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School of Industrial Process Engineering
Faculty of Materials and Chemical Engineering 'G. Natta'
Department of Materials Engineering

BIOMOLECULES GRAFTING ON TITANIUM SURFACES BY SOL-GEL TECHNIQUE

Supervisors : Prof. Roberto Chiesa
Ing. Lina Altomare
Ing. Daniele Pezzoli

Pınar Sakoğlu
752813

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Anneannem Hidayet Bala'ya ithafen,

Dedicated to my grandmother, Hidayet Bala,

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ABSTRACT

The impact of the biomaterials on the industry and people lives is of great significance. They present a unique opportunity to improve life quality of humanity through various applications. Biomaterials can be synthetic or natural materials which can be used in medical devices, in contact with biological systems, or intended to react with them.

The most important subjects for biomaterials are: toxicology, biocompatibility, healing, mechanical and performance requirements. Surface modification of the biomaterials is profitable to promote the interaction between biomaterial and host. The goal of this work is biomaterial surface functionalization to retain a material's bulk properties while modifying only its surface to possess desired recognition and specificity. Physical applications or chemical conjugations are some methods that can be applied on the surface for the immobilization of bioactive molecules, such as cell adhesive peptides, DNA, anticoagulant reagents and growth factors. For the chemical conjugation; modification techniques are silanization, sol-gel, plasma gas discaharge, vapor depositions, UV light, photoinitiated polymerization and ion beam etching. These techniques are used to provide reactive groups to the material surface for covalent immobilization on biomolecules. In this work sol-gel method had been performed.

Even though many other prospects have been investigated as methods for attaching the biomolecules on the surface of the material; sol-gel method is one of the most promising solutions. In this study, conjugation of the biomolecules on the titanium surface with sol-gel technique has been experimentally practiced, in order to improve surface characteristics of the materials for bio-medical applications. Solutions had been prepared in various chemical compositions, in order to determine the proper composition for better surface coating. Finally, characterizations of the solutions and substrate surfaces have been performed.

Keywords: Sol-gel; biomaterials; titanium; surface modifications; peptides; biomolecules, grafting

ITALIAN ABSTRACT

L'impatto dei biomateriali sull'industria e sulla vita delle persone è di grande rilievo. Essi rappresentano un'opportunità unica per migliorare la qualità della vita umana attraverso varie applicazioni. I biomateriali sono materiali sintetici o naturali che vengono utilizzati in dispositivi medici, in contatto con sistemi biologici o destinato a reagire con loro.

Gli argomenti più importanti per i biomateriali sono: la tossicologia, la biocompatibilità, la guarigione, i requisiti meccanici e prestazionali. La modifica della superficie dei biomateriali è utile a promuovere un legame tra ossa e pelle in contatto con l'impianto. La modificazione della superficie di un biomateriale con biomolecole ha lo scopo di mantenere le proprietà di bulk del materiale modificandone solo la superficie in modo tale da possedere il riconoscimento e la specificità desiderata. Applicazioni fisiche o coniugazioni chimiche sono alcuni metodi che possono essere utilizzate sulla superficie per l'immobilizzazione delle molecole bioattive, quali peptidi adesivi cellulari, DNA, reagenti anticoagulanti e fattori di crescita. Per la coniugazione chimica le tecniche di modificazione di superfici di ossidi metallici sono: silanizzazione, plasma discaharge gas, deposizioni da vapore, luce UV, polimerizzazione foto iniziata e ion etching beam. Queste tecniche sono utilizzate per fornire gruppi reattivi alla superficie del materiale per l'immobilizzazione covalente su biomolecole. In questo studio, era stata eseguita il metodo solo sol-gel.

Anche se molte altre prospettive sono state studiate come metodi per il fissaggio delle biomolecole sulla superficie del materiale; il metodo sol-gel è una delle soluzioni più promettenti. In questo studio, la coniugazione delle biomolecole sulla superficie di titanio con tecnica sol-gel è stata sperimentalmente praticata al fine di migliorare le caratteristiche delle superfici dei materiali per applicazioni biomediche. Il sol è stato preparato con composizioni chimiche diverse, per determinarne la corretta composizione per un rivestimento migliore. Infine, caratterizzazioni di superficie sono state eseguite sui campioni.

Parole chiave: Sol-gel, biomateriali, titanio, modifica di superfici, peptidi, biomolecole, grafting

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PINAR SAKOGLU

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1. INTRODUCTION

Life quality of human being is greatly related to the health of the human body. Because of this reason scientific researches on biomaterials have a great importance of human life due to their necessity of the body. These researches on biomaterials area are contributed to the implants and biomedical devices that are essential in most effective healing techniques. In recent years, the application of the biomaterials had increased due to the improvement of researches and applications.

Materials engineering involves various classes of materials with a different properties such as metals, ceramics, polymers, glass, composite materials, nanomaterials, thin films, refractory materials and biomaterials. Biomaterials area is one of the main research areas in materials engineering. The percentage of aging population is increasing in worldwide especially for the developing countries, because of this reason number of the people with physical disability is rising. This augmentation in the aging population will increase the demand for the biomaterials. If we take into account of the economical point of view, the global market for biomaterials was $25.6 billion in 2008 and it is expected to reach $64.7 billion in 2015. Moreover, biomaterial products are expensive products also due to all researches and improvements their price is getting higher. With this increase in healthcare budget, the demand for the implants also increases, which is directly affects the demand of the biomaterials. The dominate materials in biomaterials area are metals and ceramics [1, 2].

The replacement for natural bones and joints contributed 31.8% to the global orthopedic biomaterials field. This field can be categorizing in artificial hip, artificial knee and reconstructive products [2]. Over 300 000 hip and knee implants and between 100 000 and 300 000 dental implants are used each year in the United States to replace or restore function to diseased and damaged tissues [3].

1.1. Introduction to Biomaterials

Biomaterials are in use for supporting the living tissues or take place of living tissues. They are synthetic or natural materials which are used in medical devices or in contact with biological systems or intended to react with them [4, 5]. Biomaterials field is a growing field and has been extremely developed over the last fifty years and compasses aspects of medicine, biology, chemistry, and materials science. Joint replacements, bone plates, bone cement, artificial ligaments and tendons,

dental implants for tooth fixation, blood vessel prostheses, heart valves, artificial tissue, contact lenses, and breast implants are some applications of the biomaterials [6]. In brief, biomaterials are used to improve the biological systems and the biomedical devices in order to replace the organs and tissues which are not functional 100%. They are one of the multidisciplinary subjects which are in the intersections of biology and materials engineering area.

The impact of the biomaterials to people life quality and to industry is great, so it is an exciting area for researching and application for medical devices. Furthermore, they are also used to grow cells in culture, to assay for blood proteins in laboratory and processing biomolecules in biotechnology etc. All of these applications have interaction between the biomaterials and the biological systems [7, 8].

Even if the biomaterials topic can be considered new as a scientific research topics, application of them has a very long history. The first known utilization is in Egyptian mummies. The gold has been started to use by dental implants since 2000 years. The application of the bone implants which are made by bronze and copper is before Christ. Although copper has a toxic effect on body, until in the middle of the 19^{th} century it had been used as an implant material. From 19^{th} century the usage of the biomaterials improves. For example in 1880 they started to use ivory for the implants. The first metal prosthesis had done in 1938 with vanadium. Those kinds of prosthesis are used until 60ties and when they corrode in the host, they caused vital problems. In 1972 aluminum and zirconium started to be used without causing any biocompatibility problem, but since they are inert and they could not hold the tissue, mechanically they failed. This problem solved with hydroxyapatite and bioglasses. After that, in 50ies artificial heart valves and in 60ies hip joints had been applied in human body. Furthermore, in 1937 the high molecular weight polyethylene (HMWPE) used as a biomaterial for the first time. Last 30 years, more than 40 different types of materials used in order to repair and support human body. By 40ies titanium started to be used in biomedical applications [7, 8].

Currently biomaterials are in use for several medical applications such as various kinds of implants and joints, vascular grafts, aortic grafts, arterial, venous, or vascular tubing, stents, catheters, diagnostic and monitoring catheters and sensors, biosensors,dialysis membranes, tubing, or connectors, blood oxygenator tubing or membranes, ultrafiltration membranes, intra-aortic balloons, blood bags, sutures, soft or hard tissue prostheses, synthetic prostheses, prosthetic heart valves, tissue adhesives, cardiac pacemaker leads, artificial organs, endotracheal tubes, lenses for the eye such as contact or intraocular lenses, blood handling equipment, and drug delivery systems.

When a biomaterial inserted in a host, integration between the host and biomaterial is critical. Furthermore, some biomaterials such as implants has been in use for a long time in order to replace damaged tissues or improve the body functions [1]. Because of those reasons some subjects are essentially important for biomaterials such as, toxicology, biocompatibility, functional tissue structure and pathobiology, dependence of specific anatomical sites of implantation, healing, mechanical and performance requirements, industrial involvement, and regulations [7, 9].

1.2. Surface Modification of Biomaterials

Surface modification of the biomaterials is useful to promote an interaction between biomaterial and host in contact locations [10]. Osteointegration of biomaterials is a biological process that occurs when the new implant-bone forms in direct contact with implant surface. This process is osteoconductive and it is known that with various modifications of the implant surface the interaction between bone and implant can enhance in terms of intensity and speed of bone formation.

Physical applications or chemical conjugations are some methods that can be applied on the surface for the immobilization of bioactive molecules, some of them are; plasma treatment and deposition, radiation grafting, chemical reaction of the surface, ozonolysis, photoreaction, ion implantation, ion etching, solvent cast films, surface active modifiers (low and high MW), metallization, self assembly, micro-contact printing, and immobilization of biomolecules [7]. Recently most used surface modification technique for bulk materials is chemical modifications [11, 12].

On account of all these facts, the main aim of the scientific researches in these years is to improve the interaction between biomaterials and host body. The aim in biomaterial surface modification with biomolecules is to retain a material's bulk properties while modifying only its surface to possess desired recognition and specificity [13]. Functional coatings on biomaterials enhance endothelial cell adhesion and activity with a shortening of the wound healing time [14]. Chemically modification of the biomaterial surface with biomolecules can be performed by covalent coupling or self assembly techniques [15].

1.3. Surface modification of titanium and its alloys

The demand for hard tissue replacements, such as artificial hip joints and dental implants is increasing. These hard tissue implants require a material that combines mechanical strength and biocompatibility. Metals are commonly used as orthopedic and dental biomaterials due to their comparable specific strength to bone. Among the metals, titanium and its alloys are considered to have low density high strength, relatively low Young's modulus, and high corrosion resistance while relatively more compatible than the other metals [16]. Nevertheless, titanium and its alloys are considered bioinert and typically do not allow direct bonding with the bone tissue [17, 18]. The ability for the implant to integrate with the bone is important for preventing implant failure. Osteointegration can be possible with biomechanical bonding and biochemical bonding. Biomechanical bonding describes the affinity for bone growth into the implant and causes mechanical stability. This interaction, however can take

up to 1-2 years to reach maximum stability. Biochemical bonding between the implant and bone, on the other hand, represents a more immediate stabilizing reaction, as well as additional osteogenesis promotion. Performance of the titanium can be improved by surface modification with biomolecules grafting. Surface treatments to titanium surfaces increase protein adsorption and cell attachment, thereby increasing the affinity for a surface to bind [16, 19].

1.4. Introduction to Biomolecules

Biomolecules are polymers of small subunits and their atomic structures are known from their chemistry. Physics and chemistry researches can provide particular insight into the structure of the atoms and their arrangements in chemical structures, recently the focus is set on understanding the structure and function of biomolecules, essentially nucleic acids and proteins [20].

The main approaches for immobilizing biomolecules on the surface are surface adsorption, physical entrapment, ligand/receptor specific binding, and covalent attachment [21]. Biomolecules immobilizing on various materials surface is a prerequisite for developing more functionalize surface, which can be of significant use in medical, military, agricultural, and environmental monitoring, in bio-processing industries, and in biomedical application. Surface modification and biomolecules coupling procedures are easily accomplished: reactions can proceed in air, and most take place under ambient conditions. The biomolecules-modified biomaterials surfaces are stable under physiological conditions, are selective for adhesion of specific cells types, and are reusable [22].

Immobilization of the biomolecules can perform physically or chemically with covalent bonding. For the covalent immobilization of biomolecules on surface of biomaterial, the surface has been functionalized by various methods such as a surface grafting with a functional monomer initiated by UV radiation, use of chemical initiator, plasma treatment and high energy radiation [21].

A very important point in the preparation of substrates for immobilizing the biomolecules is uniformity of substrate surface. It is essential to provide uniform functionality over an extended area of the substrate surface [23]. Due to this fact, sample preparation process is very important.

Biomolecules can attach to the surface with various techniques. Surface modification techniques of surfaces are silanization, plasma gas discaharge, vapor depositions, UV light, photoinitiated polymerization and ion beam etching. Applications of these techniques are used to provide reactive groups to the material surface for covalent immobilization on biomolecules such as -SH, - COOH, - NH2, - OH [24].

Semiconductors such as silisium modified with surface-bound capture molecules have enormous potential for use in biomedical application. In this process, a film of an organo-phosphonic acid is bonded to the native or synthesized oxide-coated Ti surface as a film of the corresponding silicate. The

silicate film is functionalized to enable covalently coupling biological molecules, ranging in size from small peptides to large multi-subunit proteins, to the Ti surface [22].

The use of cross linker molecules, which may or may not be biomolecules, involves covalently coupling a majority of the linker molecules in the outer portion of the surface graft matrix. After covalent coupling to the surface, the cross linker molecules can provide the surface graft with a number of functionally active groups that has been used to covalently couple one or more primary biomolecules. The linker molecules may be coupled to the surface directly, or through coupling chemistries, such as, esterification, amidation, and acylation. Preferably, the linker molecule is at least a di-or tri-amine functional compound that is coupled to the surface graft matrix through the direct formation of amide bonds, and provides amine-functional groups that are available to react primarily with biomolecules. More preferably, cross linker molecules are polyethyleneimine (PEI), N succinimidyle 3-(2pyridyldithio) propionate (SPDP), polyallylamine (PALLA). Mixtures of these cross linkers can also be used. These molecules contain a plurality of pendant amine-functional groups that can be used to surface-immobilize one or more primary biomolecules [25].

Peptide is one of the biomolecules that can be immobilized to the biomaterials surface. There are some various techniques for immobilizing the peptides on the surface, such as silanization and sol-gel.

1.5. Peptides

Peptides have an important role in biochemical functions of life, for this reason researches about peptides are a continuously growing field of science. Peptides are responsible for communication between cells and their environments and they are commonly used in biological applications. Due to this property peptide functionalized surfaces usually provide secure interface between biological systems and biomaterials. Indeed, it is very common technique in biomaterials science. Peptides which contain the fibronectin active fragment or cell binding domain firstly have been developed to increase cell attachment to the biomaterials surfaces [26]. Many groups have exploited their properties to form molecular architectures for a variety of different applications including biosensing, controlled release/drug delivery and designed biomaterials for cell culture and tissue engineering. The arginine-glycine-asparic acid (RGD) sequence binds to key cell membrane structures within the integrin family and it has roles in cell adhesion, therefore in tissue engineering RGD-containing peptides has an important role [14]. Glycine, aspartic acid, asparagines, lysine and arginine are some of the peptides that can be used for surface immobilization and their chemical formulas can be seen in figure 1.1.

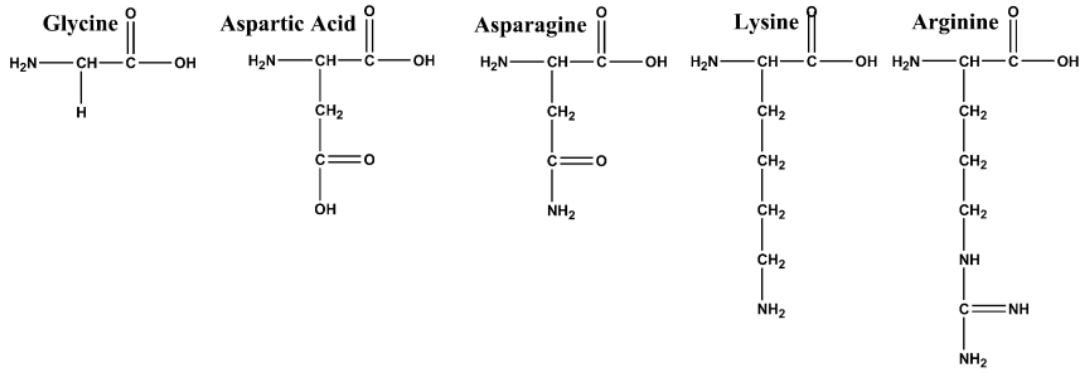


Figure 1.1 Selected set of peptides that used for immobilization on the surface [27]

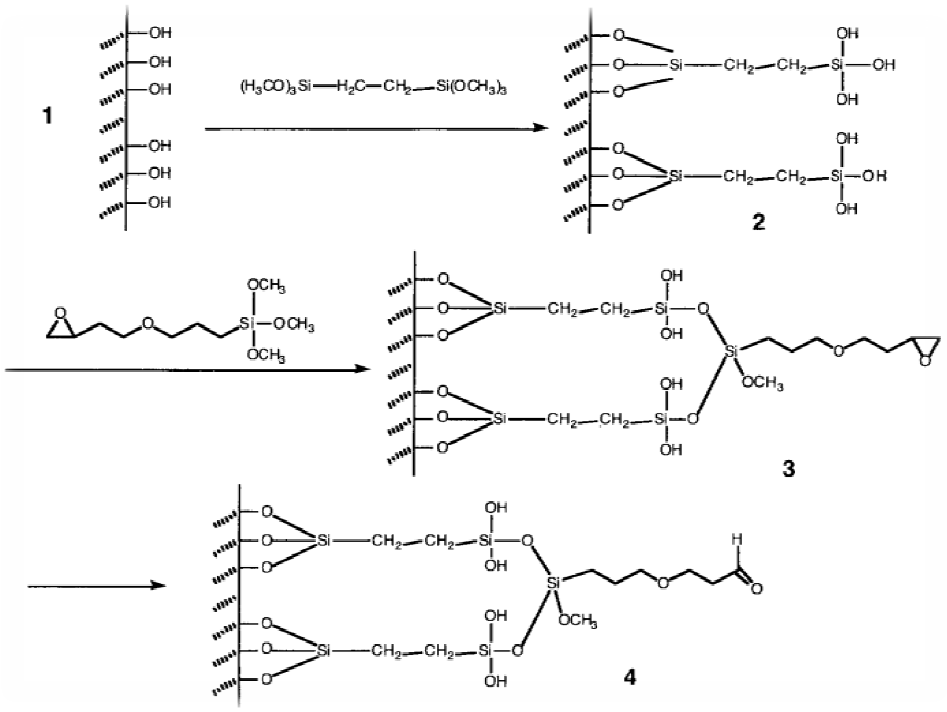
Peptides are present in every living organism and they take role in biochemical activities. They appear as enzymes, hormones, antibiotics, receptors etc. Peptides are biological molecules and short polymers of amino acid monomers bind with peptide bonds. In some cases peptides described as a polypeptide chain has less than 50 monomers, due to their size, peptides are different from proteins. Peptides have flexible polypeptide chains without one preferable conformation. Besides peptides has a N-terminus and C-terminus at the ends of the peptide. The long, continuous and unbranched peptides called polypeptide. Proteins consist of one or more polypeptides, be formed in a biologically way and are often bound to other proteins. Peptides are characterized according to their size boundaries [28, 29, 30].

In design of biomaterials that guide cell behavior for regeneration and tissue engineering, the use of short synthetic adhesion peptides like RGD should be an interesting approach. Even though there are some known disadvantages of using short peptide ligands within biomaterials, peptide usage within biomaterials is very common. Peptides have great advantageous chemical definition, access to non-native chemistries, and applicability within parallel approaches. Biomaterials which require such aspects may benefit from peptide-based applications [31].

One of the most advantageous properties of the peptides is that they are chemically defined. The definition of peptides comes into from their chemical route of production which is usually solid phase peptide synthesis. The chemical definition of peptides enable the systematic refinement of their structure, combinations of them, parallel experimental designs and precise molecular manipulations required for mechanistic investigations [31].

1.5.1. Immobilization of peptides

Immobilization of the peptides onto or within biomaterials can be accomplished with more chemical precision than proteins, due to more control on the immobilization chemistry with peptides. For the protein immobilization, the precise area interacts with the cell can be known partially, because of that chemical modifications of proteins may modify their biological activity in unpredictable ways. Furthermore, peptides can be conjugated to biomaterials through specific applications, utilizing various options including crosslinkers, addition of various peptides, UV-initiated cross-linking, amine/carboxylic acid coupling and chemo selective chemistries and native chemical ligation and one-step immobilization by using modified parylene film with formyl groups which is suitable for microplate-based immunoassay and SPR biosensor application [32]. All these techniques can be processes with chemical specificity with the covalent incorporation of proteins into biomaterial. These kinds of immobilization techniques can cause the modifications of the protein and reduction of protein efficiency, so choose of the proper peptide application process has great importance [31, 33, 34]. The current immobilization techniques sometimes are not applicable to all materials and require the presence of particular functional groups, synthetic path ways or biologically hostile environments. For this reason, the development of biocompatible linkers for surface modification of implants has been a great objective in biomaterials field [35]. As an example of the peptide immobilizing we mention HA attaching on the metal surface, in figure 1.2 there is a scheme for HA attaching of the surface with silanization technique can be seen. OH binds onto the surface after the dipping of the samples in aqueous solution, in order to functionalize Si-O bonds. After the silane treated, in figure 1.2 (a), it can be seen that condensation of trimethoxysilyl occurs, in image b condensation can be seen.



