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Primary Osteintegration in the Study of Biomimetic Surfaces

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1. Introduction

The study and use of biomaterials dates back to very ancient cultures such as that of the Mayas, the Egyptians and the Phoenicians; however the study on biomaterials as a science is to be considered recently developed, indeed dating back only to half of the nineteenth century. Archeological finds showed that Phoenicians used to tie artificial teeth to the natural ones with golden wires and that the Egyptians used different materials to build prosthesis. The first find that reached us, dates back to the Egyptian era (1000 - 600 b.C.) and is a woman's toe. The device named "Cairo Toe" is made of wood and skin, and is assembled in order to be flexible and both the shape and the wearing effect of time suggest that it helped its owner to walk; then it is considered the first functional prosthesis (Huebsch & Mooney, 2009). More finds instead, such as for hands and feet or a finger of parchment and chalk kept at the British Museum in London, are only aesthetic substitutes, since this culture used to carefully get ready for the afterlife. As far as regards oral implantology, the first find reached to us is a Maya's era mandible's splinter with three implants made of half shell which were the substitute of three missing foreteeth, datable approximately to VIII Century a.C., discovered by the archeologist Wilson Popenoe, in 1931, during some diggings in Playa de los Muertos, in Honduras. According to some studies made by the Brasilian, but native of Italy, dentistry Amedeo Bobbio during 1970, the three shells were not implanted in the relative alveolus after death, but during the life. Indeed, by the find's X-Ray, he noticed a real, "osteointegration", as we woluld say today all around the shells, certainly due to their largecontent of calcium phosphate (Bucci Sabattini, 2007).
This find represents a fundamental stage in biomaterials' history being the first osteointegrated implant which came to us. Considering the recent history, since '800, there are several documentations of efforts and experiments for orthodontic implants. The greatest development of endosseous implantology has been in the '70s with Stefano Tramonte's suggestion to use titanium to replace surgical steel as an implant's material. The Dutch School, also around the half of '70s, introduced the use of calcium hydroxyapatite inspired by previous studies on tricalcium phosphate. The osteointegrated implant methodology was initaited in the '80s by doctor Per-Ingvar Branemark, professor in applied biotechnologies at University of Gothenburg, who developed the osteointegrated implants in the oral surgery, providing inspiration for other applications. Branemark defined osteointegration as the direct structural and functional connection between living bone and the surface of a load bearing artificial implant (Brånemark et al., 1977). The basic

requirement to establish real and lasting tissue integration to biomaterials, is based on detailed understanding of hard and soft host's tissues response, the surgical preparation and implantation of the device (Albrektsson et al., 1981). This definition represents the big novelty in implantology during '80s, in fact, is no doubt that to guarantee a lasting bone healing there has to be a direct contact between bone and the alloplastic material. All recent literature shows, unmistakably, that the implant, using the "osteointegrated" methodology, is a reliable, valid and predictable solution.

2. Titanium: from bioinert material to bioactive material

The modern biomaterials' science since the '80s has been characterized by a growing emphasis on the identification of specific parameters which are critical to their performance. The union of biomaterials' science with new emerging insight from biology studies, as cell-matrix interaction, cell signaling processes, (Albrektsson & Wennerberg, 2005), creates a multidisciplinary approach to biomaterial's science. Because there are several different approaches that can be used to study a biomaterial, also its classifications are numerous. Considering the effects of biological environment on the implanted material, a biomaterial can be defined as biostable or biodegradable. Materials able to resist to the change action by the biological environment, with which they are in contact, can be classified as bio-stable; unlike materials that undergo a gradual demolition and a chemical transformation, as result of specific actions made by the host that are classified as biodegradable.
On the other hand, considering the interaction between biomaterial and body, they can be classified as:

- bioinert, material that once inserted into the host does not undergo any modification, and does not encourage any kind of specific response in the surrounding tissue;
- bioactive, materials that induce a specific response of the host tissue in the peri-implant region, due to interactions between the molecules at tissue-implant interface;
- bioabsorbable, those materials that promote a regenerative response in the host tissue, and are gradually absorbed and replaced by newly formed tissue (Park & Lakes, 1979).

In the specific case of endosseous biomaterials, the implant's effect on the new-deposition can be defined as osteoinductive or osteoconductive. Osteoinduction is the ability of a biomaterial to induce bone's new formation in heterotopic situation, which is when it is placed in a non-osteogenic tissue (Wilson-Hench, 1987). The osteoconduction is the capacity of a biomaterial to stimulate and to induce osteogenesis in a vital bone (Wilson-Hench, 1987). Osteoinductive materials are mainly used to treat large bone defects or to regenerate bone where normally would not be a spontaneous regeneration, whereas osteoconductive materials are widely used as osteointegrated endosseous implants. In the matter of osteointegrated implanotlogy, both research and industry, were focused almost exclusively on the use of a bioinert material with intermediate proprieties: the titanium. It now represents a good compromise between mechanical and biological requirements. The titanium is considered the first choice for endosseous implants due to its specific proprieties: the high mechanical strength, the high corrosion resistance and the excellent biocompatibility. Titanium's modulus of elasticity is just the half as compared to stainless steel so that it results a lower stiffness, with the same shape, which gives to the implant a greater adaptation skill to the bone's elastic proprieties. This characteristic supports the growing interest on titanium as material for all applications that simultaneously require

