base on immobilization. The results are presented in figure 54-55.

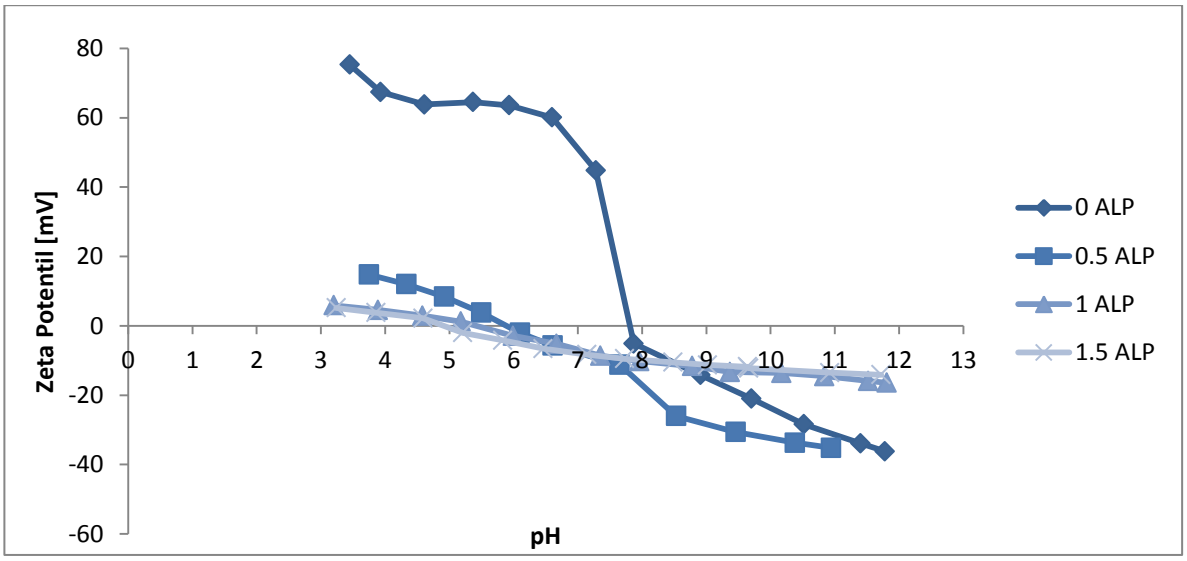

Figure 54. acid and base effect on functionalized Zirconia

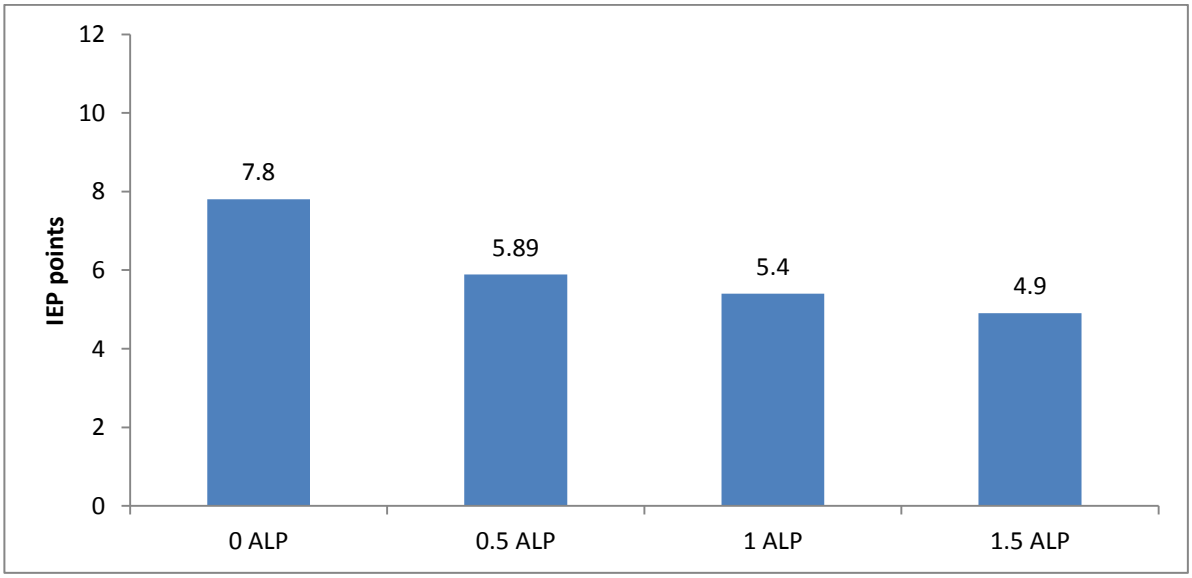

Figure 55. Effect of acid and base on IEP of functionalized Zirconia

Figure 56 demonstrates the comparison between not treated and treated functionalized Zirconia. 1 and 1.5 ALP monolayer immobilized on Zirconia shows better stability.