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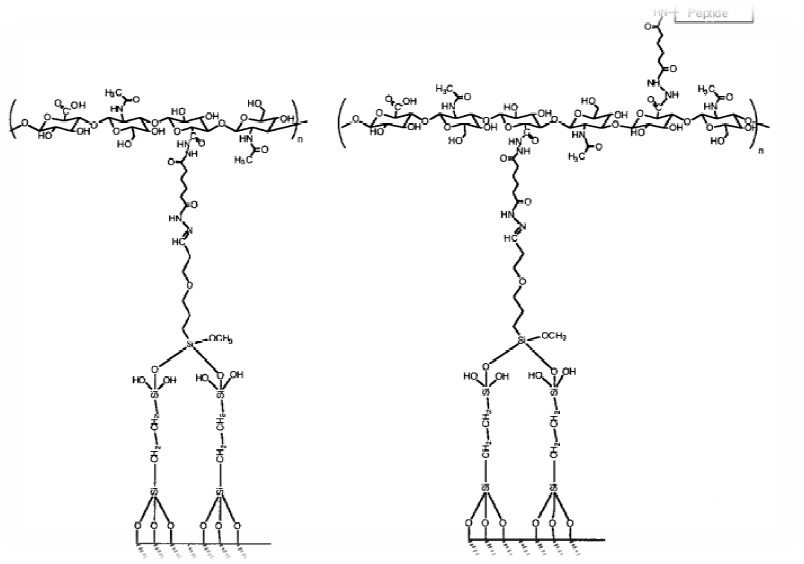


b

Figure 1.2 Chemistry scheme for attaching HA to metal surface. The figures are illustrative of the sequence of chemistry events (a) 1. Preparation of a clean basic metal oxide surface. 2. Condensation of 1,2-bis(trimethoxysilyl)-ethane. 3. Condensation of glycidyl silane. 4. Oxidation of glycidyl silane to aldehyde. (b) Condensation of aldehyde [36]

1.5.2. RGD (Arg–Gly–Asp) Peptides

The tripeptide sequence, arginine-glycine-asparic acid (RGD) is an amino acid, which is derived from fibronectin and laminin, has been commonly used to improve cell attachment and proliferation through chemical or physical conjugation onto biomaterials such as polymers, metals and ceramics [11, 37]. RGD is highly effective at promoting the attachment of numerous cell types to various materials. RGD is also present in some laminins and collagens, however RGD may be inaccessible within these molecules, and other amino acid motifs are known to serve as alternative binding modules for laminin and collagen-selective receptors. The RGD sequence can bind to multiple integrin species, and synthetic RGD peptides provide several advantages for biomaterials applications [38].

RGD peptides can be coupled to the surfaces in controlled densities and orientations. These advantages of easily synthesis, minimal cost, and tight control over ligand presentation cannot readily be achieved when using full-length native matrix proteins to functionalize material surfaces [38].

RGD peptide coating enhance biomaterials fixation by facilitating the interaction between cell and biomaterials. In many researches RGD peptide with different sequences and conformations have been tried to immobilize [39, 40]. The adhesiveness of the surface for osteblasts is increased with RGD peptides. Furthermore RGD peptides improve the rate of cell spreading compare to the other peptides [15, 37].

Interaction with RGD-containing extracellular bone matrix proteins which are absorbed on the surface of biomaterial, the integrins are believed to control to cell adhesion process in vivo. In vitro experimental studies it is clear that high concentration of RGD peptide on the surface of the implant can improve the osteoblast adhesion and activation. Even though the biological activity of implantation is not fully understood, the RGD peptide adsorption to the implant surface has an important role in osteoblast spreading and subsequent proliferation [40]. The RGD peptides linking on the substrate allow attachment to poly methylmethacrylate surfaces and improve binding to osteoblast cells and increased circulation time in the body [26].

As it is mentioned above, in order to have a quick progress of healing after the application of the biomaterial, surface immobilization of peptides, proteins and growth factors may apply. Since identification of the RGD (Arg–Gly–Asp) peptides as mediating the attachment of cells to several plasma and extracellular matrix (ECM) proteins, immobilization of RGD-containing peptides on biomaterials process started to use for improving the cell attachment. Due to its success to bind various cells through ligand and receptor, RGD is an exceptionally useful sequence for incorporating onto the biomaterials surfaces [41].

There are various techniques for immobilizing RGD peptide sequences on materials with different intrinsic abilities to promote different aspects of cell behavior; and many different ways in which RGD peptides can be presented towards cells and the manner, where peptide is immobilized on a surface can affect the peptide concentration, density, accessibility, arrangement, conformation and activity [14].

1.6. Silanization

Silanization is one of the methods which are used for covering organo-functional molecules on the surface. Silanization can be performed on metal oxide surfaces well because there are hydroxyl groups on metal oxides that they can interact with alkoxy groups on the silanization and form –Si-O-Si bond.
The methoxy (-OCH3) and the ethoxy (-OCH2CH3) groups are used as alkoxy groups for silanization. The organofunctional alkoxysilanes are classified according to their organic functions: Glycidoxysilanes, Mercaptosilanes and Aminosilanes. The Glycidoxysilanes is GPMES (3-glycidoxypropyl)-dimethyl-ethoxysilan, Mercaptosilanes is MPTS (3-mercaptopropyl)-trimethoxysilane and MPDMS (3-mercaptopropyl)-methyl-dimethoxysilane; and the aminosilanes are APTES (3-aminopropyl)-triethoxysilane, APDEMS (3-aminopropyl)-diethoxy-methylsilane, APDMES (3-aminopropyl)-dimethyl-ethoxysilane, and APTMS (3-aminopropyl)-trimethoxysilane. The reaction conditions and methods of APTES film formation are extremely important for the film on the surface. The main conditions that effect the surface are: reaction temperature, reaction time and solution concentration [39, 42].
The main aim of the silanization process is to link the functional groups with the covalent bonds on to surface in order to use them for immobilizing of the biomolecules on the surface. Reaction variables of silanizations are affecting the covalent bonding. Because of this reason, the type of the solvent, silanisation reagent and concentration of the reagent has to be optimized [42].
The silanes which are hydrolyze into multiple silano groups tend to form multilayers when the silanization contains low amount of water. Due to this control over important properties of the immobilized molecules decreases. (eg. Uniformity, orientation, surface density) [39].
Figure 1 shows the modification steps for the silanization of a pretreated titanium surface (surface A) with 3-aminopropyltriethoxylsilane (APTES) in toluene, resulting in surface B with terminal amino groups; secondly, reaction of surface B with a hetero-bifunctional cross-linker, N-succinimidyl-3-maleimido propionate (SMP), in N,N-dimethyl-formamide (DMF), resulting in surface C with exposed maleimide groups; Þnally, immobilization of a model cell-binding peptide ArgDGlyDAspDCys (RGDC) through covalent addition of the cysteine thiol (DSH) group to the maleimide group (surface D). This functionalization technique has a high fexibility, in the sense that it allows the attachment of any suitable biomolecules having a chemically accessible thiol group.

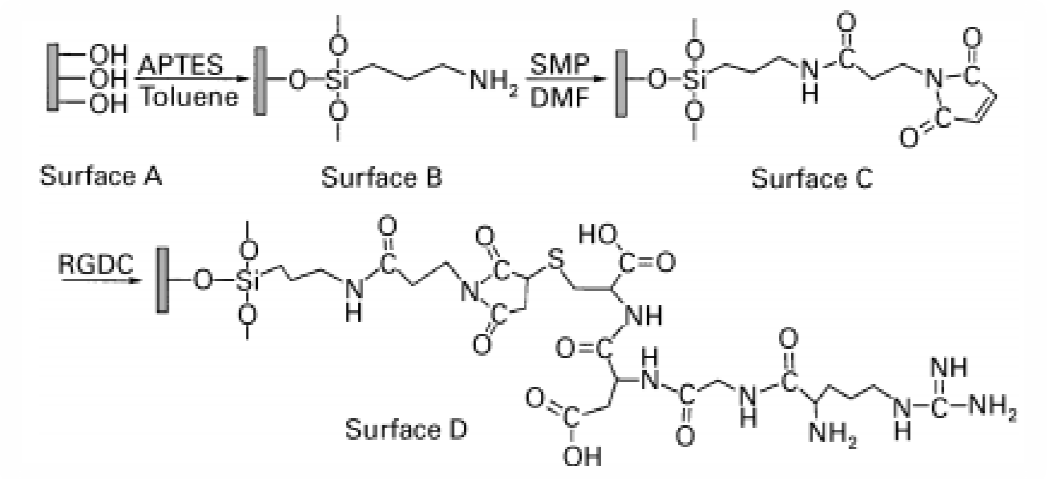


Figure 1.3 Sequence of the titanium surface modification procedure

Surface A corresponds to the pretreated titanium surface [43]

1.7. The Sol-Gel Technique

Sol-gel technique is a wet-chemical process generally used in the materials science and ceramic engineering fields. Sol- gel processing is an important method for producing amorphous, porous silica and polysilsesquioxane gels [44]. The method which is used for the fabrication of materials starting from a colloidal solution called sol and acts as an actuator for gel or an integrated network of discrete particles or polymers network. Typical precursors are metal alkoxides and metal salts such as chlorides, nitrates and precursors which are various forms of hydrolysis and polycondensation reactions [45]. Various films and coatings can been developed by sol-gel method [46].

The sol-gel process is very well-known for its simplicity and high rates, and it is simply illustrated in figure 1.4. Sol-gel is the most common technique for the nanoparticle synthesis, and it involves the simultaneous hydrolysis and condensation reaction of the alkoxide or salt. The obtained materials have several particular feature usage areas. The particles have several industrial applications; e.g., in catalysis, pigments, biomaterials, photonic devices, pharmaceuticals, and among others [6, 47].

The main advantages of sol-gel process are lower processing temperatures and higher purity and homogeneity compared with traditional glass melting or ceramic powder methods [6, 46].

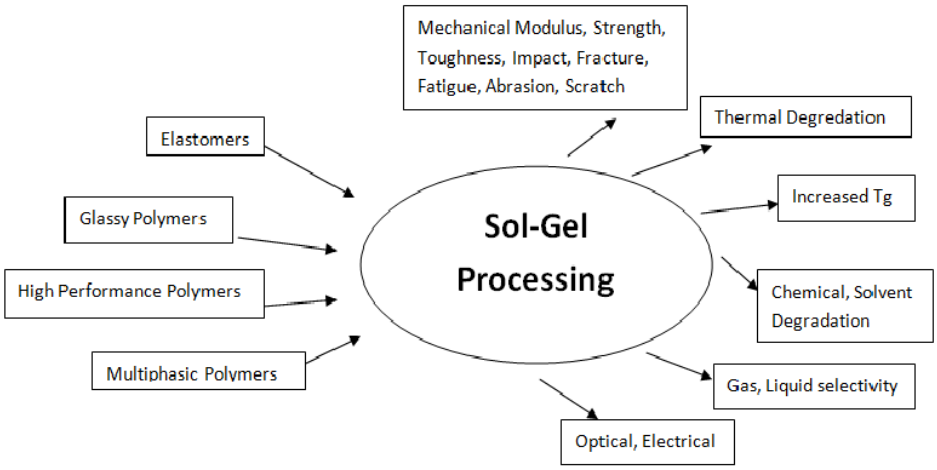


Figure 1.4 Sol-Gel Processing

It is also possible to prepare the inorganic ceramic and glass materials with sol-gel technology. The first use of this hydrolytic technique was in the 1800s, experiments were carried out on silica gels. First of all, the sol-gel process was applied for the preparation of silicate from tetraethylorthosilicate (TEOS), which to form a homogeneous solution. Nowadays various new reagents used so novel inorganic oxides and hybrid organic-inorganic materials can be synthesized with this method. Non-hydrolytic sol-gel method is also another type of sol-gel process which is a condensation reaction between a metallic or semi-metallic, and metallic or semi-metallic alkoxides to produce an oxide. (M – O – M') [6].

Currently the method is also used to produce bioactive surfaces which can provide molecular control over the incorporation and biological behavior of the cells and proteins, for biomedical applications. There are many researches on the application of the sol-gel process for production of biomaterials such as nanobioactive glass, porous bioactive glasses, and among others. Furthermore, the sol-gel process is the technique that its conditions proved suitable for preparation of the surface of the biomaterials and which provided nanoscale combinations of inorganic and organic composites natural bone is an inorganic-organic composite consisting mainly of nanohydroxyapatite and collagen fibers. The materials obtained by the sol-gel route combine the advantages of both organic and inorganic properties. Various organofunctional alkoxysilanes precursors have been used for the silica particles production [6].

The sol-gel process is compromise of the hydrolysis and condensation of metal or silicon alkoxides and is obtaining a variety of high-purity inorganic oxides or hybrid inorganic-organic materials. This process can use for the synthesis of functionalized silica with controlled particle size [6].

1.7.1. Sol-Gel Mechanisms

Sol-gel polymerization occurs in three steps. First step is the polymerization of monomers to form particles, second step growth of the particles and third step is linking of particles into chains and thickening to a gel. During these steps, there are various factors affecting the resulting silica chain, such as pH, temperature, time of reaction, reagent concentrations, catalyst nature and concentration, H_2O / Si molar ratio (R), aging temperature and time. pH, H_2O/Si molar ratio (R), and temperature have been identified as most important ones. Hence, by controlling these factors, the variation of the structure and properties of the sol-gel can be possible. Even though it is considered that sol-gel derived silicon oxide chains, under acid catalyzed conditions primarily linear or randomly branched polymers form additional branches and causes the gelation. Besides, silicon oxide chains derived under base-catalyzed conditions yield more highly branched clusters which are not join to gelation hence act as discrete clusters [48, 49, 50].

At the functional group level, there are three reactions generally describe the sol-gel process: hydrolysis, alcohol condensation, and water condensation. The reactions can be seen in figure 1.5.

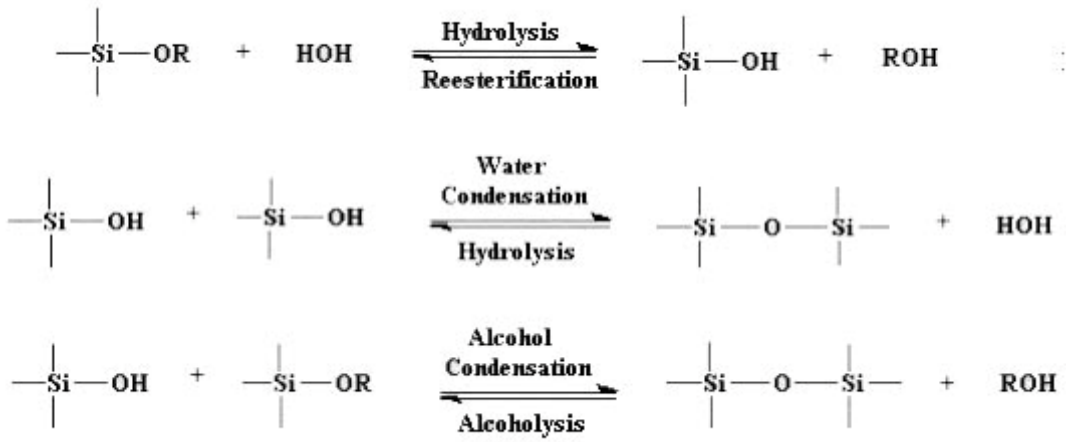


Figure 1.5 General Sol-Gel Reactions [48]

The hydrolysis reaction, through the addition of water replaces alkoxides groups (OR) with hydroxyl groups (OH). Following condensation reactions involving the silanol groups Si-OH produce siloxane bonds (Si-O-Si) and by-products are alcohol or water. Commonly, condensation arises before hydrolysis is complete. Despite, conditions such as, pH, H2O/Si molar ratio, and catalyst can force completion of hydrolysis before condensation begins.

Because of the water and alkoxides are immiscible, another solvent such as alcohol used. Alcohols are needed as sol-gel solvents because of two reasons, first permit mixing of water and non polar alkoxides, second make the solution homogenous [51]. In the presence of the alcohol, hydrolysis is simply occurs due to miscibility of alkoxides and water. Since the number of siloxane bonds increases,

the individual molecules are come together and jointly aggregate in the sol. When this happens and chain started to occur, a gel is formed. During drying, volatiles such as water, alcohol are eliminated and network shrinks as further condensation can occur. However, that the solvent additions and proper conditions for reaction can improve esterification, depolymerization reactions are always go through the reverse of the equations [48, 49].

Because of their readily reaction with water alkoxides are ideal chemical precursors. This name of the reaction is hydrolysis because a hydroxyl ion attaches to the silicon atom.

$$Si(OR)_4 + H_2O \rightarrow HO\text{-}Si(OR)_3 + R\text{-}OH$$

Based on the water and catalyst amount, hydrolysis may carry on, so all of the OR groups will changed by OH groups.

$$Si(OR)_4 + 4\ H2O \rightarrow Si(OH)_4 + 4\ R\text{-}OH$$

$[(OR)2–Si\text{-}(OH)_2]$ or $[(OR)_3–Si\text{-}(OH)]$ which are intermediate products, could be recognized the result of partial hydrolysis and those two partially hydrolyzed molecules can form a siloxane [Si–O–Si] with binding together in a condensation reaction.

$$(OR)_3–Si\text{-}OH + HO–Si\text{-}(OR)_3 \rightarrow [(OR)_3Si–O–Si(OR)_3] + H\text{-}O\text{-}H$$

$$(OR)_3–Si\text{-}OR + HO–Si\text{-}(OR)_3 \rightarrow [(OR)_3Si–O–Si(OR)_3] + R\text{-}OH$$

Water or alcohol remains free after condensation. This type of reaction can carry on building larger silicon containing molecules with polymerization. Essentially, silicon alkoxides polymerization can promote to complex branching of the polymer. Instead, under some conditions fewer than 4 OR or OH groups will be able to condensate, so little branching appears. Hydrolysis and condensation mechanisms and the conditions which affect the structure toward to linear or branched are the most critical subjects of sol-gel process. Reactions can occur in both basic and acidic conditions. [45, 48].

1.7.1.1.Acid catalyzed condensation

In the acidic conditions, firstly protonation of the alkoxides group occurs rapidly. Electron density is eliminated from the silicon atom and become more electrophilic hence more liable to attack from water. The transition declares decays by displacement of an alcohol and inversion of the silicon tetrahedron. (Fig 1.6)

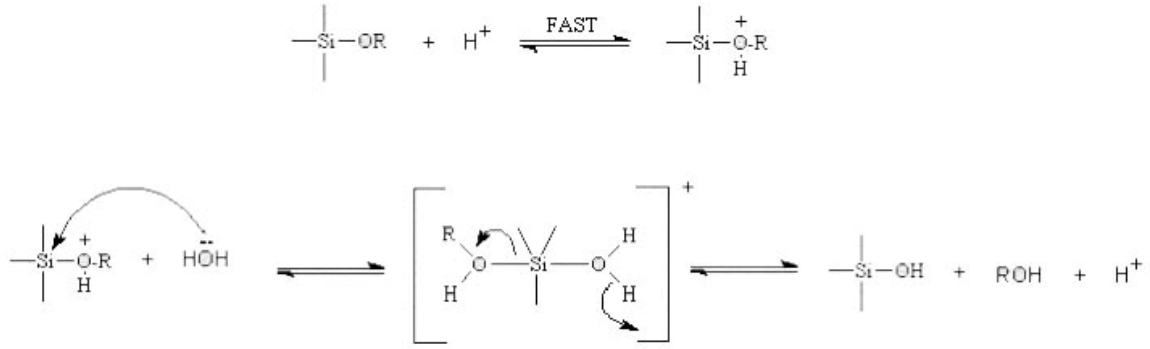


Figure 1.6 Acid Catalyzed Hydrolysis [48]

1.7.1.2.Base catalyzed condensation

The base catalyzed hydrolysis occurs slower than the acid catalyzed hydrolysis at the same conditions. Basic alkoxides oxygens likely push away the nuclesphile, -OH. Nevertheless, once an hydrolysis starts, following reactions occur stepwise and each alkoxides group more easily break and remove from the monomer then the previous one. For this reason more highly hydrolyzed silicone are more capable to attack. In the basic conditions firstly water tends to dissociate to produce hydroxyl anions, and then hydroxyl anion attacks the silicon. Basic catalyzed hydrolysis can be seen in figure 1.7.

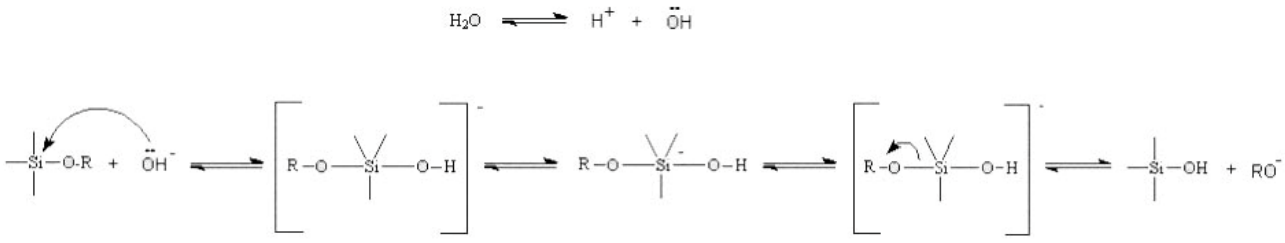


Figure 1.7 Basic Catalyzed Hydrolysis [48]

Under basic conditions, the hydrolysis reaction was found to be first-order in base concentration. Even though, as the TEOS concentration was increased the reaction deviated from a simple first-order to a more complicated second-order reaction. With weaker bases measurable speeds of reaction were

produced only if large concentrations were present. Consequently, base hydrolysis kinetics is more strongly affected by the nature of the solvent than the acid hydrolysis [48].