high mechanical characteristics, low weight and high corrosion resistance. Titanium implants can be made from commercially pure titanium (Ti cp) or its alloys. The most used one is Ti-6Al-4V (Hanawa, 1999). Titanium is classified as bioinert; because of its ability to isolate oneself from the outside through a layer of oxide that is formed spontaneously by the contact with the biological environment. Various oxides such as TiO, TiO_2 and TiO_3 are present on the surface of titanium. TiO_2 is the most stable so it is the most frequently encountered. Data shows the rapid formation of a titanium oxide layer of approximately 10 Å in less than a thousandth of a second, that increases up in thickness to 50-100 Å in a minute (Macdonald et al., 1998). The layer of oxide is inert, extremely smooth, tenacious, adherent, and if, during the implantation, the layer was damaged, it will be immediately re-established. The osteointegration protagonist is the titanium oxide, because its chemical stability prevents the surface corrosion and the spread of metal ions within tissues. These proprieties give a high degree of biocompatibility to titanium. In endosseous implants'field the aim of current researches is to create not only biocompatible, but also bioactive materials. It means that these materials can play an active role in stimulating or promote the bone apposition. In this way the implant is no longer considered as a simply bone's functional support but it helps the host tissue to form new bone. The study of bioactive materials contains a wide number of new prospects and leads to the overcoming of previous concepts of biocompatibility. When a metal implant is surgically inserted, its outer surface comes into close contact with the host tissue, and this leads to various physical-chemical and biochemical interactions which involve macromolecules and tissue molecules from biological fluids (Macdonald et al., 1998). The literature describes that biological tissues interact with the surface of an implant (0.1-1 nm), so the surface pattern plays a key role in the osteointegration. In order to improve implant osteointegration many treatments, to modify the surface characteristics, have been studied and applied (Anselme et al., 2000). The research is directed to develop treatments to improve the bone implant interface that make possible to consider titanium as a bioactive and not only as bioinert material. Three main approaches to surface modification are used: physical methods, chemical-electrochemical methods, and biochemical functionalization. Physical treatments are based on the idea that peculiar characteristics of the implant surface may facilitate osteointegration. Changes in both macro and micro architecture are designed to increase the surface contact area between implant and bone tissue; facilitating the deposition of calcium phosphate and improving bone implant's mechanical stability in terms of tensile strength and torsion strength. The modifications of titanium surface topography lead to a better response of bone tissue, because the deposition of mineralized bone within the surface irregularities increases the bond between the bone and the implant (Cooper, 2000; Thomas & Cook, 1985;, Klokkevold et al., 2001). Among the physical methods of titanium surface modification, of particular interest are the sandblasting and the coating with titanium plasma spray (TPS). Chemical and electrochemical treatments are applied on a material when changes in the chemical composition of its surface are required. Within chemical treatments, both the acid etching and the surface coatings with calcium phosphate ceramics and in particular with the hydroxyapatite, are widely considered. The electrochemical treatments produce stable, porous and oxygen enriched coatings. The oxide layer created with such treatments, can be enriched with electrolytes dissolved in the medium during the deposition process (anode / cathode). The most recent developments in the treatments of bioactive titanium and alloys

are the biochemical ones, these can be considered as a method of surface modification based on the current knowledge about biological and biochemical cells functions and differentiation.

2.1 Biomimetic implants

The biomaterials of new generation are not only biocompatible but also bioactive, stimulating specific cellular responses and activating genes that stimulate living tissues. Today, the interest is focused on biomimetic treatments, developed to promote and accelerate bone apposition directly on the implant surface (Sun & Qing, 2011). The main idea, on which this recent approach is based, is the attempt to give to the material a specific biological activity that accelerate the healing process and that promote osteointegration. Biomimetic surfaces are obtained either through electro-chemical and biochemical treatments. The main goal of biomimetic treatment is the modification of the surface composition and morphology, in order to positively influence the response of biological tissue through an appropriate cell colonization. Nowadays, there are a lot of knowledges about the mechanisms of cells adhesion to substrates. Many progresses have been done in determining the role of molecules involved in the regulation of proliferation, cell differentiation and tissue remodeling. The development of these new knowledges made possible the design of a new generation of biomaterials that can promote and support the osteoblasts adhesion to the implant and consequently its osteointegration.

2.1.1 Biomimicry to improve osteoblast adhesion

Several extracellular matrix proteins are involved in the biochemical steps necessary for cells adhesion. For this reason, the molecules contained in the non fibrillar extracellular matrix component have been extensively studied. These studies allowed the isolation and identification of their role. Glycoproteins and glycolipids exposed on the outer surface of the cell membrane play very specific tasks, such as signals receiving and cell-cell recognition, act to promote cell adhesion during the tissue formation. The cell membrane on its surface has many different receptors, some of them are ubiquitarious, it means that they are almost in all cell types, while others are characteristics of different cell types. Different are also the selectivity and affinity of receptors.

The use of biological factors to promote the adhesion of osteoblasts, such as BMPs, fibronectin and vitronectin, is not an optimal solution. It is influenced by a number of drawbacks: first of all because these proteins are complex molecules, often unstable and sometimes they are poorly soluble in a biological environment; their biological activity is influenced by the integrity of his tertiary structure (protein folding) and their use is limited by the cost of production. In addition we also have to consider the difficulty of controlling the local concentration at the interface implant-bone tissue where these molecules have to perform their biological activity (Bagno et al., 2003). To avoid these problems, the research has been directed to the identification of biologically active fragments; which come from adhesion factors or growth factors that can be easily reproduced by chemical synthesis.