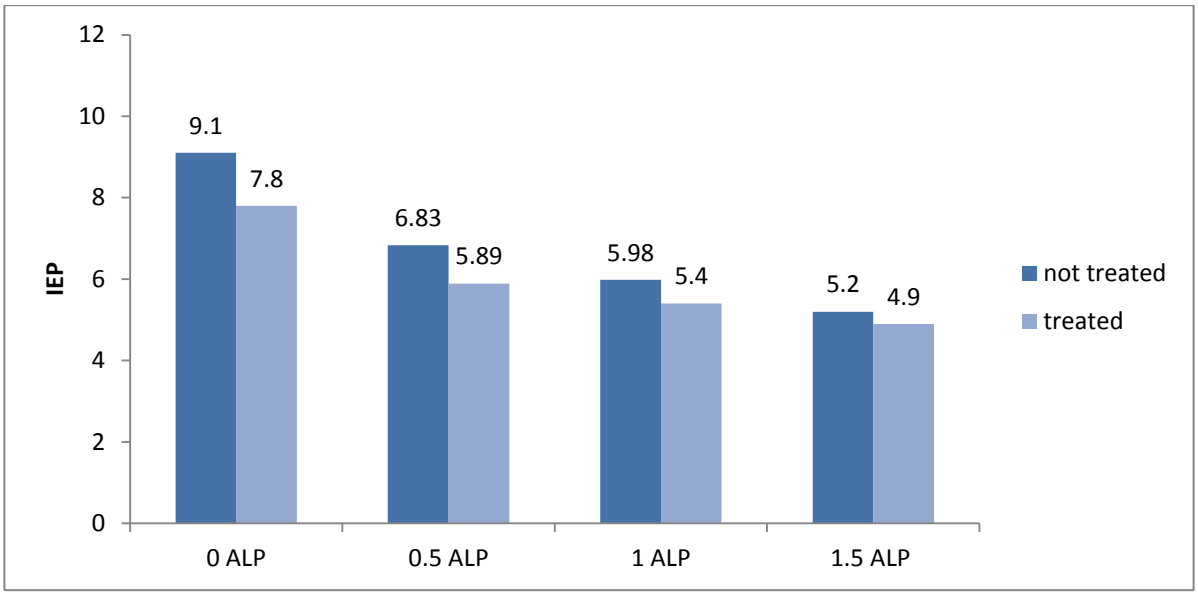

Figure 56. Comparison between not treated and treated functionalized Zirconia

The IEP changes may be due to the high agglomeration in this stage, which may influence the ALP immobilization. In general, Zirconia does not show specific stability when it is exposed to acid and base.

Table 11 demonstrates the effect of acid and base on surface area of Alumina and Zirconia. It can be concluded that there is no significant changes in surface area in both ceramics.

To acquire the better and more precise data, it is recommended to have a specific amount of acid and base in studying stability of functionalization. This may help to obtain the proper results in investigating the factor of pH.

Table 11. Surface area and total pore volume comparison between not treated and treated alumina and Zirconia with acid and base

	NOT treated surface area [$m^2 g^{-1}$]	Treated surface area (mean) [$m^2 g^{-1}$]	Total pore volume [$cm^3 g^{-1}$]	Total volume pore mean [$cm^3 g^{-1}$]
A	11.648	12.1005	0.0084403	0.0087854
AS	12.344	11.9075	0.008569	0.0086074
ASNE	11.806	11.754	0.0083161	0.0084058
ASNEA0.5	12.172	12.504	0.0086044	0.00897725
ASNEA1	10.797	12.352	0.0077712	0.0087585
ASNEA1.5	12.552	11.709	0.0086507	0.0082736
Z	6.8017	6.0122	0.0048037	0.00416415
ZS	6.2151	6.36565	0.0043118	0.00455225
ZSNE	6.8655	5.96695	0.005049	0.004131
ZSNEA0.5	6.7954	6.74615	0.0047959	0.00505235
ZSNEA1	6.004	6.28315	0.0042801	0.00446315
ZSNEA1.5	6.5326	6.3134	0.0044798	0.0044388