1.7.2. Effect of pH

Polymerization process can be divided into three pH domains: < pH 2, pH 2-7, and > pH 7. It can be seen in the figure 1.8 that hydrolysis rate has the lowest value at pH=7. Also, regardless of pH, hydrolysis occurs by the nucleophilic attack of the oxygen contained in water on the silicon atom as evidenced by the reaction of isotopically labeled water with TEOS that produces only unlabelled alcohol in both acid- and base-catalyzed systems.

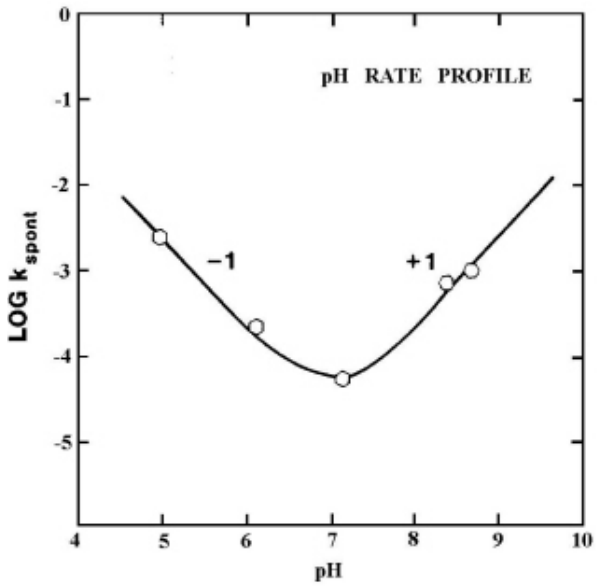


Figure 1.8 pH rate profile for hydrolysis in aqueous solution [48]

Polymerization to form siloxane bonds occurs during a water-producing or an alcohol-producing condensation reaction. The sequence of the condensation products is: monomer, dimer, linear trimet, cyclic trimer, cyclic tetramer and high order rings. This condensation process requires both availability of monomers which are in solution and depolymerization. Environmental pH affects the rate of the ring openings (depolymerization), polymerizations and monomer addition reactions. This effect can be seen on Figure 1.9, when the pH is below 2, the condensation rates are proportional to the [H+] concentration. Because the solubility of silica is low below pH, it can be seen in the figure. When the pH is between 2 and 6 condensation rates are proportional to [OH-] concentrations. Condensation preferentially occurs more highly condensed types and less high condensed and neutral. Due to the dimerization rate is low, however when dimmers started to form, they prefer to react with monomers in order to form trimes. Because of the proximity of the chain ends and substantial depletion of the monomer population cyclization occurs. Moreover further growth takes place with the addition of

lower molecular weight species to more highly condensed species and aggregation of the condensed species to form chains and networks. In this pH range, the solubility of silica is low and growth of the particles stop when the particles reach 2-4 nm diameters. When the pH is above 7, polymerization takes place same as in pH 2 to pH 6 but in the pH range, condensed species are ionized and as a result mutually repulsive.

Growth of particles occurs with the addition of monomer to the higher condensed particles, more than by aggregation of particles. Considering high solubility of silica and greater size dependence of solubility above pH 7, particles grow in size and decrease in their number because highly soluble small particles dissolve and precipitate on large particles which they are less soluble. When the solubility difference becomes indistinguishable between the particles, growth stops. Obviously particle size is temperature dependent in the higher temperatures. Moreover in this pH range, the particle growth rate strongly depends on the particle size distribution [48].

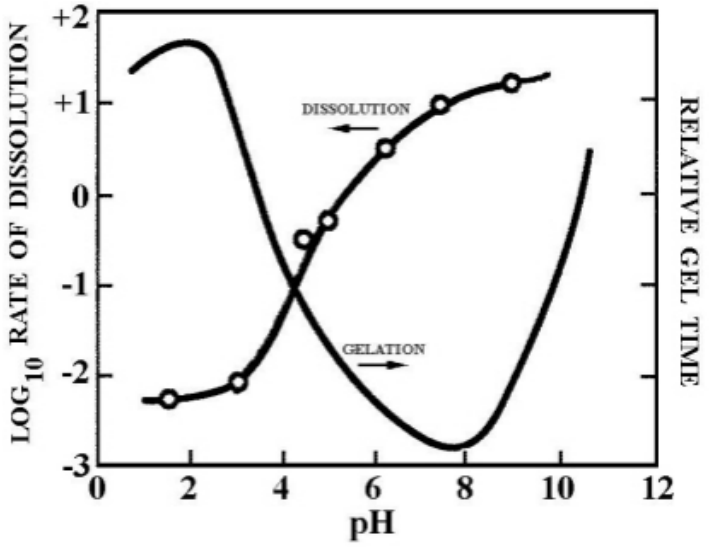


Figure 1.9 Dissolution rate and relative gel time as a function of pH [48]

1.7.3. TEOS

As its name says, sol-gel process involves inorganic chain evolution with the formation of a colloidal suspension which is sol and gelation of the sol in order to form a chain in continuous liquid phase which is gel. For synthesizing these colloids contains a metal or metalloid element, the precursors surrounded by different reactive ligands. The most popular ligands are metal alkoxides because they react with the water easily. Commonly used metal oxides are the alkoxysilanes, such as tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS). Howbeit, aluminates, titanates and borates are also widely used in the sol-gel process, often mixed with TEOS [48].

In this work TEOS which is commonly used metal oxide in sol-gel process used. Chemical formula of TEOS is $Si(OC_2H_5)_4$, or $Si(OR)_4$ with the alkyl group R = C_2H_5. In figure 1.10, molecular structure of TEOS can be seen. Besides tetraethoxysilane other names of TEOS are tetraethyl orthosilicate; ethyl silicate; silicic acid, tetraethyl ester; silicon ethoxide. In figure 1.11, TEOS in water atmosphere and polymerized sol-gel illustrated.

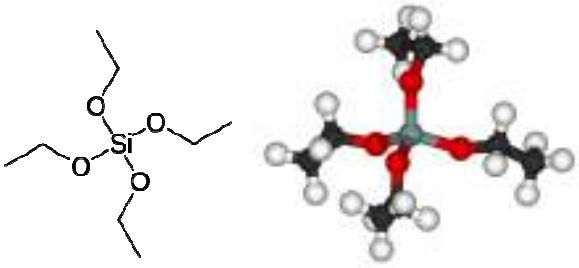


Figure 1.10 Tetraethoxysilane [52]

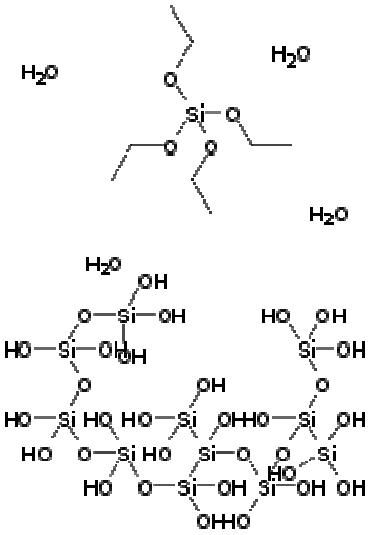


Figure 1.11 Sol-gel Silicate Bonds: TEOS, water and polymerized sol-gel [45]

1.7.4. Effect of H_2O/ Si Molar Ratio (R)

The hydrolysis reaction has been performed with R values ranging from 1 to over 50, depending on the wanted polysilicate product. An increased value of R is expected to accelerate the hydrolysis reaction. Furthermore higher values of R caused more complete hydrolysis of monomers before condensation happens.

Even thought increased values of R generally accelerate hydrolysis reaction, when R is increased the silicate concentration is reduced. When the silicate concentration reduces the hydrolysis and condensation rates reduces and it causes longer gel times. In figure 1.12 we can see this effect.

At the end, therefore water is the by-product of the condensation reaction, high values of R accelerate siloxane bond hydrolysis.

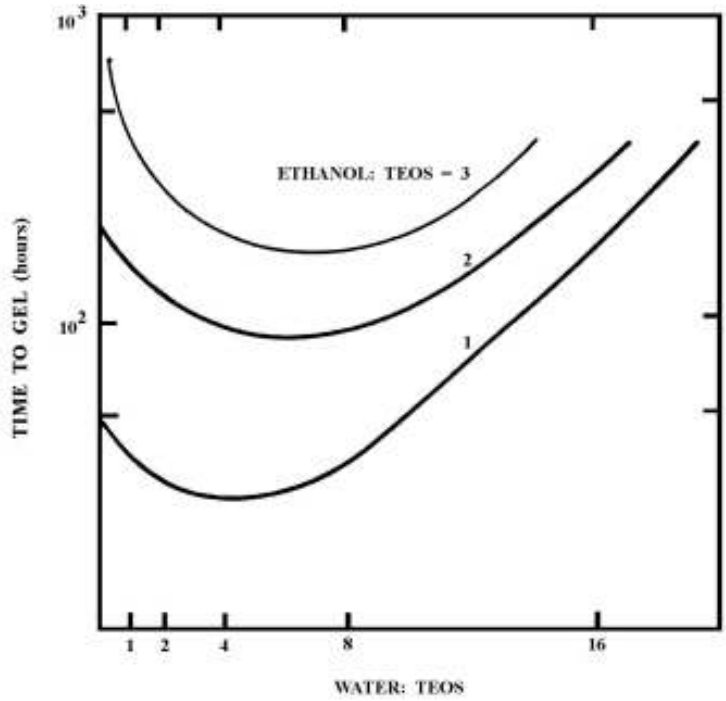


Figure 1.12 Gelation time as a function of H2O:TEOS Ratio (R) [48]

1.8. Aim of the work

For determination of interaction between biomaterials and hosts, surface modification of biomaterials with biomolecules has a significant role. Surface of biomaterials can be modified in order to promote biocompatibility adhesion and cell interaction with appropriate modification techniques. Therefore, modification of the surfaces is vital in development and design of new biomaterials for bio-medical application. Moreover, the lifetime of the biomedical device can be improved by proper surface treatments [36]. Many techniques are developed in order to modify biomaterials surfaces such as plasma polymerization, covalent binding of poly (ethylene glycol) (PEG), heparinisation to improve blood compatibility, calcium phosphate deposition, and peptide functionalisation.

Biomolecules are any molecules that are produced by a living organism and some of them can be used in order to modify surface of biomaterials for bio-medical applications. DNA's proteins and peptides are some of the biomolecules that can be attached to the biomaterials surface with various techniques.

For the immobilizing of the biomolecules main approaches are surface adsorption, physical entrapment, ligand/receptor specific binding, and covalent attachment. Biology provides numerous examples where peptides are responsible for communication between cells and their environments. Immobilization of the peptides on the biomaterials surface improves cellular interactions between biomaterial and host [30, 39, 53].

Slinazation and sol–gel are most common biomolecules immobilization techniques. Sol-gel chemistry provides new and interesting possibilities for the promising encapsulation of heat-sensitive and fragile biomolecules mainly; it is an inherent low temperature process and biocompatible [54].

Due to this well known property of the peptides and advantages of the sol-gel tecniques, in this work we aimed to functionalize the titanium surface by sol-gel technique in order to support peptide immobilizing. Surface functionalization is aimed to perform with N succinimidyle 3-(2pyridyldithio) propionate (SPDP) grafting on the surface by sol-gel technique. The important point for this work is determination of chemical composition of the solution.

2. MATERIALS AND METHODS

2.1. Materials and sample preparation

The experiments have been performed using rectangular commercially pure, titanium grade 2 samples. Firstly, titanium plate was cut in 7cm-2cm rectangular samples. Before all experimental steps start, the samples have been treated by acidic solution in order to purify the surface and clean the titanium oxides. In the beginning with HNO_3 (Nitric Acid) (30%) and HF (Hydrofluoric Acid) (10%), acid solution is prepared and is put in the plastic beaker. All the samples were waited in the acid solution in around 1 minute and then they moved to another beaker which is full of water. The samples waited under the tap water and waited dropping water on them one night in order to clean all the acid from the surfaces. After one day the samples are put in drier at 25 ^{0}C and wait 2 hours to get dry. After they get dried, their surfaces were checked by eye and they were ready for the treatment.

2.2. Sol-Gel Preparation

In this experimental study, sols were prepared by mixing 3-aminopropyltrimethoxysilane (AMS) with tetraethylorthosilicate (TEOS). All the sol-gel experiments were carried out on the AMS-TEOS. In order the see the effects on the reactions various AMS ratios, various hydrolysis ratios and two different acids used during solutions preparation. Hydrolysis ratio is the molar ration H = H_2O / (TEOS + ¾ Additive), the factor ¾ is considering the fact that the additive contains only three hydrolysable groups in relation to TEOS which contains four hydrolysable groups [45]. In our case additive is AMS so the calculation of the H is; H = H_2O / (TEOS + ¾ AMS) and the experiments were carried on H=4, H=5, H=6 and H=7.

Furthermore, during the sols preparation different AMS percentage used and they can be seen in table 2.1.

Table 2.1 Molar AMS Ratios

AMS = 15 % (TEOS + AMS)
AMS = 20 % (TEOS + AMS)
AMS = 25 % (TEOS + AMS)
AMS = 30 % (TEOS + AMS)
AMS = 40 % (TEOS + AMS)
AMS = 50 % (TEOS + AMS)

The preparation method of the solution has a magnificent importance; three different techniques are used for preparing the sols.

In the first technique, firstly TEOS and ethanol were put in a beaker and they started to stir with magnetic stirring. Water added as a solvent and then acid added in the solution. After acid added concerning to achieve pH=2, we waited 30 minutes and determine the pH. When pH reached 2, AMS started to add and gelation started to occur after 1 hour stirring, due to the fact that this technique didn't used.

In the second technique, the sol had been started to prepare in two different beakers. TEOS and ethanol put in the first beaker and magnetic stirring started in the meanwhile AMS and ethanol put in another beaker and started magnetic stirring. In order to reach pH=2, acid started to add in the second beaker, drop wise. For the H=4, after 0,4 ml acid addition precipitations can be seen by eye because of that water added and precipitations solved and all acid amount added in the second beaker. After this, pH of the sol determined, it is aimed to have pH=2. At the end, solution in the second beaker added in the first beaker and sol become white immediately. After 24 hours stirring, gelation started to occur.

In the last technique, water, acid and ethanol were put in a beaker made magnetic stirring. Firstly, AMS added drop by drop in the beaker, and waited until the reaction occurs and gas discharge finished, the waiting time was around 20 minutes. When the sol was transparent again without any gas reaction products, all amount of TEOS was added in the beaker and continue to magnetic stirring for 24 hours.

Experiments were not performed on the second sol-gel preparation technique studied previously because off longer preparation time and sooner gelation time. Due to its practical application and success third technique used for the preparation of the all sols mentioned below.

Sol-gel preparation has been done in two different steps. First of all, two types of sols prepared with 15%, 20% and 25% AMS ratio with HNO_3 and HCl, for H=4, H=5, H=6 and H=7. Names of the sols can be seen in table 2.2, 2.4, 2.6, 2.8, 2.10, 2.12 below and chemical composition of the sols can be seen in the table 2.3, 2.5, 2.7, 2.9, 2.11, 2.13. Secondly, sols prepared with 30%, 40% and 50% AMS ratio with HCl for H=5 and H=6.

Table 2.2 Sols with 15 % AMS Ratio with HCl

Name of the Sol	AMS Ratio	H	Acid
A15H4	AMS = 15 % (TEOS + AMS)	4	HCl
A15H5	AMS = 15 % (TEOS + AMS)	5	HCl
A15H6	AMS = 15 % (TEOS + AMS)	6	HCl
A15H7	AMS = 15 % (TEOS + AMS)	7	HCl

Table 2.3 Moles of the chemicals in the sols with AMS = 15 % (TEOS + AMS) and HCl

Name	TEOS	AMS	Ethanol	H_2O	HCl	Density (g/ml)
A15H4	0,05	0,009	1,307	0,226	0,010	0,774
A15H5	0,05	0,009	1,391	0,286	0,010	0,823
A15H6	0,05	0,009	1,374	0,339	0,010	0,825
A15H7	0,05	0,009	1,356	0,396	0,010	0,827

Table 2.4 Sols with 15 % AMS Ratio with HNO3

Name of the Sol	AMS Ratio	H	Acid
B15H4	AMS = 15 % (TEOS + AMS)	4	HNO_3
B15H5	AMS = 15 % (TEOS + AMS)	5	HNO_3
B15H6	AMS = 15 % (TEOS + AMS)	6	HNO_3
B15H7	AMS = 15 % (TEOS + AMS)	7	HNO_3

Table 2.5 Moles of the chemicals in the sols with AMS = 15 % (TEOS + AMS) with HNO3

Name	TEOS	AMS	Ethanol	H_2O	HNO_3	Density (g/ml)
B15H4	0,05	0,009	1,414	0,226	0,010	0,821
B15H5	0,05	0,009	1,397	0,283	0,010	0,823
B15H6	0,05	0,009	1,379	0,340	0,010	0,825
B15H7	0,05	0,009	1,362	0,396	0,010	0,828

Table 2.6 Sols with 20 % AMS Ratio with HCl

Name of the Sol	AMS Ratio	H	Acid
A20H4	AMS = 20 % (TEOS + AMS)	4	HCl
A20H5	AMS = 20 % (TEOS + AMS)	5	HCl
A20H6	AMS = 20 % (TEOS + AMS)	6	HCl
A20H7	AMS = 20 % (TEOS + AMS)	7	HCl

Table 2.7 Moles of the chemicals in the sols with AMS = 20 % (TEOS + AMS) with HCl

Name	TEOS	AMS	Ethanol	H_2O	HCl	Density (g/ml)
A20H4	0,05	0,013	1,291	0,238	0,013	0,779
A20H5	0,05	0,013	1,370	0,297	0,013	0,826
A20H6	0,05	0,013	1,352	0,356	0,013	0,828
A20H7	0,05	0,013	1,333	0,416	0,013	0,830

Table 2.8 Sols with 20 % AMS Ratio with HNO3

Name of the Sol	AMS Ratio	H	Acid
B20H4	AMS = 20 % (TEOS + AMS)	4	HNO_3
B20H5	AMS = 20 % (TEOS + AMS)	5	HNO_3
B20H6	AMS = 20 % (TEOS + AMS)	6	HNO_3
B20H7	AMS = 20 % (TEOS + AMS)	7	HNO_3

Table 2.9 Moles of the chemicals in the sols with AMS = 20 % (TEOS + AMS) with HNO_3

Name	TEOS	AMS	Ethanol	H_2O	HNO_3	Density (g/ml)
B20H4	0,05	0,013	1,395	0,238	0,013	0,824
B20H5	0,05	0,013	1,377	0,297	0,013	0,827
B20H6	0,05	0,013	1,359	0,356	0,013	0,829
B20H7	0,05	0,013	1,340	0,416	0,013	0,831

Table 2.10 Sols with 25 % AMS Ratio with HCl

Name of the Sol	AMS Ratio	H	Acid
A25H4	AMS = 25 % (TEOS + AMS)	4	HCl
A25H5	AMS = 25 % (TEOS + AMS)	5	HCl
A25H6	AMS = 25 % (TEOS + AMS)	6	HCl
A25H7	AMS = 25 % (TEOS + AMS)	7	HCl

Table 2.11 Moles of the chemicals in the sols with AMS = 25 % (TEOS + AMS) with HCl

Name	TEOS	AMS	Ethanol	H_2O	HCl	Density (g/ml)
A25H4	0,05	0,017	1,271	0,250	0,018	0,784
A25H5	0,05	0,017	1,345	0,313	0,018	0,829
A25H6	0,05	0,017	1,326	0,375	0,018	0,832
A25H7	0,05	0,017	1,307	0,438	0,018	0,835

Table 2.12 Sols with 25 % AMS Ratio with HNO_3

Name of the Sol	AMS Ratio	H	Acid
B25H4	AMS = 25 % (TEOS + AMS)	4	HNO_3
B25H5	AMS = 25 % (TEOS + AMS)	5	HNO_3
B25H6	AMS = 25 % (TEOS + AMS)	6	HNO_3
B25H7	AMS = 25 % (TEOS + AMS)	7	HNO_3

Table 2.13 Moles of the chemicals in the sols with AMS = 25 % (TEOS + AMS) with HNO3

Name	TEOS	AMS	Ethanol	H_2O	HNO_3	Density (g/ml)
B25H4	0,05	0,017	1,374	0,250	0,018	0,828
B25H5	0,05	0,017	1,355	0,313	0,018	0,830
B25H6	0,05	0,017	1,336	0,375	0,018	0,833
B25H7	0,05	0,017	1,317	0,438	0,018	0,835

After those sols prepared, sols with the H = 4 does not work well and gelation occurred immediately. Because of this we continue to work with H = 5 and H =6 for AMS ratio 30 %, 40 % and 50 %. Furthermore according surface characterization of the samples it is seen that using HCl and HNO_3 acid does not make any difference for the coating on the surface. Due to this it is continued to work only with HCl acid. The name of the sols can be seen in Table 2.14, 2.16 and 2.18 and the chemical composition of the sols can be seen in table 2.15, 2.17, 2.19.