These fragments, called as bioactive peptides because they are necessary to perform biological activity, have many advantages over native proteins: they are stable, soluble, can be obtained by chemical synthesis with relatively low costs, moreover they ensure an extremely high level of purity and their biological activity does not depend on tertiary structure. Since the identification of the sequence Arg-Gly-Asp (RGD), as cell adhesion

mediator (that is present in many plasma proteins and extracellular matrix proteins, including fibronectin, vitronectin, collagen I, osteopontin and bone sialoprotein) a research field has been focused on the development of bioactive materials, obtained by deposition of synthetic peptides, containing the RGD sequence, on the biomaterials. The aim is to promote cell adhesion to the implants surfaces. Transmembrane receptors, belonging to the superfamily of integrins, are able to recognize the RGD sequence and to mediate cell adhesion; in particular, a high affinity of the RGD sequence for integrin α5β1 has been shown. Because of the importance of the affinity between integrins and adhesion proteins, and also because the same integrins are owned by many cell types, the problem of a non-specific cell adhesion to RGD modified implant surface has been introduced (Puelo & Nanci, 1999). Some research groups are trying to overcome this problem using synthetic peptides, with a particular conformation. The used peptides are longer than the short tetra, penta and hexapeptides (Rezania et al., 1997). Other groups are considering the use of no RGD peptides that may have greater specificity for bone cells. Nowadays, many studies are aimed to the development of surfaces functionalization with adhesion peptides; which are selective for osteoblastic cells. This approach take advantage of the characteristic mechanism of osteoblast adhesion based on the interaction between heparan sulfate proteoglycans, on the cell membrane, with heparin binding sites in the extracellular matrix proteins. Peptide mimicry studies concerning the amino acid sequences binding to heparin, made on several proteins (eg. human vitronectin, apolipoprotein E, B-100 and platelet factor IV), led to the identification of highly conserved signal sequences type XBBXBX XBBBXXBX (B is a basic amino acid and X is a non-basic amino acid) (Cardin & Weintraub, 1989). Subsequent studies have identified the minimal sequence for the osteoblast adhesion via heparan sulfate proteoglycans. The sequence proposed is the tetrapeptide Lys-Arg-Ser-Arg (KRSR), which replicates the motif BBXB. In addition, has been demonstrated that the osteoblastic cells interact with the RGD and KRSR peptides through two distinct types of molecules that are integrins and heparan sulfate proteoglycans. In fact, the interaction between the membrane integrins and the peptide RGD does not inhibit the interaction of osteoblastic cells with the peptide KRSR (Dee et al., 1998).

A significant feature of the sequence KRSR seems to be its selective action on osteoblasts; indeed has been demonstrated a significant increase of bone cells adhesion on a support patterned with this sequence, instead there were no appreciable results for endothelial and fibroblasts cells (Dettin et al., 2002).

The mechanism of adhesion, mediated by integrin, is not specific to osteoblasts; in fact the sequences containing the RGD motif are able to promote the adhesion of several cell types (eg. Fibroblasts) as well as osteoblasts, instead mechanism of adhesion mediated by proteoglycans is specific for osteoblasts. Further investigations have been done to identify potential peptide sequences binding to membrane heparan sulfate proteoglycans by the motif XBBXBX and XBBBXBBX in vitronctin, fibronectin, sialoprotein, bone thrombospondin and osteopontin. This led to the identification of several peptides (HVP) contained in the sequence (339-364) of human vitronectin (Dettin et al., 2002). Also in this study has been shown that the new peptides identified are able to promote osteoblast adhesion via membrane proteoglycan. In particular, peptide sequence (351-359) has a higher activity than RGD peptides and fibronectin. Another approach is to use two different peptides on the same surface, one for the interaction with integrins and the other one for interaction with heparan sulfate proteoglycans. In this way, both mechanisms of osteoblasts adhesion are exploited and a better cell adhesion to the material should be reached (Rezania & Healy,

1999). The release of one or more of these factors, which have an important physiological role on osteogenesis at the bone-implant interface, promote the bone formation.

2.1.2 Biomimicry to improve hydroxyapatite deposition

The hydroxyapatite (HA) is considered the best osteoconductive material. It takes directly part in bone formation and in particular in the mineralization step, providing substances necessary to the tissue. It is widely used as coating for titanium and its alloys; it is applied by plasma spray technique. These coatings chemically modify the titanium surface, prompting a close interaction with the surrounding tissue mediated by chemical bonds (Davies, 2003; Brossa et al., 1993; Klein et al., 1991). The main problem with this approach is the coating long-term stability which may be subject to phenomena of delamination and hence loss of adhesion with the substrate (Kim, 1996). The excellent results obtained by the osteoconductive coatings in terms of osteoinegration capacity led to the study and the development of alternative methods of coating. An anodizing technique, known as Anodic Spark Deposition or Anodic Spark Discharge (ASD) has been considered, as a starting point for the development of treatments designed to improve osteointegration (Ishizawa, 1995a, 1995b, 1997). With this technique it is possible to obtain porous surfaces rich in oxygen, with a relatively thick oxide layer, enriched with electrolytes dissolved in the medium during the anodic deposition process (Schreckenbach, 1999). The functionalized surfaces presented in this study were developed by a three steps ASD (Sandrini, 2003, 2005); in particular, this functionalization consists of two following steps of ASD that is made in solutions containing phosphate and calcium ions, followed by a step of alkaline attack. This biomimetic treatment is able to provide a thin titanium oxide layer, which is nanoporous and contain calcium and phosphorus (Zhu, 2001; Chiesa 2003). Further chemical treatments of surface modification have been used to enrich the surface with-OH groups, which act as preferential sites for precipitation of hydroxyapatite that is the main component of the bone mineral phase. It has been shown that these surfaces induce an enhanced primary osteointagration that leads to a reliable and durable implants osteointagration.