Table 2.14 Sols with 30 % AMS Ratio with HCl

Name of the Sol	AMS Ratio	H	Acid
A30H5	AMS = 30 % (TEOS + AMS)	5	HCl
A30H6	AMS = 30 % (TEOS + AMS)	6	HCl

Table 2.15 Moles of the chemicals in the sols with AMS = 30 % (TEOS + AMS) with HCl

Name	TEOS	AMS	Ethanol	H_2O	HCl	Density (g/ml)
A30H5	0,05	0,021	1,317	0,330	0,022	0,834
A30H6	0,05	0,021	1,297	0,396	0,022	0,837

Table 2.16 Sols with 40 % AMS Ratio with HCl

Name of the Sol	AMS Ratio	H	Acid
A40H5	AMS = 40 % (TEOS + AMS)	5	HCl
A40H6	AMS = 40 % (TEOS + AMS)	6	HCl

Table 2.17 Moles of the chemicals in the sols with AMS = 40 % (TEOS + AMS) with HCl

Name	TEOS	AMS	Ethanol	H_2O	HCl	Density (g/ml)
A40H5	0,05	0,033	1,248	0,375	0,034	0,845
A40H6	0,05	0,033	1,225	0,450	0,034	0,848

Table 2.18 Sols with 50 % AMS Ratio with HCl

Name of the Sol	AMS Ratio	H	Acid
A50H5	AMS = 50 % (TEOS + AMS)	5	HCl
A50H6	AMS = 50 % (TEOS + AMS)	6	HCl

Table 2.19 Moles of the chemicals in the sols with AMS = 50 % (TEOS + AMS) with HCl

Name	TEOS	AMS	Ethanol	H_2O	HCl	Density (g/ml)
A50H5	0,05	0,05	1,151	0,438	0,051	0,859
A50H6	0,05	0,05	1,124	0,525	0,051	0,863

2.3. Dip Coating

The compounds and compounds attached to the silicate can be applied to the substrate by conventional methods such as spraying, dipping, coating, brushing and other methods that can form a uniform and reproducible coating or layer on the substrate used for immobilizing biomolecules. Such substrate can be glass, quarts, silica or various metals, like titanium as we used in this study [23]. In this study dip coating technique has been used in order to cover the titanium surfaces with silica-oxide. Dipping has been done by the mechanical coating machine (Fig.2.1) with two different velocities. Firstly, sample properly fixed with the arm, after that the length of the sample which will be dipping in the sol determined and all the data entered the dipping device. The samples dipped in the sol one time and waited 5 seconds in the sol. Due to determine the effect of the dipping velocity two different velocities has been used: 50 mm/min and 100mm/min. After dip coating, samples were leaved in oven at 60 ^{0}C for 2 hours in order to complete dry.

Figure 2.1 Dip coating of the titanium grade 2 samples

2.4. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS)**,** also known as to as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS**)**, is a non-invasive, well-established technique in physics that can be used to determine the size of molecules and particles. DLS typically determine the size in the submicron region but with the latest technology it can determine the particles lower than 1 nanometer. With a laser beam is sending through a liquid with suspended particles, scattering of those particles occurs in all directions, and develop a scattering-angle-dependent intensity pattern. While the particles are experiencing Brownian motion, the intensity pattern also fluctuates randomly. The developed scattering pattern is speckled in appearance, and the speckles can be classified into two categories: speckles composed of light that is scattered from a single particle, and speckles composed of light that has scattered off of several particles. These two kinds of scattered speckles distinct in one important way, if the incident light is polarized perpendicularly to the plane on which the scattering angle is measured, singly scattered speckles are tall, narrow vertical streaks intersecting the horizontal plane. Multiply scattered speckles are roughly round in shape, much smaller, and with respect to the horizontal plane they are located randomly. Speckles arise from scattered laser light that stayed relatively polarized, and also has not been destructively interfered with by other less well preserved scattered light. Consequence, scattering destroys the polarization of the incident light, resulting in significantly smaller speckles, which are relatively short, lived and rapidly moving. Analyzing the intensity fluctuations at a particular scattering angle can be important information about the particles scattering the laser beam, including the hydrodynamic radiuses of the suspended particles [55].

2.4.1. Utility of DLS for solutions

- DLS can analyze samples containing very broad distributions of species of widely differing molecular masses (*e.g.* a native protein and various sizes of large aggregates)
- DLS can detect very small amounts of the higher mass species (<0.01% in many cases).
- Because there is no chromatographic separation or dilution involved (it is a batch-mode measurement), one does not have to worry that protein aggregates are being lost within a chromatographic column or from dissociation by dilution.
- DLS is the able to study samples directly in their formulation buffers or at high protein concentrations (50 mg/ml or more).

Another very important advantage of the DLS is it can be a way to detect precursors long before the visible particles. A usual problem with proteins is the appearance of visible particulates over time and these large particles grow from trace amounts of much smaller precursor aggregates. Because of this reason it is important to determine the aggregates month before by DLS. Thus this technique can provide a quick assay to help track down and prevent formation of visible particulates, and problem can be solved successfully [56].

Besides all this advantages, in some cases it is difficult to precisely quantity the amount of any aggregates which may be present in the solution. Although, DLS is a very good technique for relative comparisons such as indication formulation, sample treatment or purification process produces more aggregates [56]. For this study DLS analyzes of the sols performed with Malvern Zetasizer Nano Series ZS, in order to determine the size of the silica particles related to AMS ratio. Characterization of nanoparticles, colloids and proteins can be performed by Zetasizer Nano ZS, particle / molecular size absolute molecular weight and zeta potential can be determined in between 0-90 °C temperature rang and 0.3nm – 10.0 microns (diameter) range. In our case particle size were determined at room temperature.

2.5. Scanning Electron Microscopy (SEM)

The Scanning electron microscope (SEM) permits the observation and characterization of heterogeneous organic and inorganic metals on a nanometer (nm) to micrometer scale (μm). SEM can image and analyze the bulk specimen. SEM is used to investigate the samples morphology at wide range of magnifications, from about 10 times more than 500,000 times, about 250 times the magnification limit of the best light microscopes. SEM uses high energy electrons in order to produce signals on the surface of specimens. Electrons are accelerated through a voltage difference between cathode and anode (1eKv-50eKv). The range between 1eKv and 5eKv is called low voltage SEM.

The basic components of the SEM are the lens system, the electron gun, the electron collector, the visual and photo recording cathode ray tubes.

In the SEM the area to be analyzed is irradiated with a greatly focuses electron beam which comes from the special source. The electron beam may swept in raster across the surface of the specimen to form images or it may be static to obtain analyze at one position. The signals types produced from the interaction of the electron beam with the sample include secondary electrons, backscattered electrons, characteristic x-rays or various energies. The various signals derived from specific emission volumes, can be used to analyze many properties of the sample such as surface topography, crystallography, and chemical composition.

The most used electron source of the SEM is the tungsten filament. The tungsten filament is connected to a source of current and electrons are passed through the wire. While the electrons passing through the filament heats up and electrons start to be emitted from the electron source. In this type of analyze with outside electron source, heating occurs. The accelerated electrons have kinetic energy and this energy dissipated as a variety of signals produced by the interaction between electrons and sample. The particular signals has secondary electrons, backscattered electrons and diffracted backscattered electrons, photons, light and hear.

The most common imaging signals are the secondary and backscattered electrons because these vary primarily as a result of surface topography difference. Secondary electrons are usually used for morphology and topography analyzed and backscattered electrons are used for composition analyzes. The secondary electron emission limited to a quite small area volume close to beam impact area for several chooses of the beam energy, allows images to be obtained at a resolution approximating the size of the focus electron beam. Characteristic x-rays are emitted as a result of electron bombardment. Analyze of the characteristic x-radiation emitted from samples can yield both qualitative identification and elemental analyzes from particular regions of a specimen approximately 1 μm diameter and 1μm depth under normal conditions. SEM analyses are non-destructive; the X-rays produced by electron interactions do not cause the volume loss on the sample surface, due to this property of the SEM it is possible to analyze the same materials repeatedly [57, 58].

The samples were analyzed by SEM, Cambridge Stereoscan 360 capable of imaging between 5X and 50.000X with a accelerating voltage 20kV in high vacuum conditions. The interface between coating and bulk material, the border of the samples and coated surfaces were analyzed at different magnifications after the surface treatments.

2.6. Glow Discharge Optical Emission Spectroscopy (GDOES)

Glow discharge optical emission spectrometry (GDOES) is a method used for solids' elemental analysis. With the GDOES the bulk analysis of samples and profile analysis of coating on the samples and surface modified materials can be performed. As GDOES can also perform bulk analysis on raw materials, it provides a single analytical technique for a wide variety of surface treatment at coating applications. Firstly from radio frequency plasma, positively charged argon ions (adjacent to the sample surface) are accelerated and bombard to the surface of the negatively-polarized sample. When the argon-ions collide with the surface, surface material is evaporated and emits light due to excitation by the surface near plasma condition. Then the emitted light is element-specific and used for semi-quantitative to quantitative elemental analysis as a function of time which is related to the depth. Sputtering rates are in between 10nm/s and 50nm/s. The technique is not restricting to conductive materials. During analyzing the samples local heating of the sample can occur. The GDOES analyze are processed in vacuum environment. The analyze processes are in the range of 0,5-1 nm. The main strength of this technique is the continuous profiling of chemical (elemental) composition as a function of depth up to 100 m. The main disadvantages of GDOES are the high sample damaging and the low spatial resolution [59, 60]. For the experimental work of this study the samples were analyzed after the sol-gel treatments by GDOES. The samples were analyzed after the treatments by Horiba Scientific Pulsed RF-GDOES GDA 750 analyzer. The analyses were performed setting a voltage of 700 V with an argon pressure between 2.3hPa and 3.6 hPa.

2.7. Grafting

Physiosorption, crosslinking and grafting, are the techniques by which the association of monomers and polymers. The term physiosorption emphasizes that it is related to physical attractive forces. The process is a reversible one and is achieved by the end functionalized molecules on to the solid surface, where crosslinking is the association of polymers through a chemical bond. In most cases, the crosslinking is irreversible. [61]

Grafting is the covalent attachment process and irreversible. The bonding of reactants to another structure occurs during grafting. Surface Grafting is a grafting reaction system involving a surface and a grafting medium containing a monomer. The system of grafting is based on forming free radicals.

The surface is activated with free radicals and placed in contact with the monomer, in solution under very specific temperature and time conditions. The effect of activation is to create surface radical species, from which the monomer polymerization reaction proceeds.

The substrates that can be modified by the grafting method include metals such as titanium/titanium alloys, TiNi shape memory alloys, aluminum oxide, platinum/platinum alloys, stainless steels,

pyrolytic carbon, silver or glassy carbon; polymers such as polyamides, polycarbonates, polyethers, polyesters, polyolefins including polyethylenes or polypropylenes, polystyrenes, polyurethanes, polyvinyl chlorides, polyvinylpyrrolidones, silicone elastomers, fluoropolymers, polyacrylates, polyisoprenes, polytetrafluoroethylenes, and rubber; minerals or ceramics such as hydroxapatite; human or animal protein or tissue such as bone, skin, teeth, collagen, laminin, elastin or fibrin; organic materials such as wood, cellulose, or compressed carbon; and other materials such as glass, or the like. For biomedical area, the substrate is a biomaterial for use in a number of medical devices such as vascular grafts, aortic grafts, arterial, venous, or vascular tubing, vascular stents, dialysis membranes, tubing, or connectors, blood oxygenator tubing or membranes, ultrafiltration membranes, intra-aortic balloons, blood bags, catheters, sutures, soft or hard tissue prostheses, synthetic prostheses, prosthetic heart valves, tissue adhesives, cardiac pacemaker leads, artificial organs, endotracheal tubes, lenses for the eye such as contact or intraocular lenses, blood handling equipment, apheresis equipment, diagnostic and monitoring catheters and sensors, biosensors, dental devices, drug delivery systems, or bodily implants of any kind [25, 61].

2.7.1. SPDP Crosslinker

SPDP Cross-Linker is called 3-(2-Pyridyldithio) propionic acid or N-hydroxysuccinimide ester. SPDP crosslinker is a cleavable, water insoluble, amino and thiol (sulfhydryl) reactive hetero bifunctional protein crosslinker. The SPDP crosslinking reagent crosslinks protein or peptides via reactive moieties consisting of an N-hydroxysuccinimide (NHS) ester that reacts with primary amines, as found in lysine side chains or the N-terminus of proteins and peptides; on the other end of the SPDP crosslinking molecule pyridinyldisulfide reacts with sulhydryls to yield a reversible disulfide bond which can be reduced with reducing agent [62].

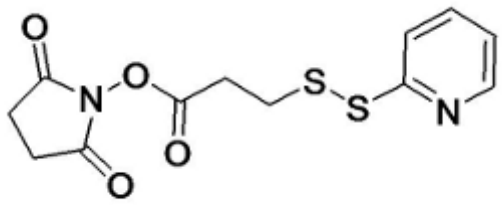


Figure 2.2 3-(2-Pyridyldithio) propionic acid [63]

The amine-reactive portion of SPDP reagents is the N-hydroxysuccinimide (NHS) ester. Reactions are most commonly performed in phosphate, carbonate/bicarbonate, or borate buffers at pH 7-8. In our study it was performed in borate buffer. Other buffers can be used provided they do not contain primary amines. The rate of reaction and degradation by hydrolysis increases by with increasing pH.

NHS-ester reagents like SPDP have limited aqueous solubility and must be dissolved in organic solvent before adding them to a reaction mixture.

The sulfhydryl-reactive portion of SPDP reagents is the 2-pyridyldithio group, which reacts optimally with sulfhydryls between pH 7 and 8.1. The reaction results in displacement of a pyridine-2-thione group, the concentration of which can be determined by measuring the absorbance at 343nm. Reaction buffers must be free of thiols and disulfide reducing agents until quenching or reduction of the 2-pyridyl disulfide is desired. Two basic strategies can be used to form cleavable crosslinks between proteins with SPDP reagents, depending whether one or neither protein already possesses sulfhydryl groups (-SH) in addition to primary amines. Both conjugation methods result in crosslinks that contain a disulfide bond in the spacer arm, which can be broken by reduction with reducing agent [62, 63, 64].

2.7.2. β – Mercaptoethanol

2-Mercaptoethanol is also known β-mercaptoethanol with a chemical formula $HOCH_2CH_2SH$ and its shortcuts are BME, 2BME, 2-ME or β-met. It is a hybrid of ethylene glycol, $HOCH_2CH_2OH$, and 1,2-ethanedithiol, HSCH2CH2SH. 2-Mercaptoethanol may be prepared by the action of hydrogen sulfide on ethylene oxide. It is illustrated in figure 2.3.

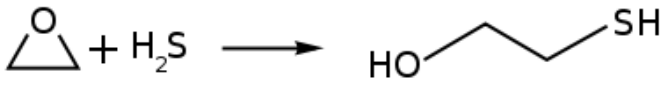


Figure 2.3 Preperation of β-Mercaptoethanol may by the reaction of hydrogen sulfide on ethylene oxide

βME, is used to reduce disulfide bonds and can act as a biological antioxidant by scavenging hydroxyl radicals. It is widely used because the hydroxyl group confers solubility in water and lowers the volatility [65, 66].

2.7.3. Conjugation of SPDP

SPDP has to be diluted in borate buffer before grated on titanium surface otherwise a white precipitate forms on the SPDP addition. Due to this firstly, borate buffer prepared. The solution contains the 0,2 M borate buffer (pH = 8,4) and 1M NaCl. 6,183 gram of Boric Acid (H_3BO_3) with a molecular weight 61,83 gram and 0,542 gram of NaCl with a molecular weight 58,44 were added to 500ml of H_2O. pH was adjusted to 8,4 by adding 660 µl of 10 M NaOH. 50ml of the resulting solution was placed in a tube and 2,93 gram NaCl added, pH was adjusted to 8,4 with the usage of 10 M NaOH. In figure 2.4, reaction scheme for SPDP conjugation to the surface can be seen. NH_2 molecules were bound on the surface through silica oxide and SPDP binding to the N atoms also can be seen in the figure 2.4.

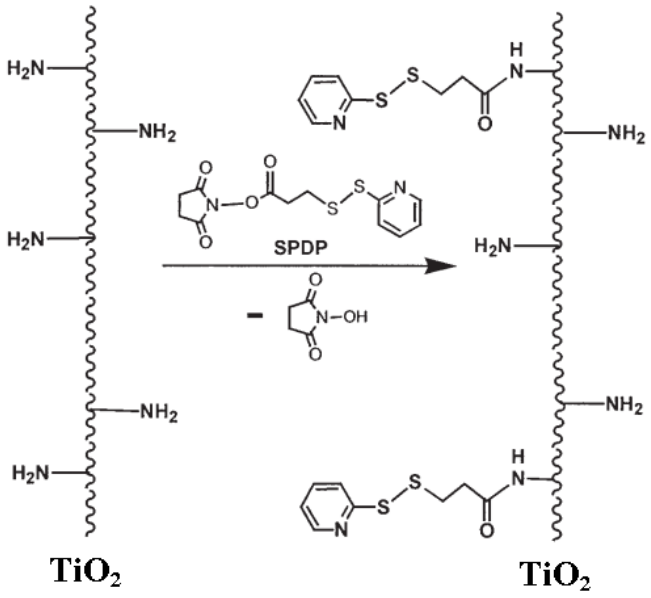


Figure 2.4 Reaction scheme for SPDP conjugation to Ti surface

N-Succinimidyl (3-(2-pyridyldithio)-propionate) (SPDP) was dissolved in ethanol and dimethhyl-sulfoxide (DMSO) added to final concentration. 6.24 µgr (20 µmol) of 10 M SPDP with a molecular weight 312.3 gram were dissolved in 667 µl ethanol and 26,6 µl DMSO added to a final concentration. After that, 1,42 ml borate buffer added in the final solution. Higher concentrations couldn't be used because SPDP precipitates upon dilution 1:4 with borate buffer.

SPDP solutions prepared 30 M, 15 M, 10 M, 5 M, 2,5 M and 1 M in order to determine the molarity for homogenous SPDP grafting on the surface. According to the analysis with each molarity the molarity of the last solution optimized 10 M and the reaction performed in the same conditions and spectrophotometer analyzes performed in order to compare the results and optimized the best molarities of the solution. After the preparation of the 10 M solution, Ti samples were incubated for 3 hours with a drop of 100 µl of 10 µM SPDP at 25 ^{0}C. Then samples washed in water and ethanol twice.

2.8. Spectrophotometer

Spectrophotometers measure the amount of the light that a sample can absorb. For quantitative analysis, spectroscopic absorption methods of analysis is one of the most common and strong tools. The method is used to determine concentrations of chemical variability which can give colors either directly or after some chemical addition. The use of a spectrometer is analyzing the extent of absorption of various wavelengths of visible light. The spectrophotometer operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector [67, 68].

Spectrophotometers which are aimed to measure the absorption of radiant energy have the basic components, as it is illustrated in figure 2.5. The main four parts are: a stable source of radiant energy; filter or monochromator as a wavelength selector to isolate an aimed wavelength from the source transparent container for the blank and sample; a radiation detector in order to convert the radiant energy received to a measurable signal; and a readout device that displays the signal from the detector [68].

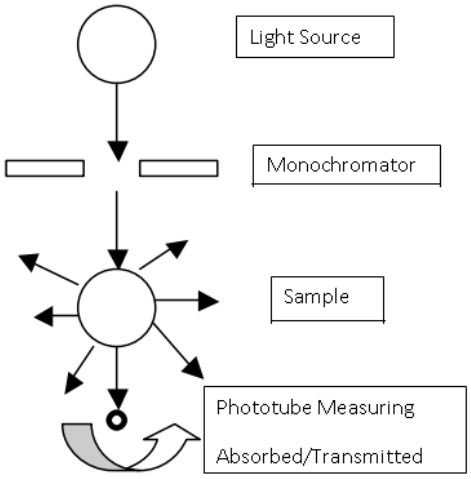


Figure 2.5 Components of spectrophotometer [68]

2.8.1. Spectrophotometric Measurement

Absorption spectroscopic methods of analysis are based on the fact that compounds absorb light radiation of a specific wavelength. In the analysis, the amount of light radiation absorbed by a sample is measured. As it is mentioned above the light absorption is directly related to the concentration of the colored compound in the sample. The maximum absorption wavelength is known for different compounds [68].

The energy source supplies a stable source of light radiation, and the wavelength selector permits separation of radiation of the desired wavelength from other radiation. Light radiation passes through a glass container with sample. The detector measures the energy after it has passed through the sample. The readout device calculates the amount of light absorbed by the sample displays the signal from the detector as absorbance or transmission [68].

Spectroscopic method was used to quantify the SPDP content of Ti surfaces. The kinetics of the conjugation reaction between Ti and SPDP were directly analyzed by the monitoring of the absorbance change at 343 nm corresponding to the release of 2-thiopyridone from the reaction between SPDP and βME. The SPDP reacts with the surface and S-S bond has been broken and 2-thiopyridone releases, βME reacts with this by product and 2-thiopyridone can be analyzed with 343 nm. The Ti surfaces conjugated with SPDP were incubated with 10 µl drop of 1% βME on the surface and the release of 2-thio-pyridone was measured at 343 nm by reading the absorbance by the spectrophotometer Thermo Scientific NanoDrop 2000 which can measure sample purity, DNA, RNA and protein concentrations.

3. RESULTS AND DISCUSSION

3.1. Analyzes of the sols

3.1.1. Dynamic Light Scattering

Dynamic Light Scattering (DLS) was performed with Zetasizer Nano series SZ in order to determine the dimension of the silica particles in the sols.

In figure 3.1, the graph of the size distribution of particles diameter for A20H5 is showed, and a mono-model distribution can be seen on the fig 3.1 and the average diameter size for this solution is 6,635 nm.