3. Experimental procedure

The biomimetic surfaces above described were tested to evaluate the osteogenic primary response and the osteointegration of implants through two separate experiments. In both trials the outbreed male New Zealand White rabbit was chosen as animal model. The rabbits, weighing 4.3 ± 0.2 kg, were at reproductive age and skeletal maturity, using as index the successful welding of the femoral growth metaphysis. European and Italian regulations on animal experimentation (Italian DL 27 January 1992 N°116 -European union 86/609 CEE) were strictly followed during the entire studies (Health Ministry Authorisation 21/01/2004; 19/01/2007; 16/04/2010). As anatomical site for the implantation, the distal epiphysis of the femur was chosen. It is mainly characterized by spongy bone with a periosteal thin coating made up of compact bone tissue, the exception is for the articular edge that is covered of articular hyaline cartilage. Since that the surfaces to be tested are designed mainly for oral implantology, the anatomical site chosen for the in vivo trials presented a type of bone tissue similar to maxillary and mandibular bones. Surgical procedures were performed aseptically under general anesthesia (Domitor, Pfizer, New York, NY 0.1mL/kg; Ketavet 100, Gellini, Latina, Italy 0.3mL/kg; Isoflurane-Vet, Me´rial, Duluth, GA). In particular, after arthrotomy and dislocation, the trocheal grove was exposed and a precise hole was created, using a low

rotational drilling speed and continuous internal cooling, strictly parallel to the long axis of the femur (samples HVP(351-359) trials: 3.04 mm diameter and 14.08 mm length; samples ASD trials: 3.00 mm diameter and 13.00 mm length). Both the studies are designed on a bilateral approach. The soft tissues suture was done in separate layers using interrupted sutures. After surgery, the samples position was assessed by X-ray. All the materials tested in the studies described herein are subject to patents.

Regarding the trials for the functionalization with the HVP peptide (351-359) nine rabbits were used; for each animal, one peptide-grafted cylinder (HVP) was inserted in the left femur, whereas one nongrafted cylinder (CTRL) in the right femur as internal control. On the basis of time points, rabbits were randomly divided into three groups: the first was sacrificed 4 days after surgery (4D group), the second 9 days after surgery (9D group), and the third 16 days after surgery (16D group).

Regarding the trials for the ASD treatments, ASD1 and ASD2 surface treatments that involved two consecutive ASD processes carried out in different electrolyte solutions at different voltage ranges, and followed by an alkali etching processes were used. The first ASD was performed in a solution containing phosphate anions and calcium cations; the second ASD was performed in a solution containing only calcium cations. ASD 1 and ASD 2 differ only for a final chemical treatment: ASD 1 was obtained in NaOH solution, instead ASD 2 was obtained by a final chemical treatment in KOH solution. An acid-etching treatment ETC was used as internal control. Eight implants for each surface (ETC, ASD 1, and ASD 2) were inserted into the left and right femoral epiphysis of the rabbits, avoiding implanting the same materials in the counterlateral; a total of 24 implants were placed. On the basis of time points, rabbits were randomly divided into two groups: the first was sacrificed 2 weeks after surgery (2W); the second 4 weeks after surgery (4W).

To assess the new bone apposition and the osteointegration fluorochromic bone vital markers were used; these are fluorescent substances that allow to highlight areas where bone growth occurs during the administration period. Fluorochrome labels, when bound to calcium ions, can be incorporated at sites of mineralization in the form of hydroxyapatite crystals. As result, the fluorescent label demarcates the mineralization front at the time of administration and can be detected in histological sections without any further staining or decalcification (van Gaalen et al., 2010; Rahn, 2003). The florochromic bone markers used in these studies were: Calcein Green (CG, 5 mg/kg BW; Sigma), Calcein Blue (CB, 30 mg/kg BW; Sigma), Xylenol Orange (XO, 90 mg/kg BW; Sigma). These fluorochromes generate different color clearly distinguishable from each other (green, blue and red) and thus can be used sequentially, in order to highlight the bone neodeposition respectively to each marking period. For the HVP trials CG was administered for 2 days after surgery to all groups; XO was administered on the seventh and eighth day after surgery to the animals of the 9D group only; CB was administered on days from 9th to 15th after surgery, to the animals of the 16D group only. For the ASD trials CG was daily administered for the first week after surgery to all groups; CB was daily administered for the second week after surgery to all groups; AR was daily administered for the fourth week after surgery to 4W animals. At the end of the experimental trials, the implants and surrounding bone were immediately excised and excess tissue was removed. The implant containing tissue blocks were promptly fixed in paraformaldehyde 4%, dehydrated in alcoholic solutions of increasing concentration (70 up to 100%), treated with xylene, and finally embedded in polymethylmethacrylate resin. The histological and histomorphometrical analysis were performed by a motorized microscope (Nikon Eclipse 90i, Tokyo, Japan), equipped for polarized light and

fluorescence. Parameter calibrations for magnification and image acquisition were fixed on the basis of standardized conditions imposed before the histomorphometric. The following parameters were considered (Parfitt et al., 1987; Recker, 1983; Schnitzler & Mesquita, 2006):

- Bone-to-implant contact (%) [Bc]: it was calculated as the ratio between the length of the bone profile in direct contact with the implant surface and the length of the implant profile.
- Mineral apposition rate for single label (μm/day) [MAR-SL]: it was calculated as the ratio between the average thickness of the marking band and the duration of the administration period for each bone marker.
- Mineralizing surface versus bone surface (%) [MS/BS]: it was calculated as the ratio between the surface marked with the vital marker (under fluorescence) and the whole bone surface within the area of interest (under polarized light). This parameter was measured for each vital marker administered.

Histomorphometric data were statistically checked by means of one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests, with the statistical tool SPSS v.18.0. Data are reported as means ± standard deviations at a significance level of $p<0.05$.

3.1 Results

Observation of X-rays and histological sections has confirmed the correct positioning of the specimens in the anatomical sites, in fact they are centrally located in the distal femoral epiphysis, surrounded by trabecular bone. The Goldner's trichrome stain excluded the interposition of fibrous connective tissue at the bone-implant for all the tested surfaces.

With regard to trials for the functionalization with the HVP peptide (351-359) is detectable, through the polarized light analysis, already in 4D group, a thin trabeculation of newly formed bone closely associated with the implant surface, which thickens in the 9D group. In the newly formed bone numerous round shape osteocytes, characteristic of woven fiber bone, were detected. In agreement with these observations, at the experimental time of 4D, from the analysis of bone vital markers is noticeable an osteogenic activity at the interface that is confirmed by the polarized light analysis (Fig. 1).

For each parameter considered the results of static and dynamic quantitative histology (Cacchioli et al., 2009) are detailed below in the text and plotted in the figure (Fig. 2).

The bone-to-implant contact (Bc) shows an upward trend over time, from 4D to 16D, in both CTRL and HVP. This increase is statistically significant, both for CTRL and HVP going from 4D to 9D ($p <0.01$), instead statistically significant only for HVP from 9D to 16D ($p <0.01$). The bone-to-implant contact is always higher for HVP implants with a statistical significance at 9D ($p <0.05$) and 16D ($p <0.01$). The increase that occurs in Bc from 4D to 9D, for both HVP and CTRL implants, indicates that this is the time interval at which a widest bone neodeposition occurred (Fig. 2). This observation is supported by data on the mineral apposition rate, because it showed a statistically significant ($p <0.01$) upward trend both for HVP and CTRL surfaces, from 4D to 9D, where it presented its peak. Then there is a statistically significant ($p <0.01$) decrease, both for CTRL and HVP surfaces from 9D to 16D.

HVP groups have to have a higher mineral apposition rate than the CTRL groups at all experimental times (4D, 9D and 16D) (Fig. 2).

The Calcein Green - Mineralizing surface vs. Bone surface [CG-MS/BS] was measured in all groups HVP and CTRL at the end of each experimental time. Since the CG marker was administered within two days after the surgery in all experimental groups, and measured