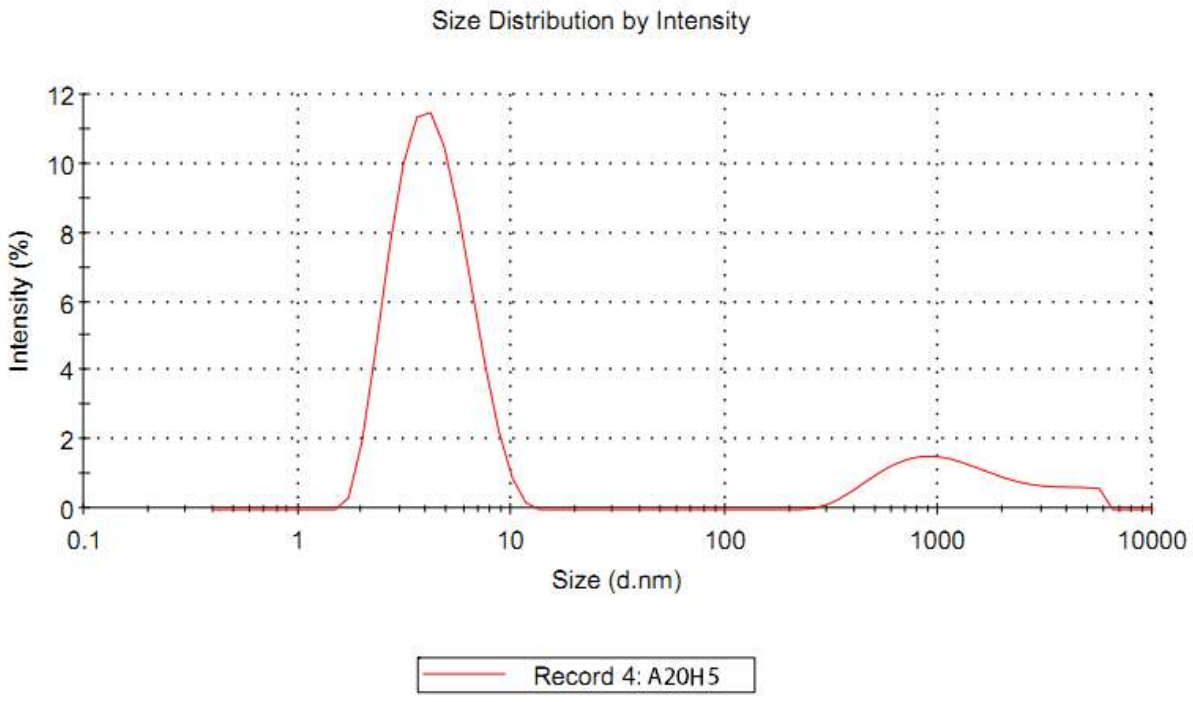


Figure 3.1 DLS result of sample A20H5

In figure 3.2, the graph of the size distribution of particles diameter for A25H5 is given, mono-model distribution can be seen for the diameter size and the average diameter size for this solution is 132,6 nm.

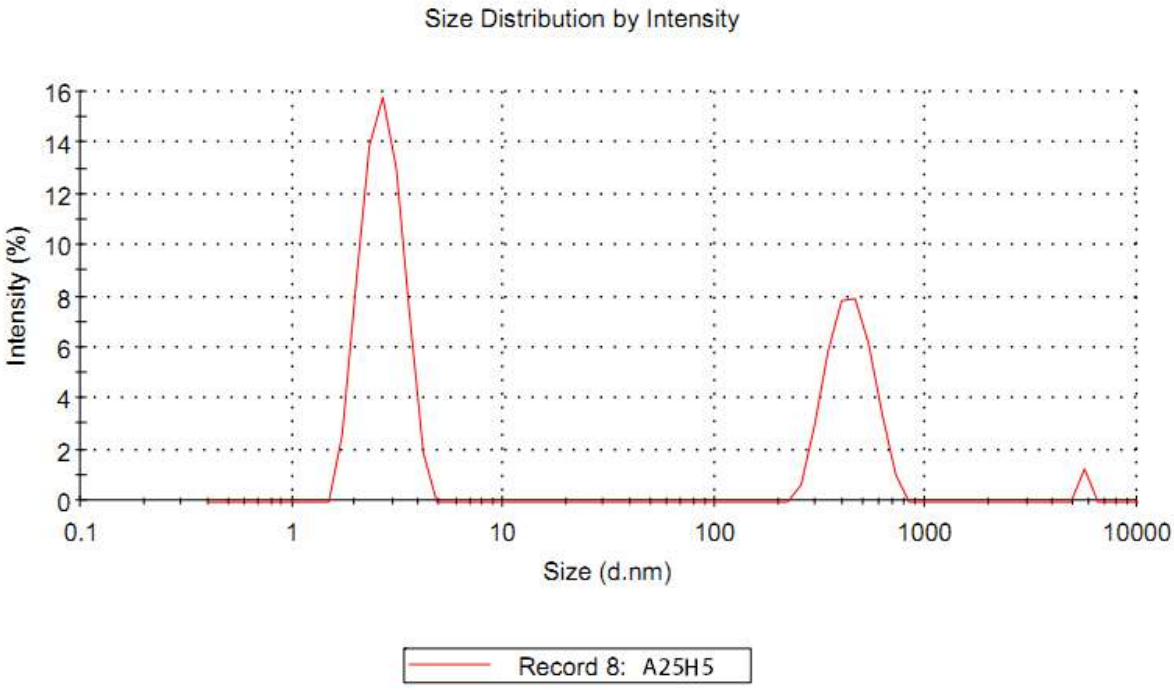


Figure 3.2 DLS result of sample A25H5

In figure 3.3, size distribution of particles diameter for A30H5 is given, mono-model distribution can be seen for the diameter size and the average diameter size for this solution is 11,02 nm.

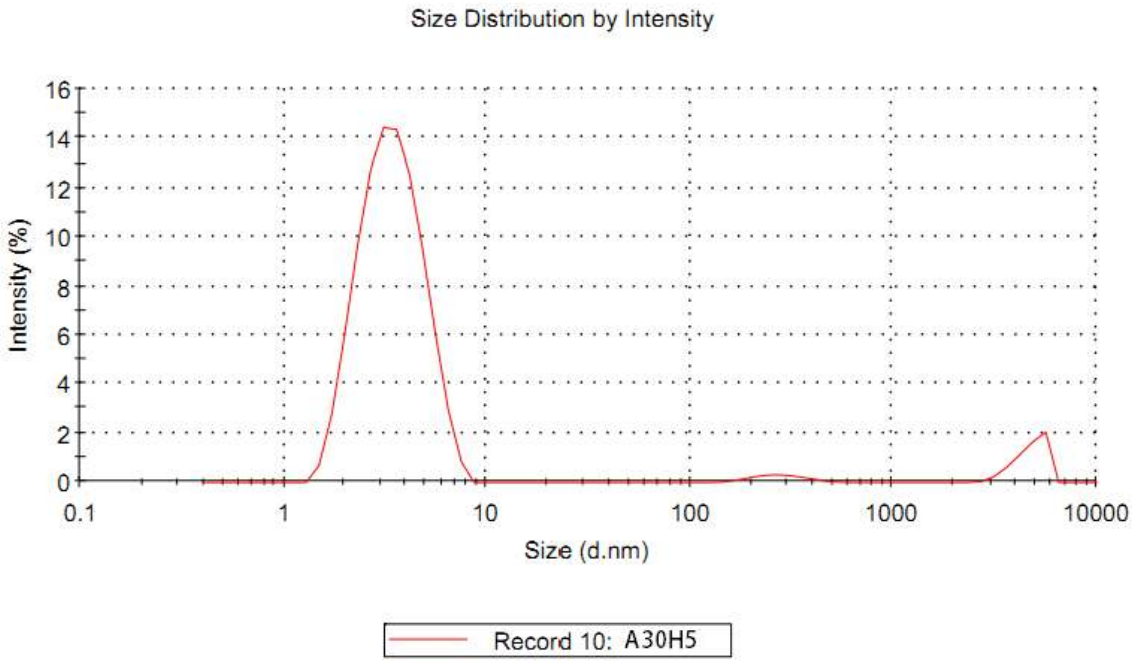


Figure 3.3 DLS result of sample A30H5

The size distribution of particles diameter for A40H5 can be seen in figure 3.4, due to the one peak there is mono-model distribution for the diameter size and the average diameter size for this solution is 4,333 nm.

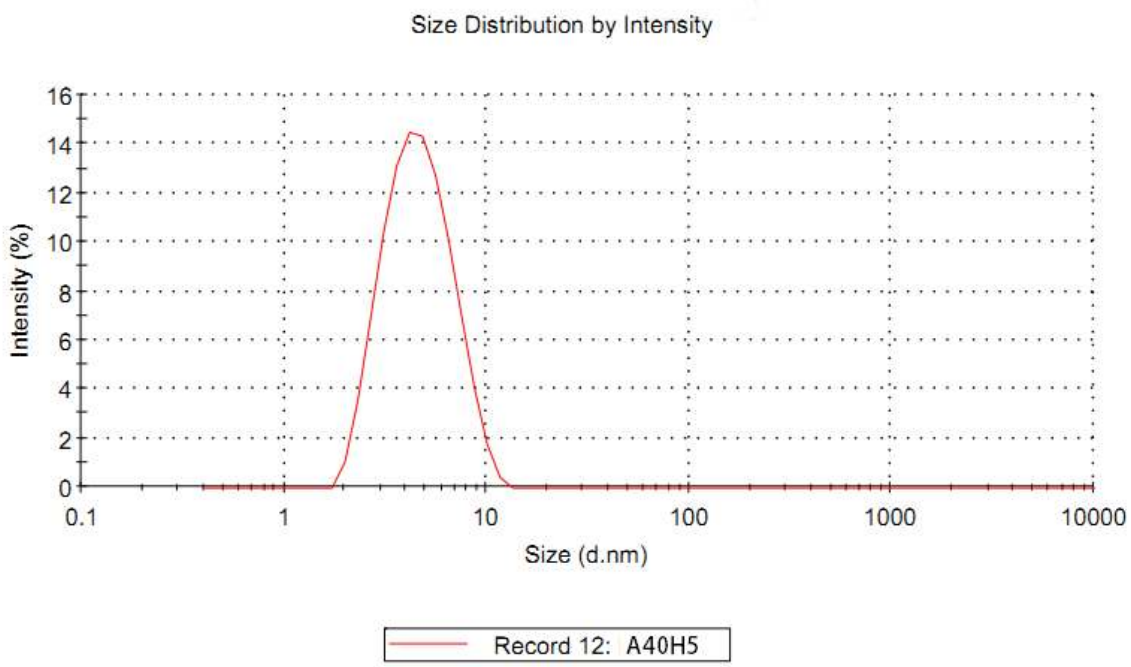


Figure 3.4 DLS result of sample A40H5

In figure 3.5, mono-distribution of the particles diameter for A50H5 is given, the average diameter size for the sample is 6,041 nm.

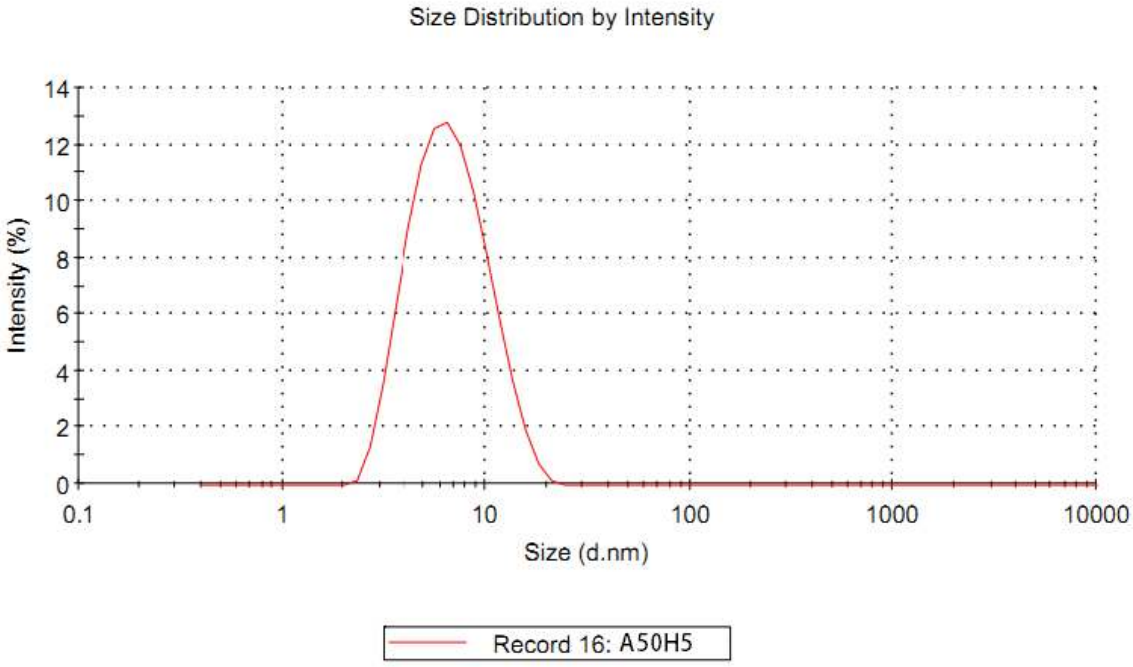


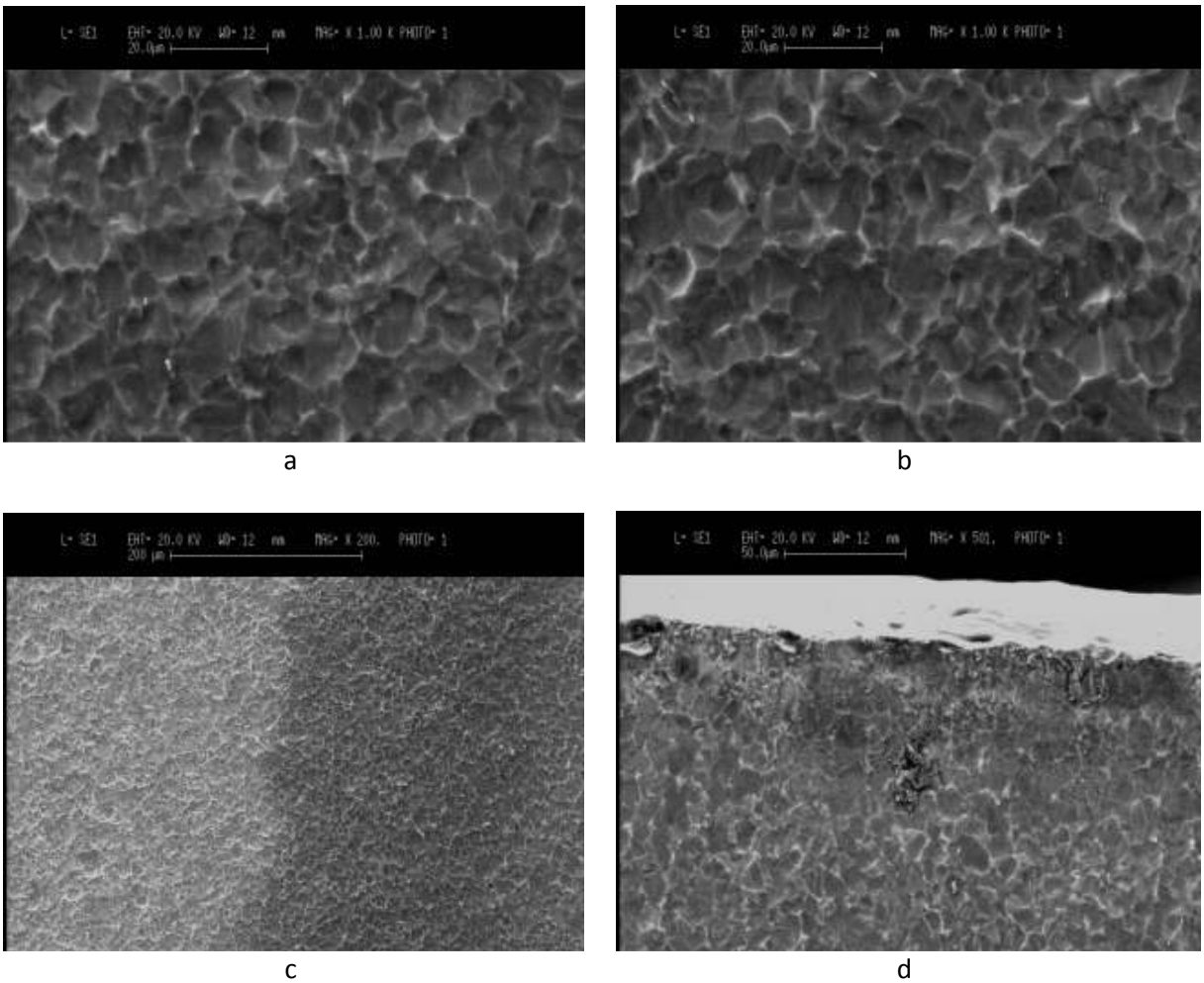
Figure 3.5 DLS result of sample A50H5

For the sols A40H5 and sol A50H5, we see mono-model distribution with one peak in the figure 3.4 and 3.5 and they show that the particles are intensely in one size and most probably those sols have more stable particle size than the others. Instead the result for the sol A20H5 and A25H5 has also mono-model distribution but less precise which can be seen in figure 3.1. Due to the DLS results, we believed that A40H5 and A50H5 are more stable in the sense of particle size.

3.2. Surface Characterization

3.2.1. Scanning Electron Microscope Analyzes

In the figure 3.6 it is possible to see surface of the sample A15H5 with dipping velocity 50 mm/min. In the image a and b we see the surface of the coating with 50mm/min velocity under 1000X magnification, the image c shows the interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification.

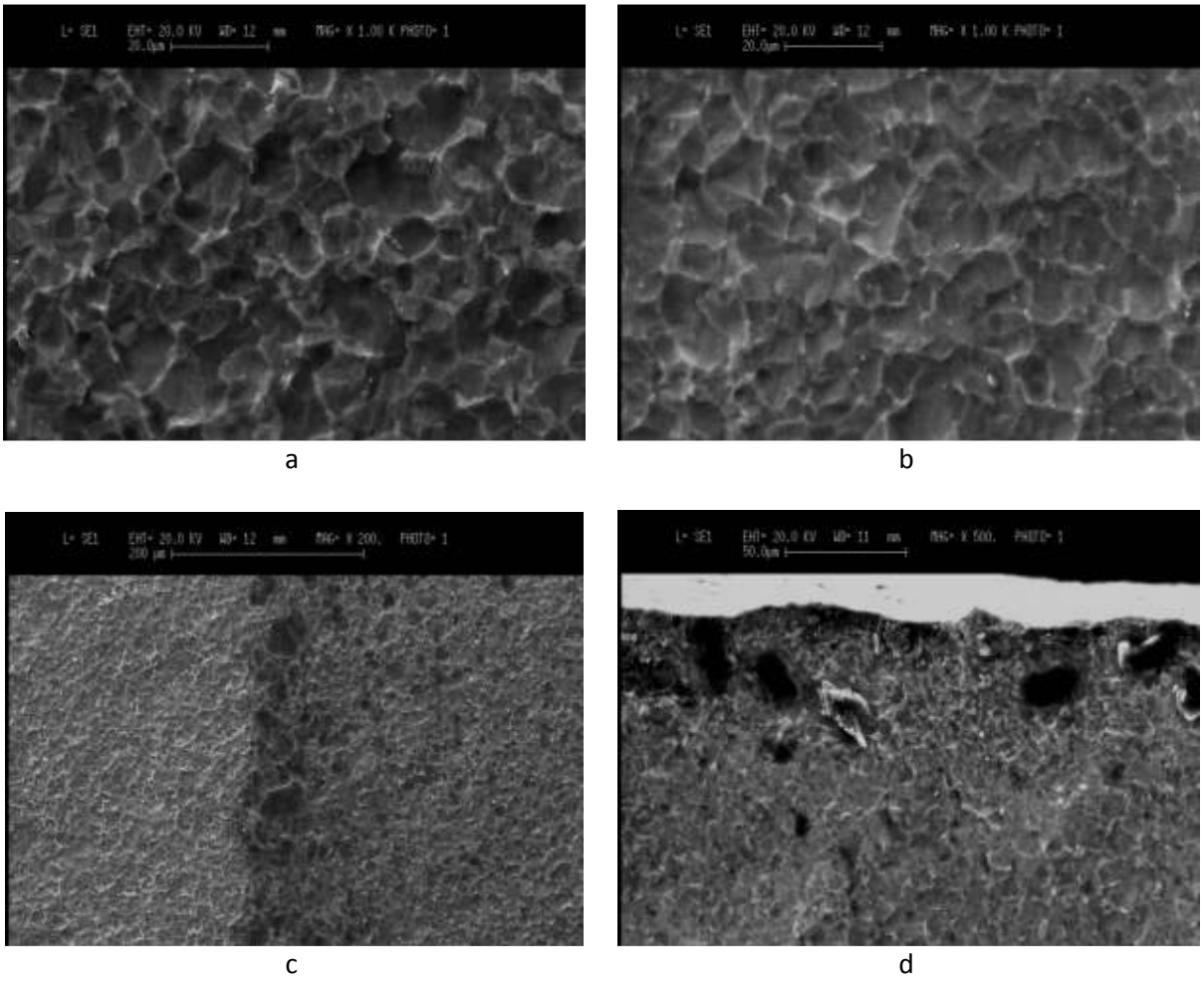


a b

c d

Figure 3.6 SEM images of the sample A15H5 with dipping velocity 50 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.7 it can be the surface of the sample A15H5 with dipping velocity 100 mm/min. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the interface between titanium and coating, and it is possible to see some cracks on the interface; and in the image d non homogenous coating at the border can be seen with 500X magnification can be seen.