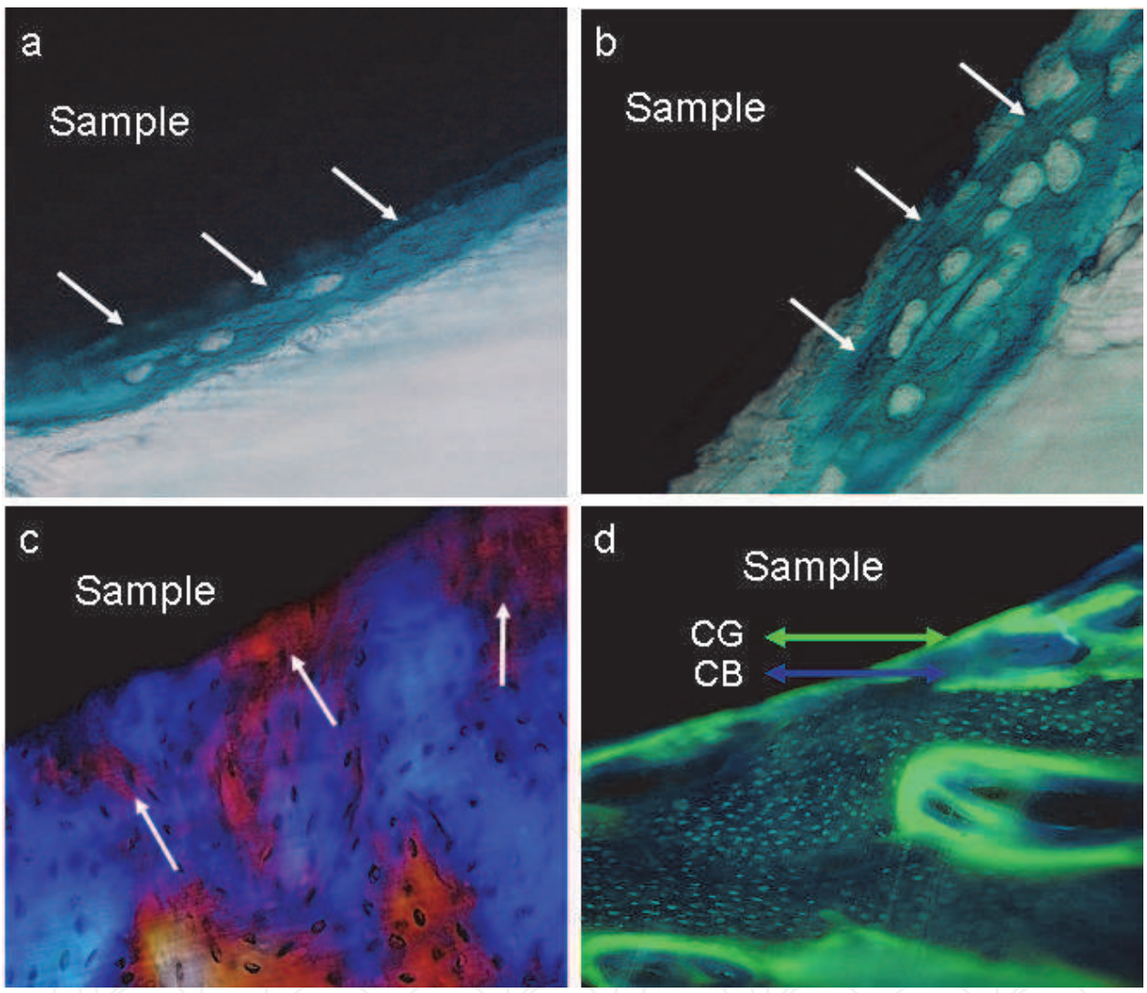

Fig. 1. Histological results of HVP (351-359) trials. (a-b) Goldner's trichrome stain (original magnification, 40x). Primary new bone directly apposed to the interface. (a) The picture corresponds to an HVP samples belonging to the 9D group; (b) the picture corresponds to an HVP samples belonging to the 16D group. Arrows show newly formed bone characterized by round shape osteocytes. (c) Polarized light microscopy with compensation plate (original magnification, 40x). Woven fiber bone located at the interface in the HVP sample, belonging to 16D group. Arrows indicate the woven fiber bone. (d) Fluorescent microscopy of CG and CB fluorochromic bone marker (original magnification, 20x). The picture represents the direct bone apposition to the interface the days immediately after surgery (Calcein green bone marker) and the following bone apposition, marked with Calcein Blue in the HVP sample belonging to 16D groups.

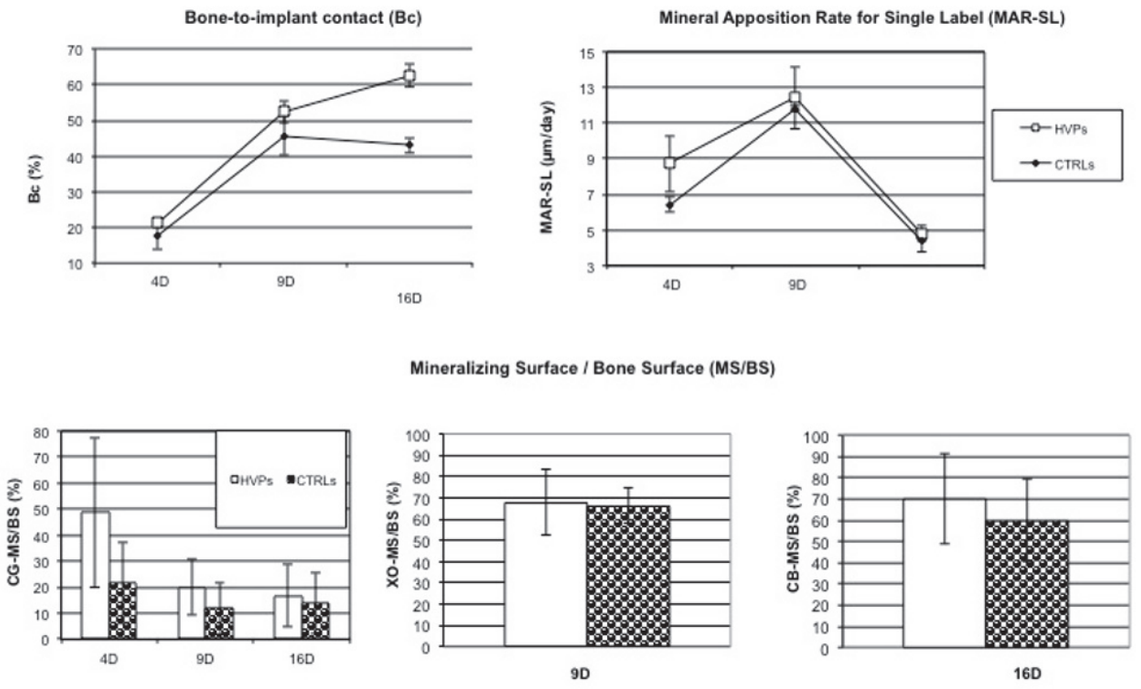

Fig. 2. Static and dynamic histomorfometric results of HVP (351-359).