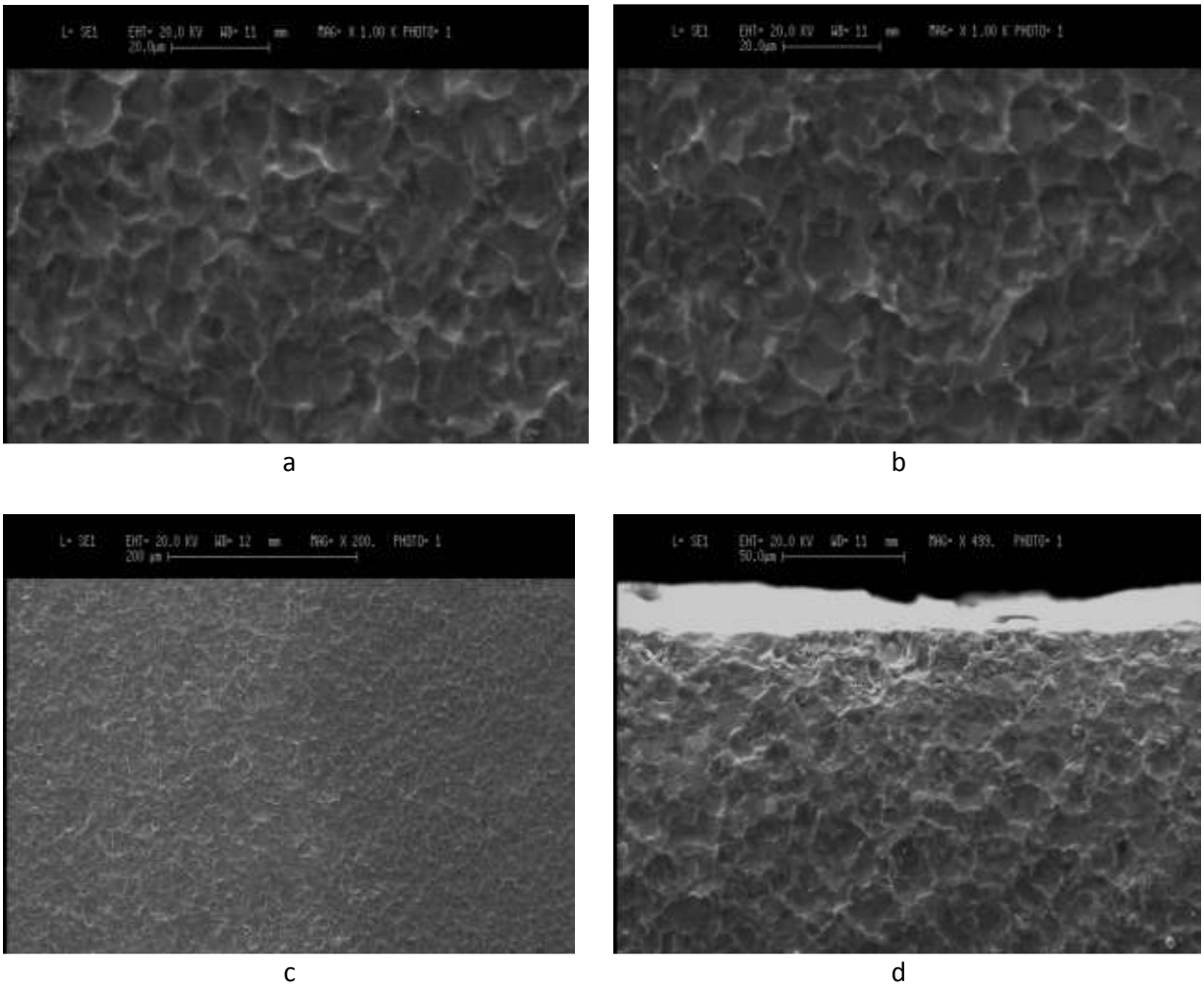


a b c d

Figure 3.7 SEM images of the sample A15H5 with dipping velocity 100 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

It can be seen in figures 3.6 and 3.7 that dipping velocity affects the coating on the surface. Even if the coating has been done with the same sols, we see less homogenous surface with dip coating velocity 100 mm/min especially on the border and interface cracks can be seen.

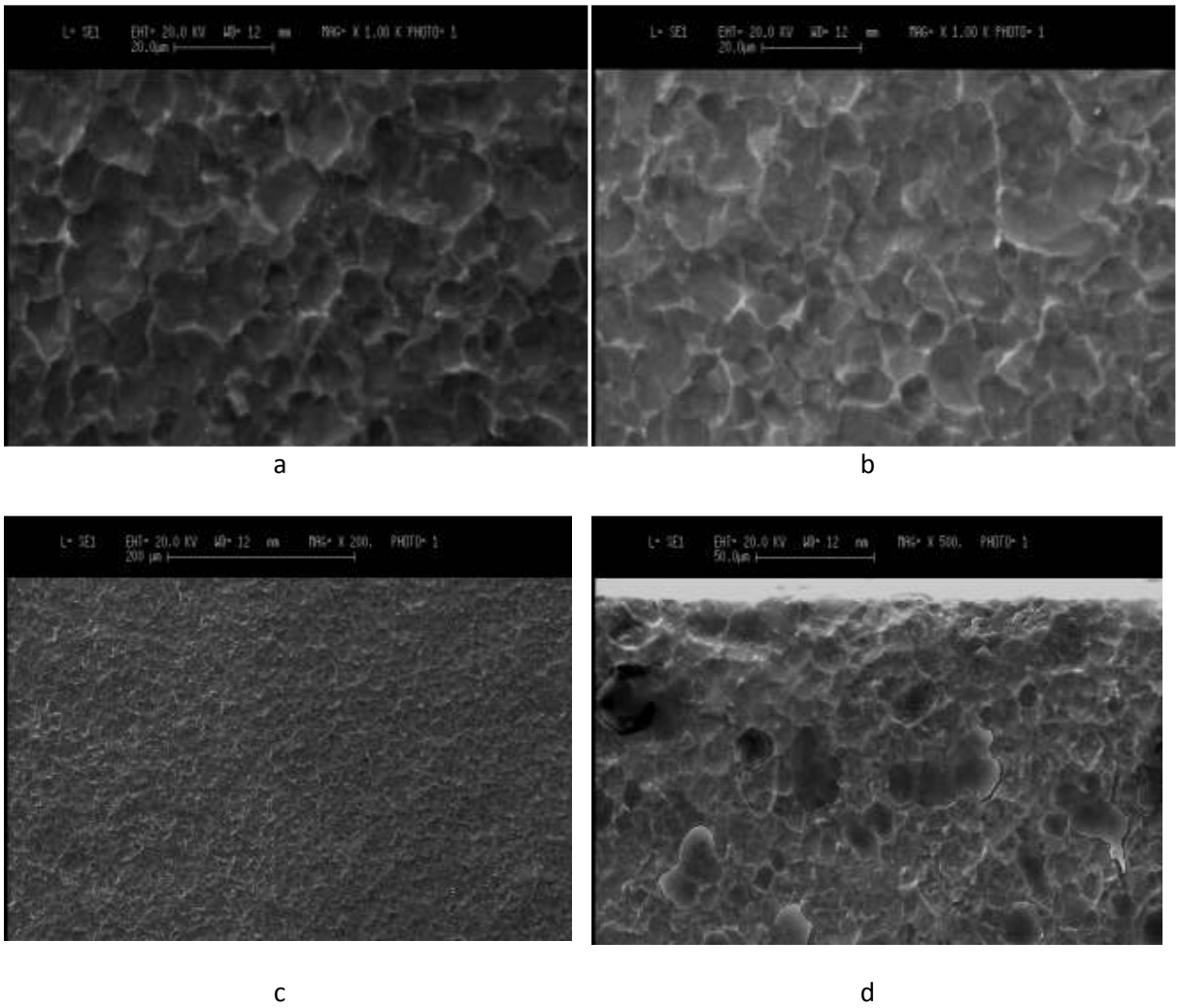
In the figure 3.8 surface of the sample B15H5 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification. The results imply that the sol with the HCl and HNO_3 with the same AMS ratio have the similar surface properties, due to this we continued to analysis only sols prepared with HCl.



a b c d

Figure 3.8 SEM images of the sample B15H5 with dipping velocity 50 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.9 surface of the sample B15H5 with dipping velocity 100 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification. Also here it is possible to see some cracks probably due to speed of 100mm/min that has been excluded of the following experiments.

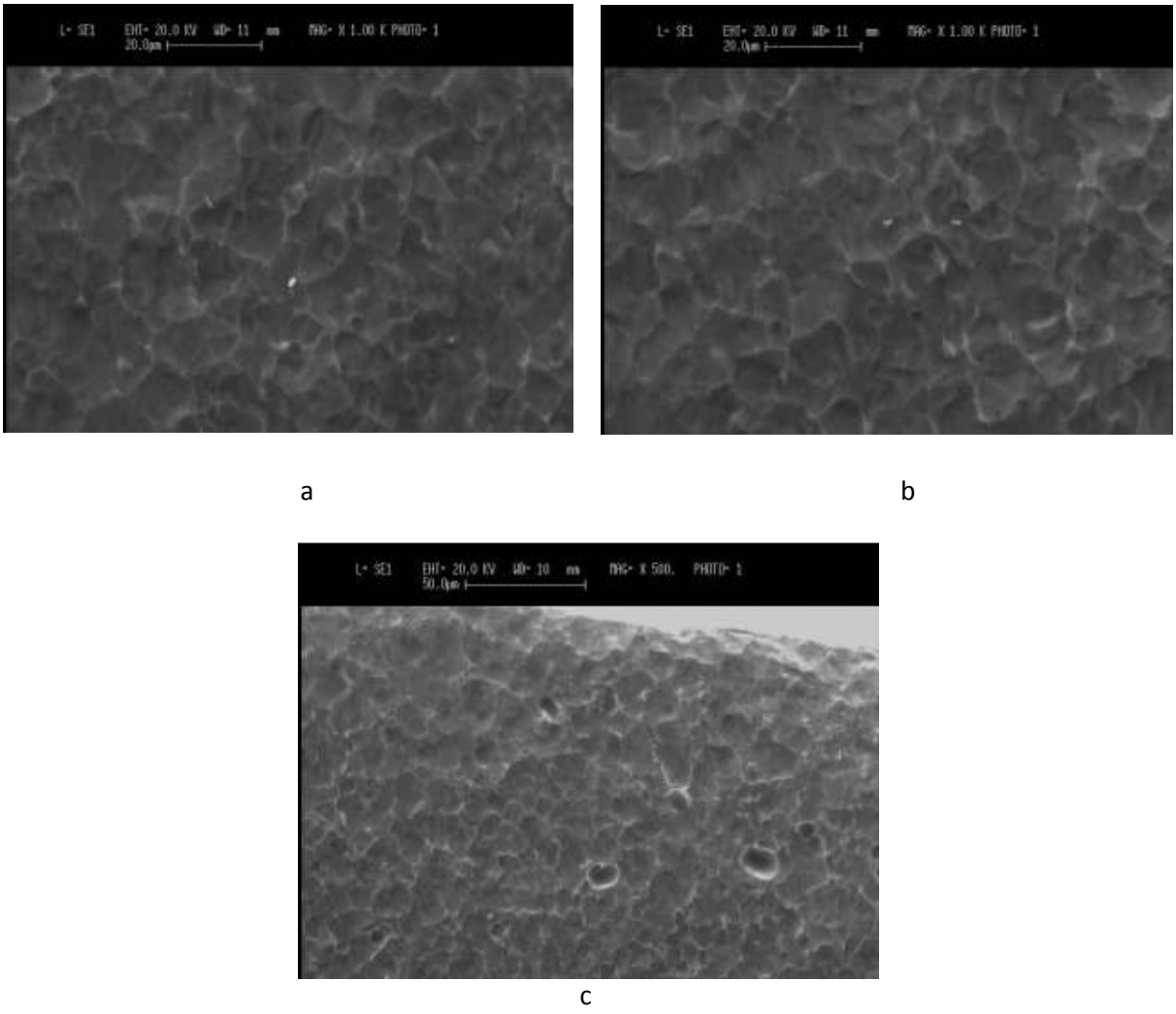


a b

c d

Figure 3.9 SEM images of the sample B15H5 with dipping velocity 100 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.10 surface of the sample A20H6 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the coating at the border with 500X magnification. In images a and b, homogeneous coating can be seen, on the edge it is possible to see some cracks.

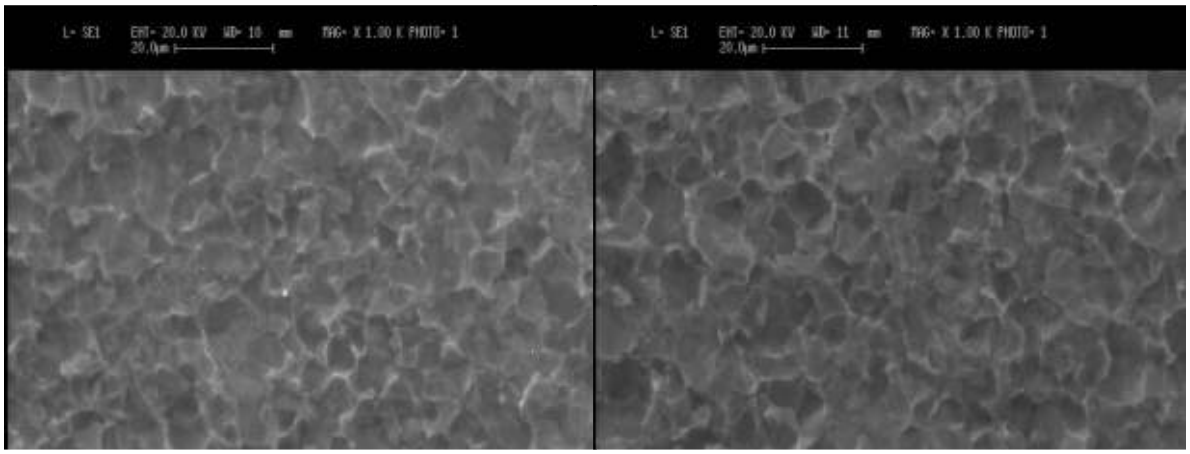


a b

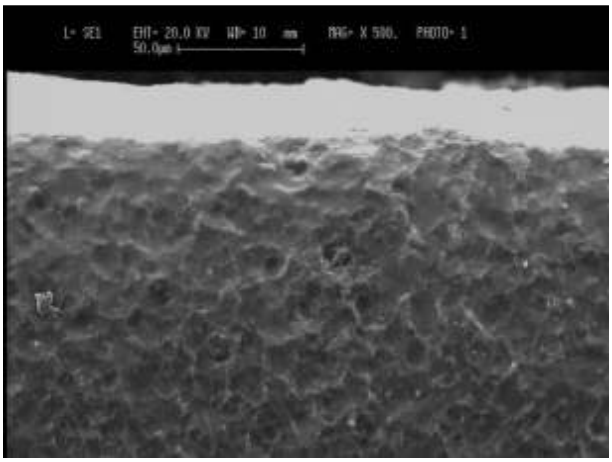
c

Figure 3.10 SEM images of the sample A20H6 with dipping velocity 50 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Border of the coating. (500x)

In the figure 3.11 surface of the sample B20H6 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the coating at the border with 500X magnification. In these images we see the similarity of the surfaces between B20H6 and A20H6.



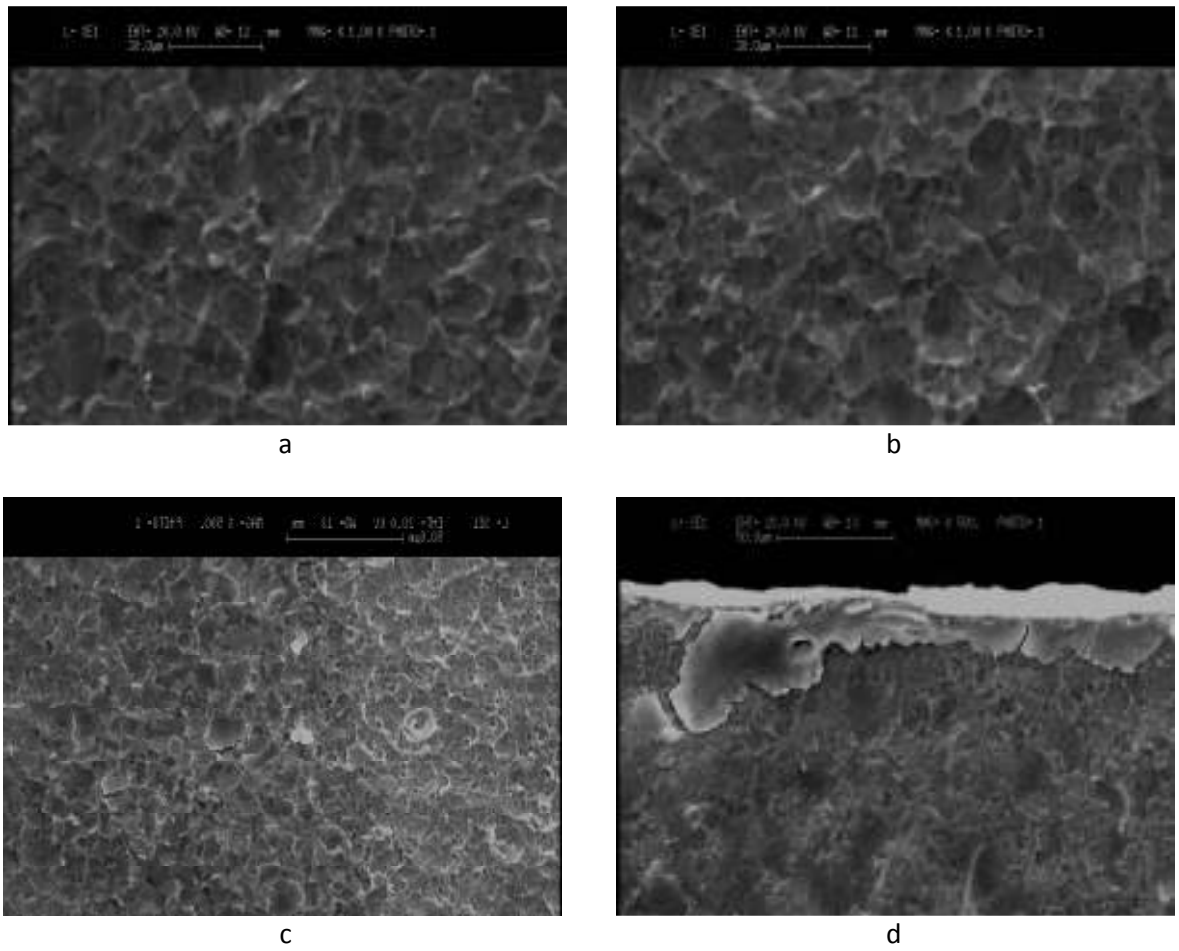
a b



c

Figure 3.11 SEM images of the sample B20H6 with dipping velocity 50 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Border of the coating. (500x)

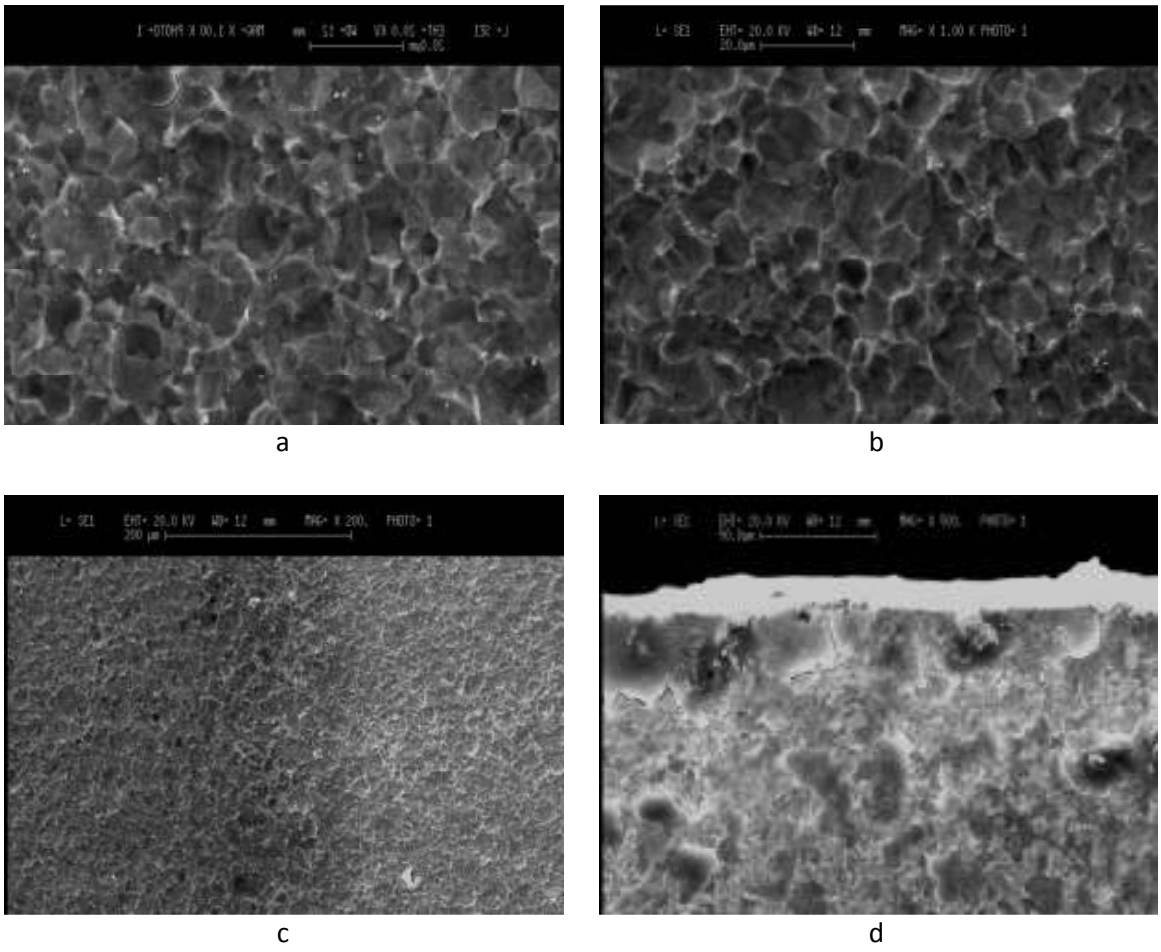
In the figure 3.12 surface of the sample A25H6 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification.



a b c d

Figure 3.12 SEM images of the sample A25H6 with dipping velocity 50 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.13 surface of the sample A25H6 with dipping velocity 100 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification.



a b c d

Figure 3.13 SEM images of the sample A25H6 with dipping velocity 100 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.14 surface of the sample B25H6 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the non homogenous interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification.

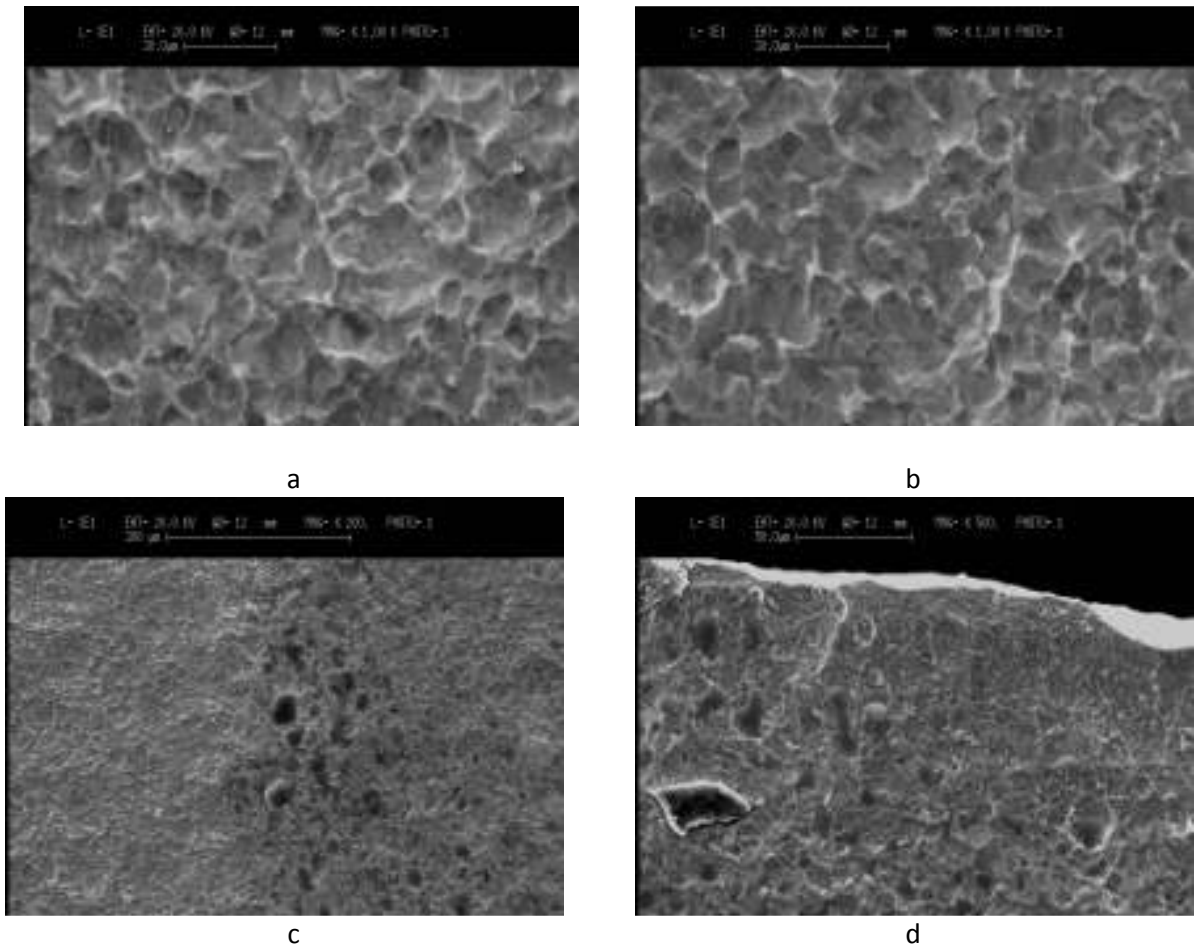


Figure 3.14 SEM images of the sample B25H6 with dipping velocity 50 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.15 surface of the sample B25H6 with dipping velocity 100 mm/min can be seen. In the image a and b we see the surface of the coating with 1000X magnification, the image c shows the interface between titanium and coating, and in the image d non homogenous coating at the border can be seen with 500X magnification.

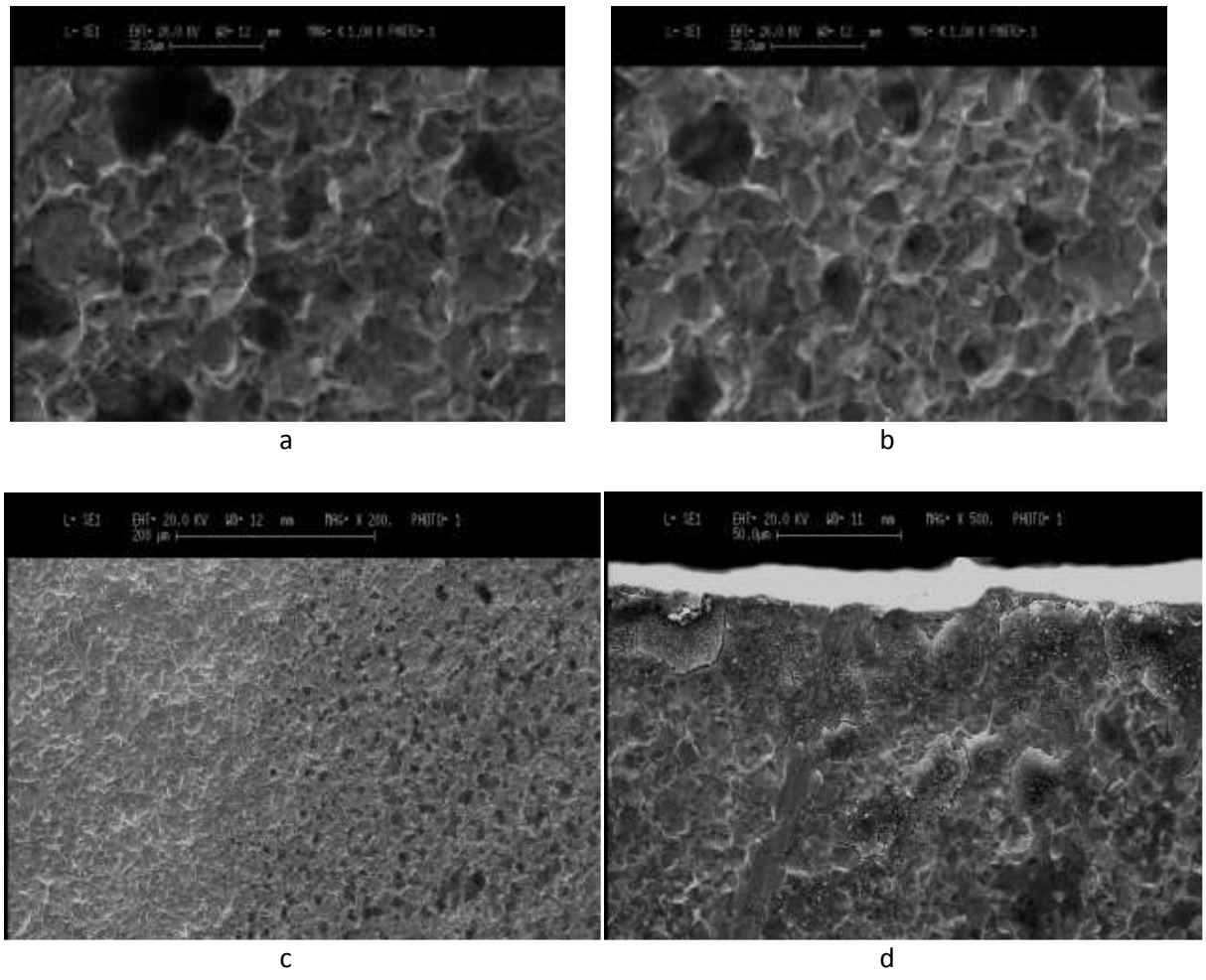


Figure 3.15 SEM images of the sample B25H6 with dipping velocity 100 mm/min. a) Picture of the coating. (1000x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

The results implies that the sol with the HCl and HNO_3 with the same AMS ratio have the similar surface properties, due to this we continued the analysis only sols prepared with HCl. Moreover, In Figure 3.14 and 3.15, it can be seen that when the coating velocity increases, amount of cracks also increases on the surfaces especially on the borders they can be seen clearly. Due to this we continue our experiments only with dipping velocity 50 mm/min. Following SEM images are with 20 %, 30 %, 40 % and 50 % AMS with H=5.

In the figure 3.16 surface of the sample A20H5 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 100X and 1000X magnification, the image c shows the interface between titanium and coating, and in the image d coating at the border can be seen with 500X magnification.

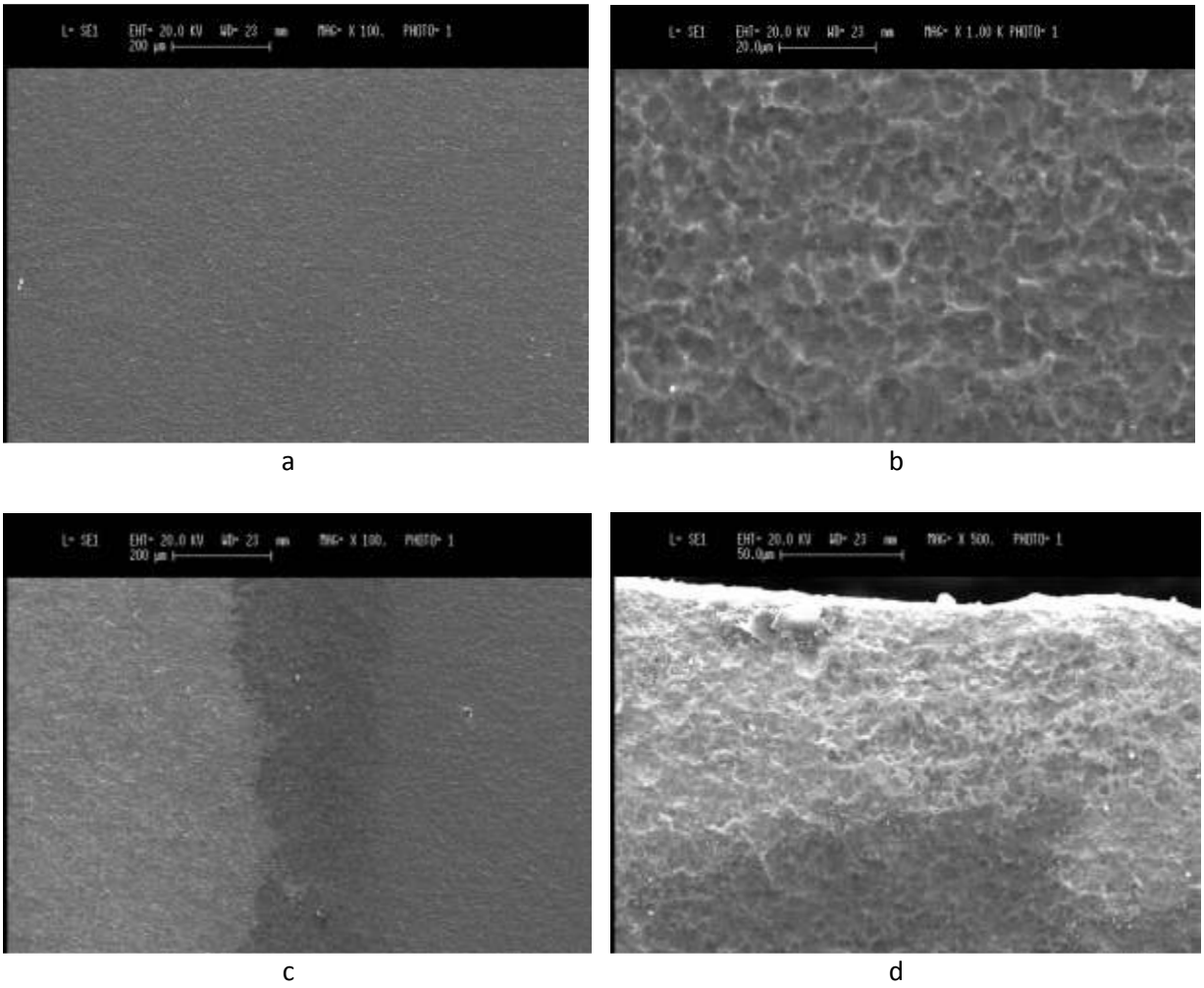


Figure 3.16 SEM images of the sample A20H5 with dipping velocity 50 mm/min. a) Picture of the coating. (100x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.17 surface of the sample A30H5 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 100X and 1000X magnification, the image c shows the interface between titanium and coating, and in the image d coating at the border can be seen with 500X magnification.

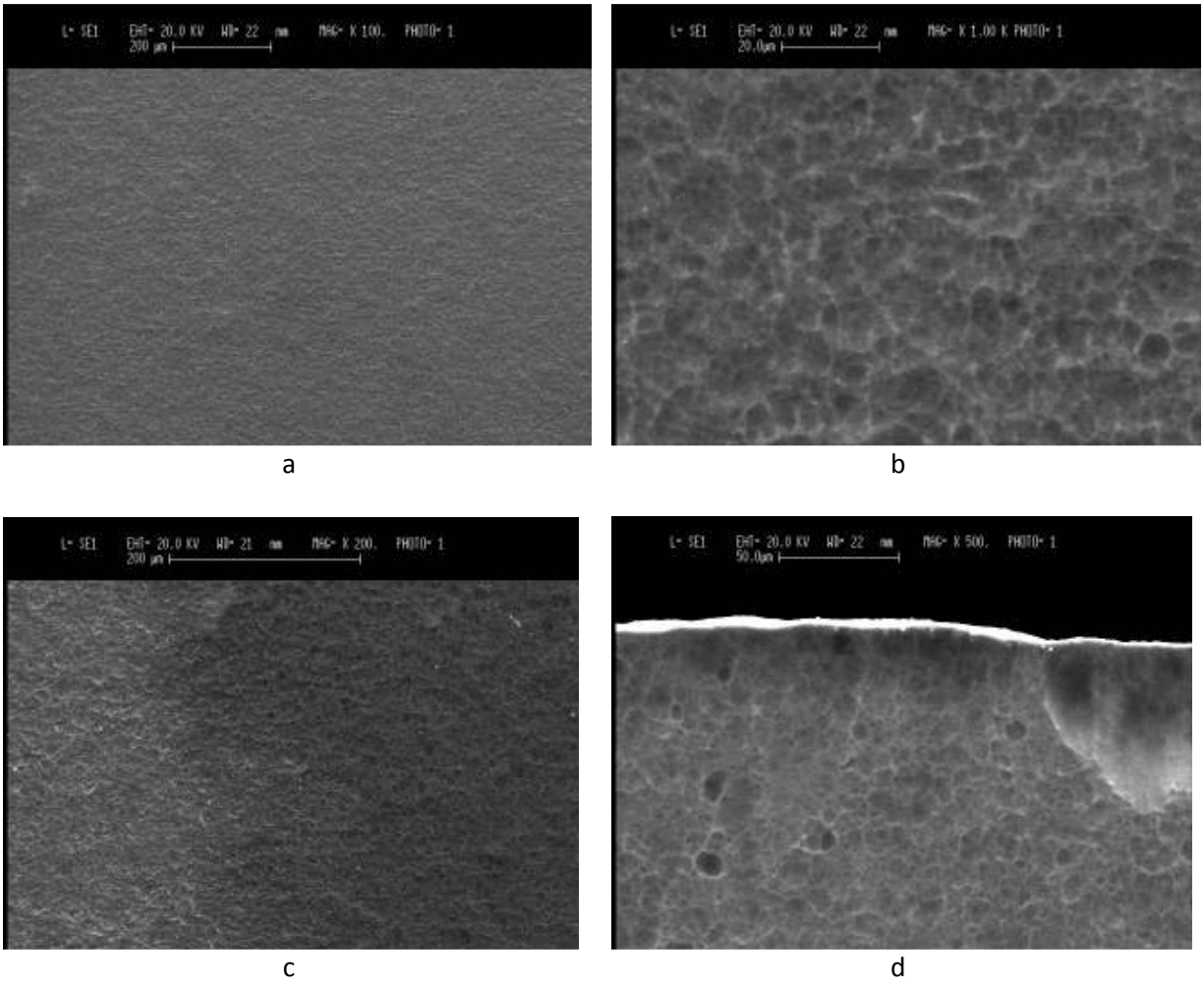


Fig. 3.17 SEM images of the sample A30H5 with dipping velocity 50 mm/min. a) Picture of the coating. (100x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.18 surface of the sample A40H5 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 100X and 1000X magnification, the image c shows the interface between titanium and coating, and in the image d homogenous coating at the border can be seen with 500X magnification.

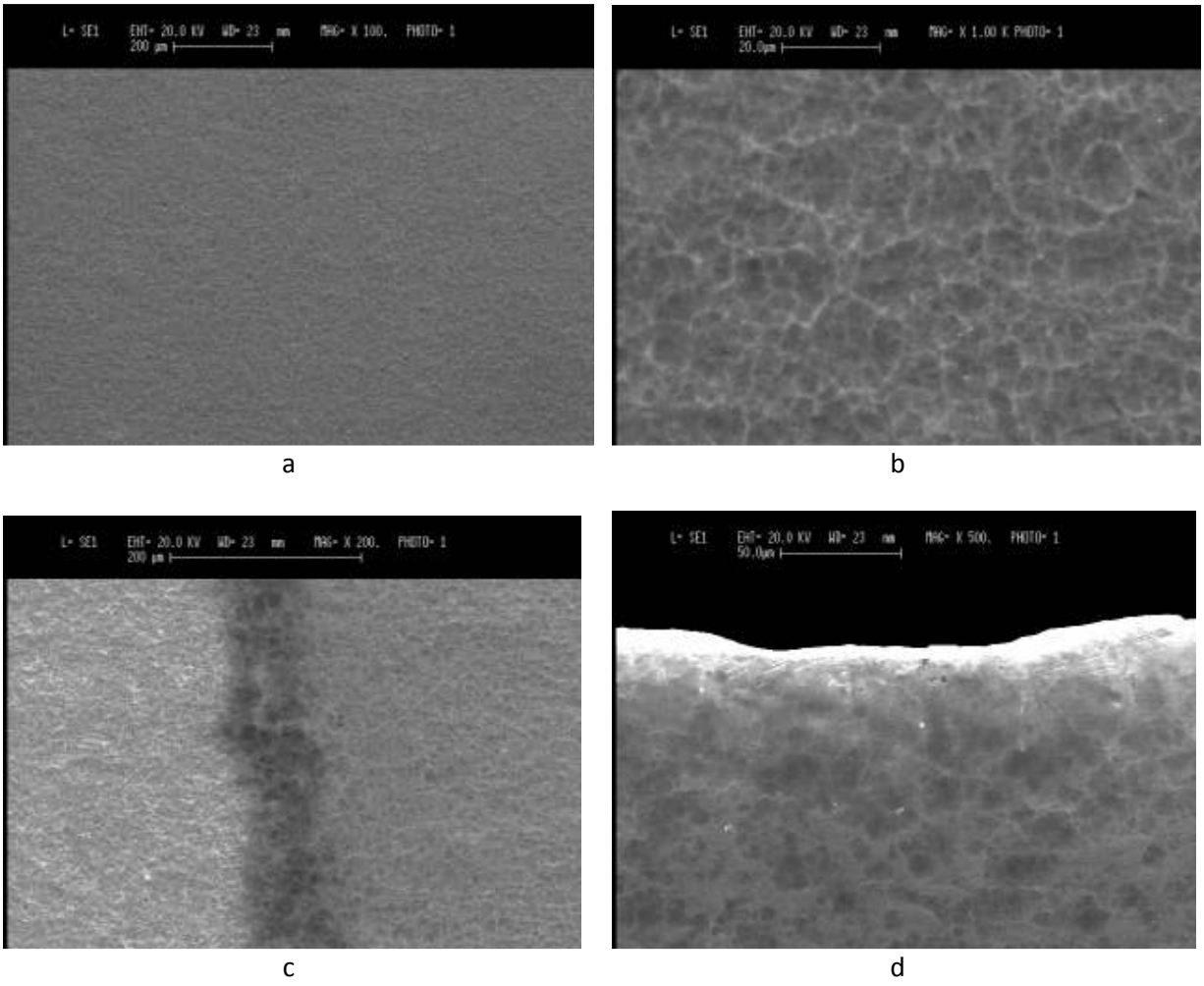


Fig. 3.18 SEM images of the sample A40H5 with dipping velocity 50 mm/min. a) Picture of the coating. (100x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

In the figure 3.19 surface of the sample A50H5 with dipping velocity 50 mm/min can be seen. In the image a and b we see the surface of the coating with 100X and 1000X magnification, the image c shows the interface between titanium and coating, and in the image d homogenous coating at the border can be seen with 500X magnification.