after 4D, 9D and 16D, respectively, its evaluation, relatively to the 9D and 16D groups, is an indirect index of the bone remodeling activity occurred from CG administration until animal sacrifice. For the 4D group, data showed, a statistically significant difference ($p < 0.01$) between CTRL and HVP. For both the 9D and 16D groups, data collected showed the tendency of groups HVP to present higher values compared to CTRL, although these differences are not statistically significant as it is highlighted at 4D. This could be interpreted as result of the remodeling activity occurred nearby at the interface. The Xylenol Orange - Mineralizing surface vs. Bone surface [CG-MS/BS] was measured for the 9D group, since the XO marker was administered to this group only. The comparison between CTRLs and HVPs average values did not show any significant difference. The Calcein Blue - Mineralizing surface vs. Bone surface [CG-MS/BS] was measured for the 16D group, since the CB marker was administered to this group only. The comparison of averages shows, even if not supported by statistical significance, a higher values trend of the HVP than the respective CTRL surfaces (Fig. 2).

With regard to trials for the treatment with ASD, for the 2 weeks group newly grown bone tissue in direct contact with the sample surface was already clearly visible at this time point. In polarized light microscopy this newly formed bone tissue has the morphology of primary woven bone, that is the first tissue to fill quickly and evenly the gap between the bone and the implant. This is an important support to ensure the implant stability and consequently allow an earlier loading of the implant. For the 4 weeks group the remodeling activity on the newly formed bone tissue was detectable, in fact it is observed an increase in the thickness of trabecular woven bone and at the same time, portions of bone with parallel fiber organization structured as secondary bone were present (Fig. 3).