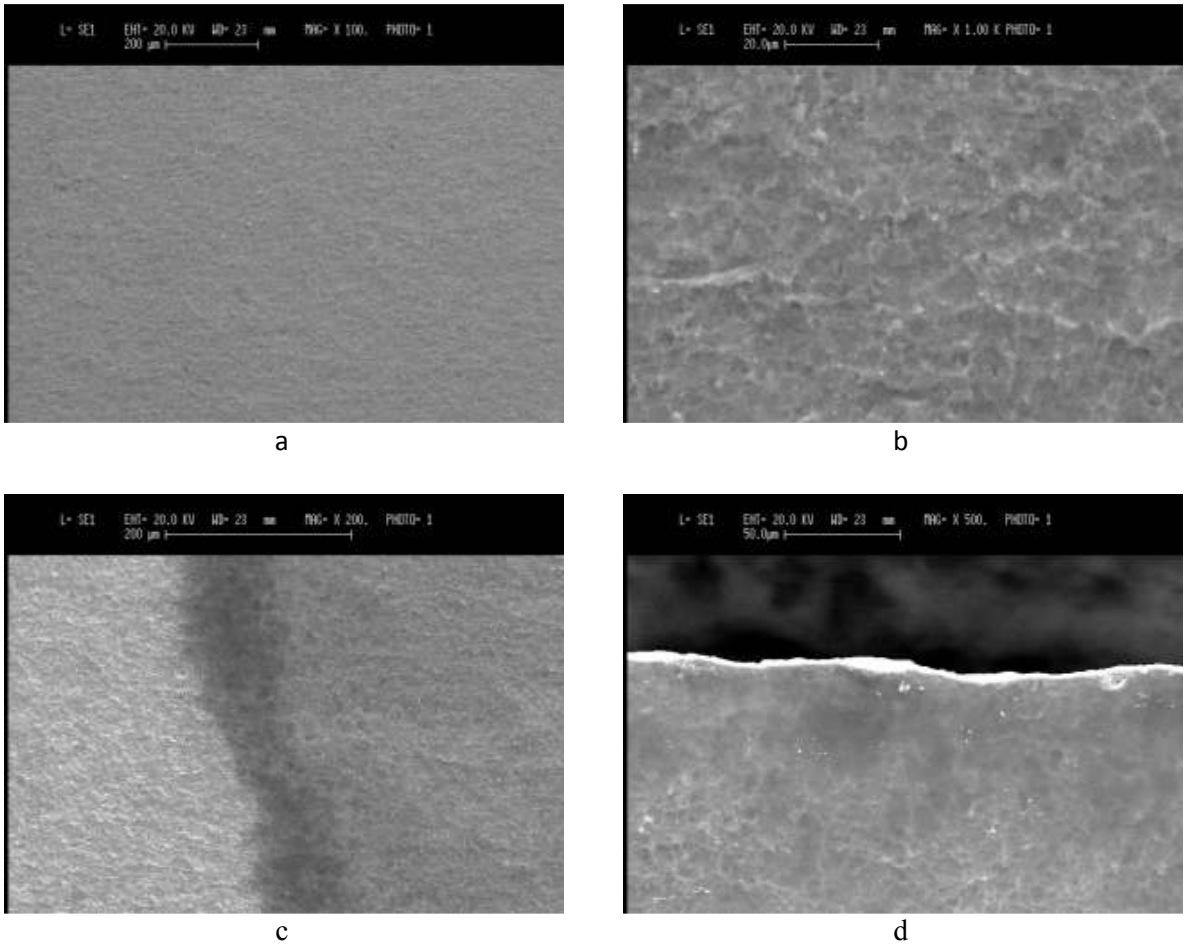


Fig. 3.19 SEM images of the sample A50H5 with dipping velocity 50 mm/min. a) Picture of the coating. (100x) b) Picture of the coating. (1000x) c) Interface between pure titanium and coating. (200x) d) Border of the coating. (500x)

3.2.2. Glow discharge optical emission spectrometry (GDOES) before grafting

The relative intensity of chemical elements had been evaluated by GDOES analysis. The result of GDOES analysis for A40H5 and A50H5 is given in figure 3.20 and 3.21. It was possible to quantify the intensity of the present chemical species on the surfaces before SPDP grafting.

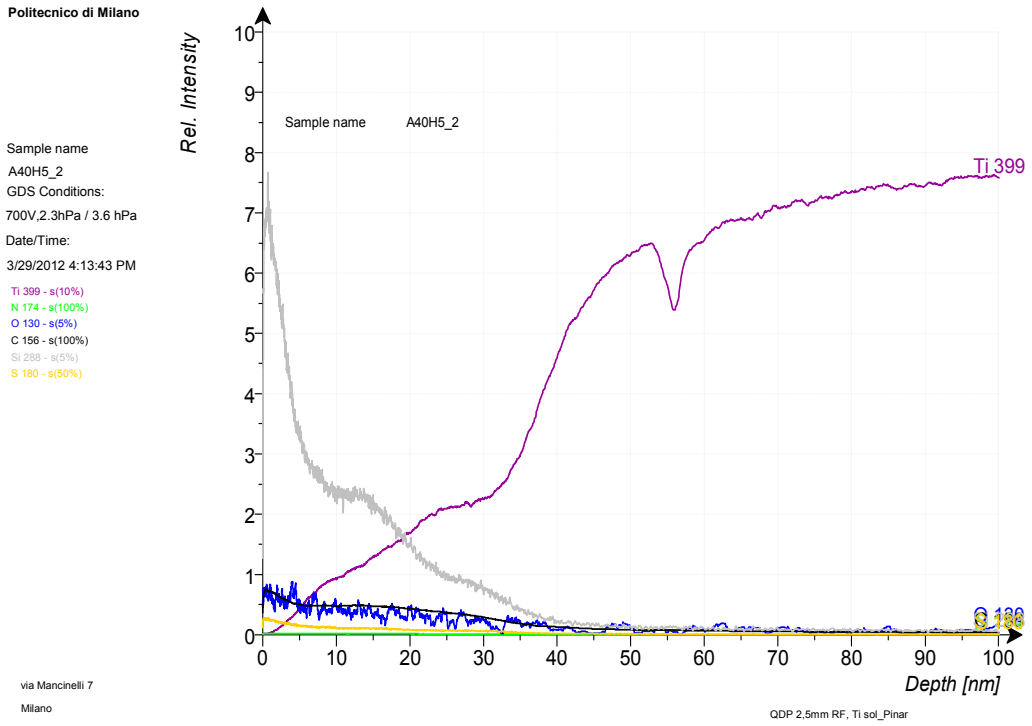


Figure 3.20 GDOES analysis of the sample A40H5

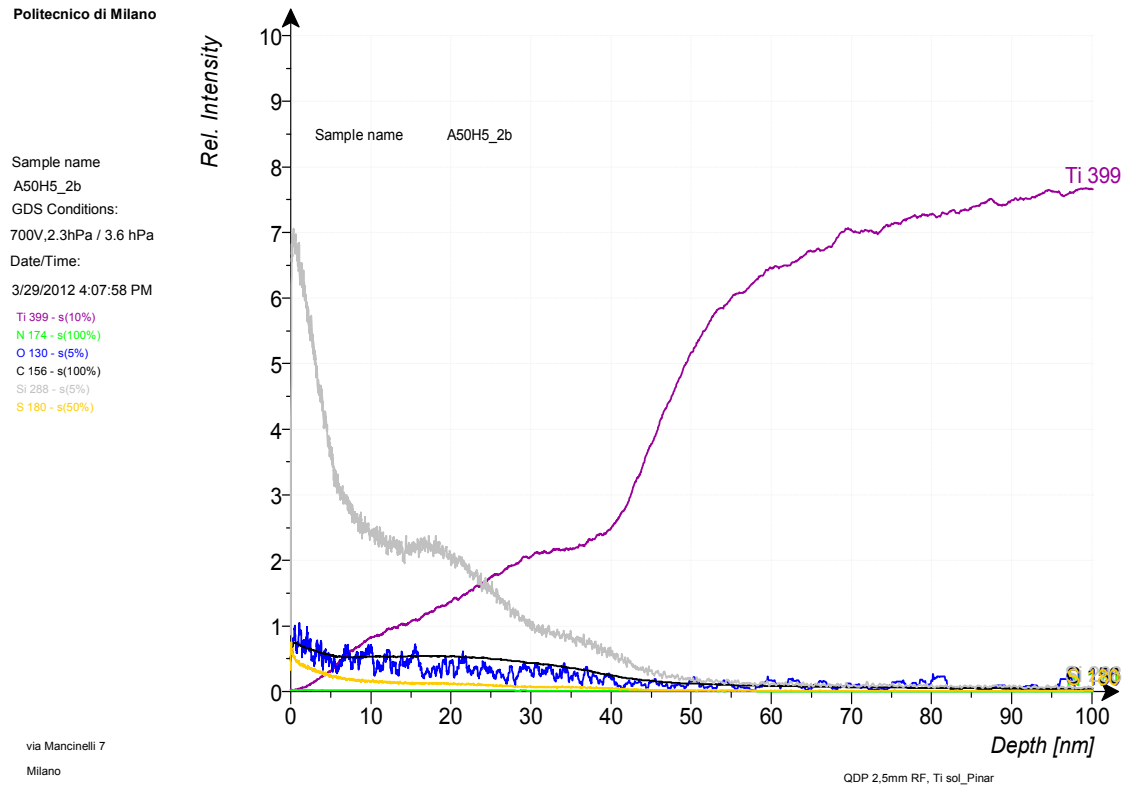


Figure 3.21 GDOES analysis of the sample A50H5

3.2.3. Spectrophotometer

In this part of the experimental work the kinetics of the immobilizing reaction between sample surface and SPDP were directly analyzed by the monitoring of the absorbance change at 343 nm corresponding to the release of 2-thiopyridone from the reaction between SPDP and βME. The surface conjugated with SPDP was quantified by incubating 10 µl 1% βME on the surface and the release of 2-thio-pyridone was measured at 343 nm by reading the absorbance with Nanodrop spectrophotometer.

The sample with 15% AMS ratio was like the blank analyze. The sample with 20% AMS ratio has absorbance lower absorbance; in table 3.1 we can see that absorbance increases with increasing of AMS ratio.

Table 3.1. Absorbance values of samples

Name of the sample	nm	Average Absorbance (Abs)
A20H5	343	0,002667 ± 0,000943
A30H5	343	0,003667 ± 0,001247
A40H5	343	0,03666 ± 0,000943
A50H5	343	0,253 ± 0,019815

3.2.4. Glow discharge optical emission spectrometry (GDOES) analysis of grafted surfaces

The result of GDOES analysis after SPDP grafting for A40H5 and A50H5 is given in figure 3.22, and 3.23 and the relative intensity of the sulphur component increase on the A50H5 sample and this augmentation can been in figure 3.24.

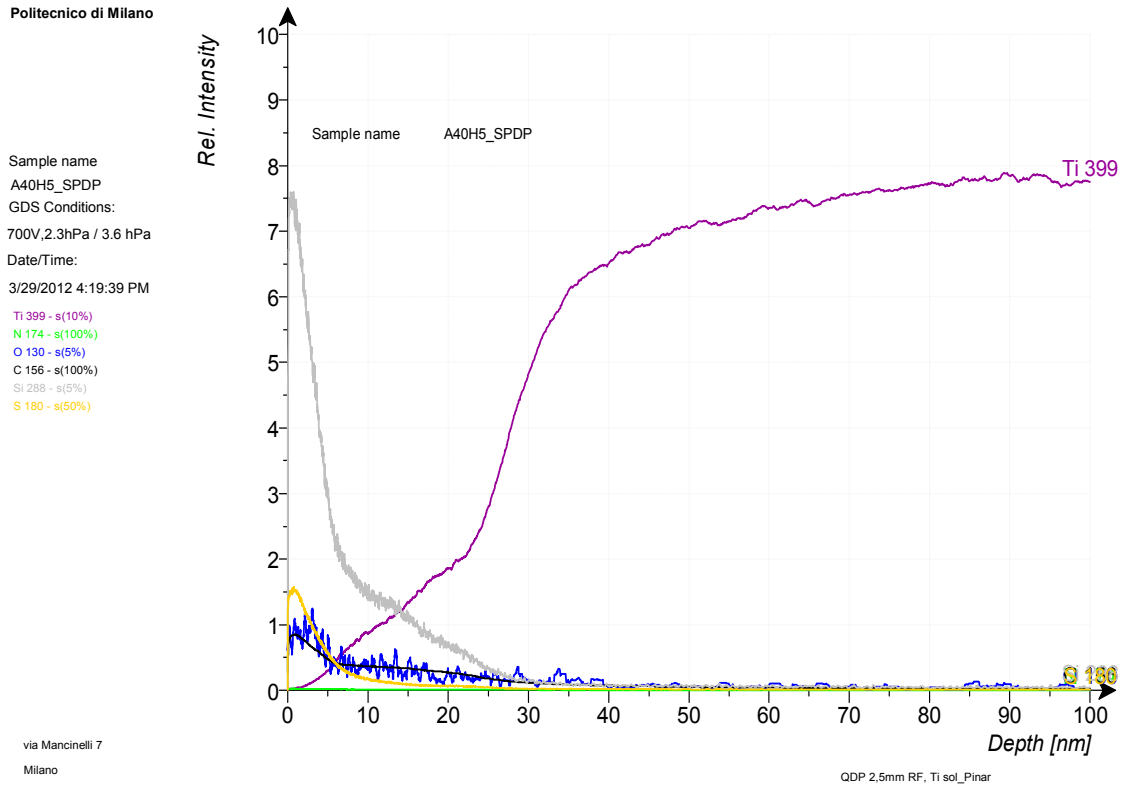


Figure 3.22 GDOES analysis of the sample A40H5 after SPDP grafting

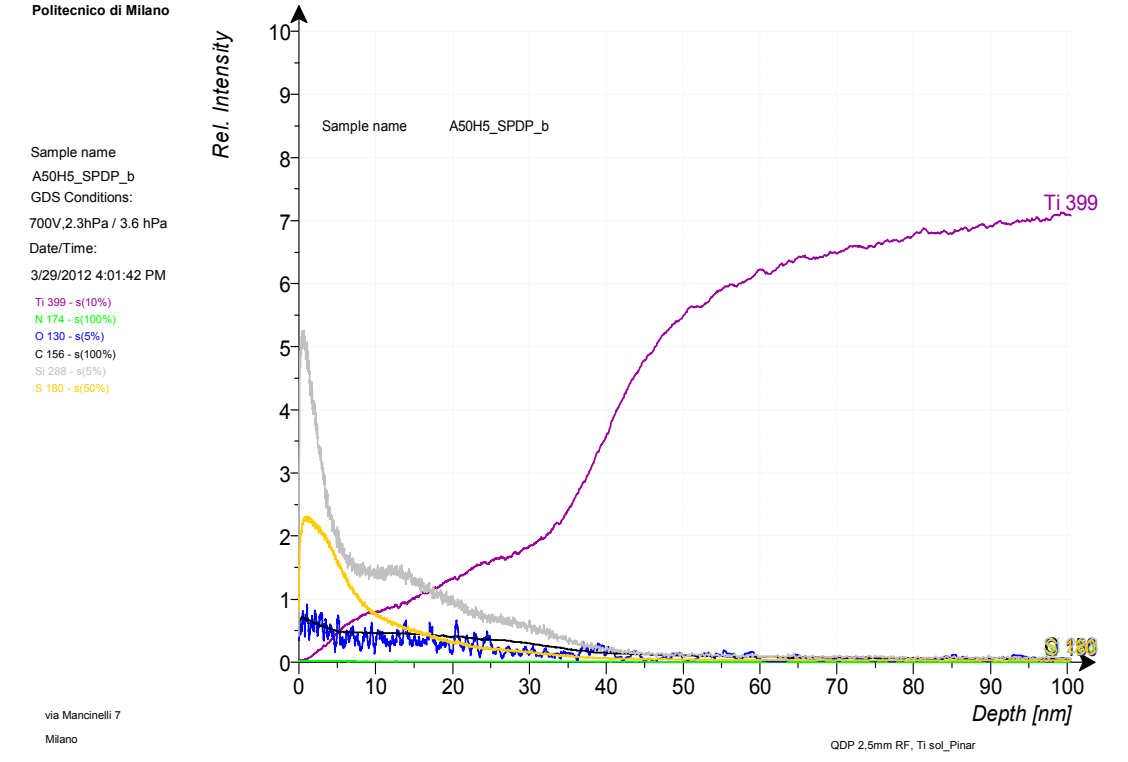


Figure 3.23 GDOES analysis of the sample A50H5 after SPDP grafting

In order to be able compare the intensity and depth of the sulphur on the surfaces an overlay graph illustrated (fig. 3.24). It is clear from the graph that due to SPDP grafting the increasement of S intensity can be seen for all samples. The graph emphasize that, intensity of the S on the surface for A50H5 with SPDP grafting is higher than the other samples, moreover it can be seen from the graph that the depth of the S for A50H5 is higher than the other samples.

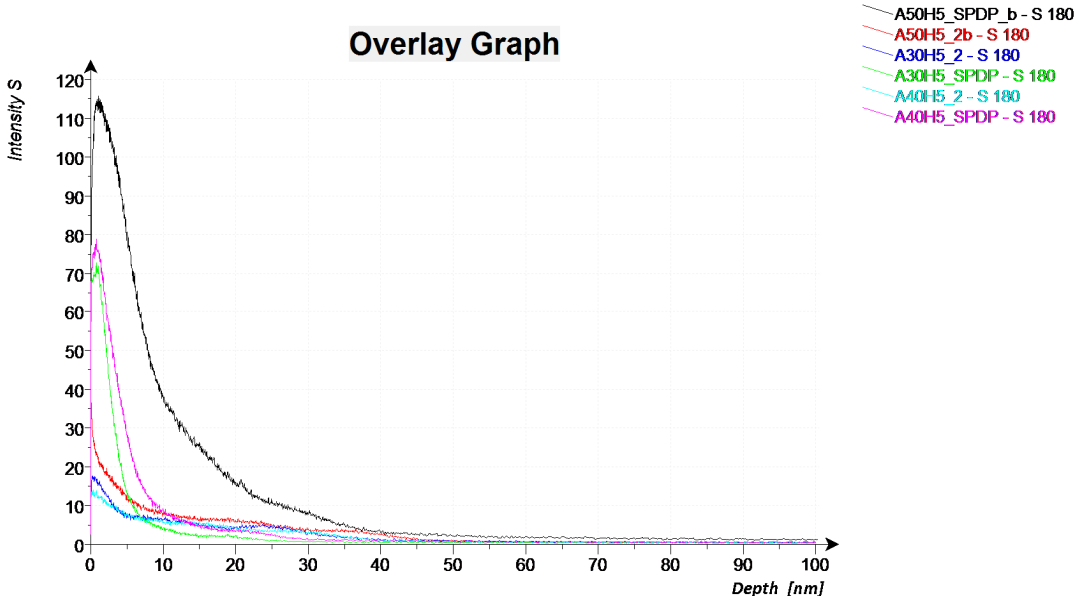


Figure 3.24 Overlay graph of the S intensity for the samples

4. CONCLUSIONS AND FUTURE WORK

4.1. Conclusions

In this study, a controllable method for the immobilization of biomolecules on the titanium surface by chemically grafting with sol-gel technique was performed. The aim of this work was functionalize the titanium surface for various bio-medical applications obtained through biomolecules grafting. The biomaterial substrate for use in a number of medical devices such as vascular grafts, vascular stents, biosensors, dental devices, drug delivery systems, or bodily implants of any kind [25]. Surface functionalization is aimed to perform with N succinimidyle 3-(2pyridyldithio) propionate (SPDP) grafting on the surface by sol-gel technique.

In the experimental work, it has been studied to improve surface properties with SPDP immobilization in order to improve peptide conjugation with sol-gel techniques. Due to achieve the purpose of producing stable coatings on the titanium surface the sols prepared with different water ratios,

(H = H_2O / (TEOS + ¾ Additive)) and AMS ratios. For the sols prepared with H=4 gelation occur after 1 hour stirring because probably they didn't have enough water as a solvent, due to this, we continued to work with H=5.

The changes in the AMS ratio with increasing values showed a similar tendency to that of the grafted SPDP concentration on the surface. The highest absorbance value, 0,253 with 343 nm, and most homogenous surface was obtained on the sample which was coated by sol A50H5 with a highest AMS ration 50 %. This result can be attributed to the dependence of AMS ratio on the SPDP.

The results of the SPDP concentration measurement revealed that the silica, used as an initiator for grafting SPDP, were effectively introduced on the titanium surface by dip coating and silica and NH groups' intensity on the surface is depended on the sol which has been used. The grafted SPDP on the titanium surface was determined by the concentration formed on the titanium surface by the GDOES, and spectrophotometer analysis. According to the results, SPDP was grafted on the titanium surface coated with the 30%, 40% and 50 % AMS ratio sols. The immobilization of various types of peptides on the surface can perform with the usage of SPDP as a spacer.

The advantage of the method used in this work is easy application; it is easy to graft biomolecules with this technique because grafting can be performed at room temperature without any specific atmosphere. Using this method, various functional groups can be introduced onto biomaterial surfaces and their surface properties can be controlled as well. These functional groups can be further utilized to covalently immobilize various biomolecules such as enzymes, proteins, and DNAs on the

biomaterials surface [21]. According to the type of biomolecules grafted on the surface, biomaterials surface can promote or not cell or bacterial addition on specific cells.

4.2. Future work

The method which has been used in this study is an easy chemical surface functionalization technology. The method is quite simple, economic and does not require special atmospheric conditions. After SPDP grafting performed, surface of the substrate was ready for bonding to any biomolecules. The whole grafting process took around 3 hours or so, which is quite fast and the process can be improved by more researches.

In this work we tried to prepare the proper surface for biomolecules conjugation especially peptides, RGD. Future researches on this field, should aim to improve and optimize SPDP grafting conditions and perform biomolecules grafting on the SPDP. The results obtained with this experimental study, promote SPDP grafting may perform with dipping the samples in the SPDP instead of drop wise grafting, in order to make reaction more stable and have more certain results.

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