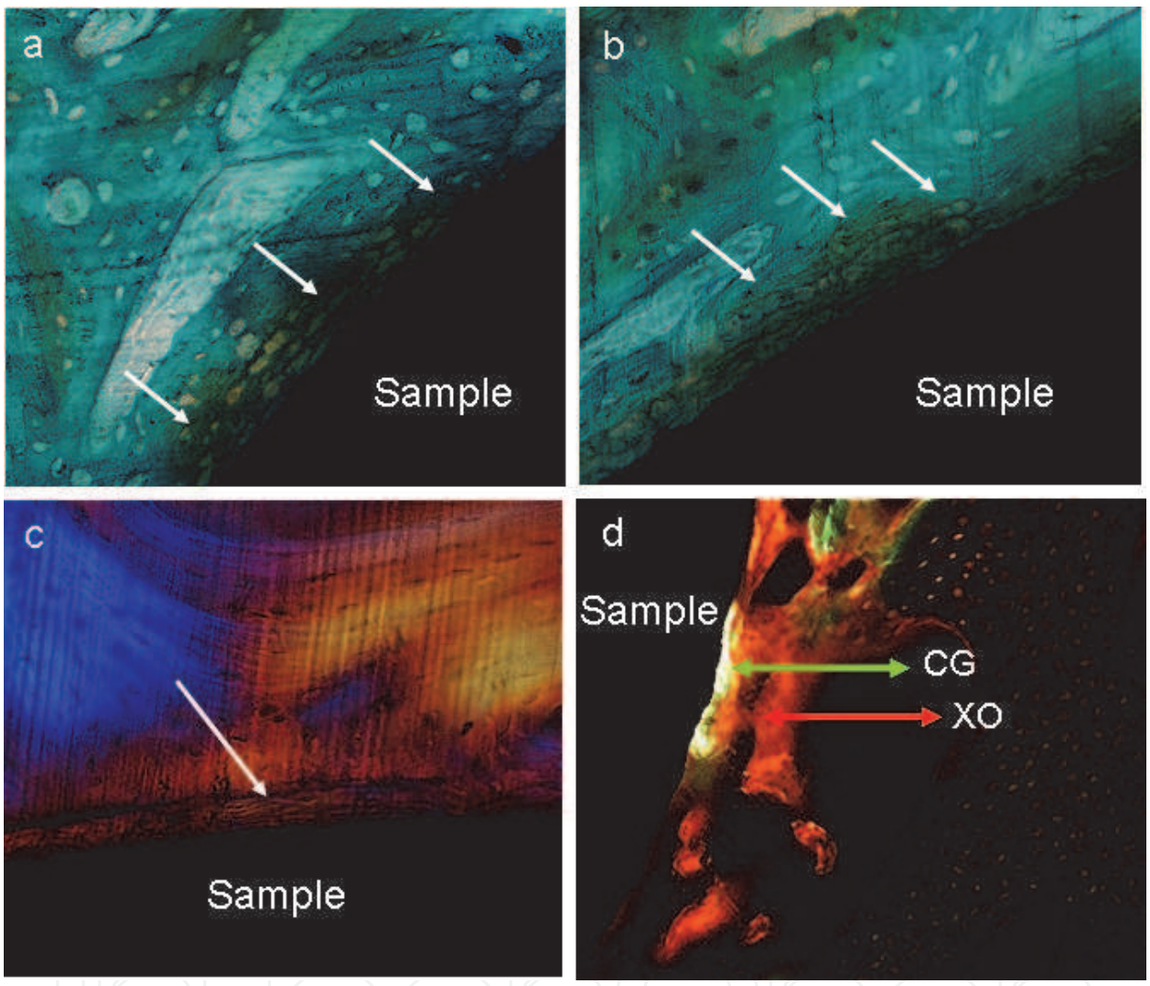

Fig. 3. Histological results of ASD trials. (a-b) Goldner's trichrome stain (original magnification, 40x). Primary new bone directly apposed to the interface. (a) The picture corresponds to an ASD samples belonging to the 2W; (b) the picture corresponds to an ASD samples belonging to the 4W group. Arrows show newly formed bone in (a) and the result of remodeling activity in (b). (c) Polarized light microscopy with compensation plate (original magnification, 40x). Woven fiber bone and parallel fiber bone located at the interface in the ASD sample, belonging to 4W group. Arrow indicates the woven fiber bone. (d) Fluorescent microscopy of CG and XO fluorochromic bone marker (original magnification, 20x). The picture represents the direct bone apposition to the interface the days immediately after surgery (Calcein green bone marker) and the following bone apposition, marked with Xylenol Orange in the ASD sample belonging to 2W group.

For each parameter considered the Results of static and dynamic quantitative histology (Ravanetti et al., 2010) are detailed below in the text and plotted in the figure (Fig. 4).

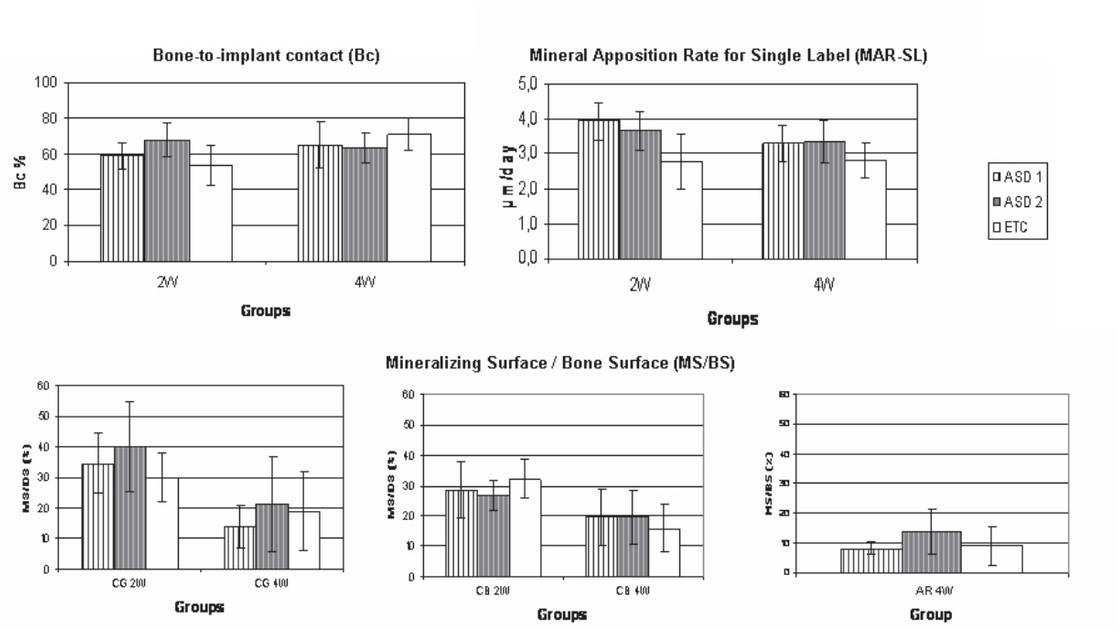

Fig. 4. Static and dynamic histomorfometric results of ASD trials.

The bone to implant contact, in the 2Wgroup, shows the achievement of a greater contact for the surface ASD2 as compared to ETC (ASD 2 vs. ETC $p < 0.01$). The ASD1 surface has slightly higher contact than the ETC, but this is not statistically significant.
For the 4W group, all the tested surfaces achieved a similar bone to implant contact, without statistical significance. From 2W to 4W, for both the ASD surfaces, no significant increase resulted, while a significant increase is detected for the ETC surface (2W ETC vs. 4W ETC $p < 0.01$). The mineral apposition rate concerning the first week after surgery, indicating a higher bone deposition rate in both ASD surfaces as compared to ETC surface (ASD1 vs. ETC $p < 0.01$; ASD2 vs. ETC $p < 0.05$) (Fig. 4). The mineral apposition rate concerning the fourth week after surgery indicates a decrease in mineral apposition rate for the ASD 2 and ASD 1 surfaces, while for the ETC surface the rate detected at 2W was maintained. Despite these changes, the mineral apposition rate on the fourth week has maintained the same trend of the first week, in fact the surfaces ASD 1 and ASD 2 resulted higher than ETC, but without statistical significance (Fig. 4).
The Calcein Green - Mineralizing surface vs. Bone surface [CG-MS/BS] was measured in both experimental times 2W and 4W, since the Calcein Green was administered the first week after surgery to all experimental groups. For the 2W group, the bone activity for ETC samples appears to be slightly lower than for ASD 1 and ASD 2 samples, but no statistically significant difference were detected. For the 4W group, a significant decrease in absolute value and a similar situation among surfaces was observed; this decrease is due to remodeling activity and it was statistically significant only for ASD1 and ASD2 surfaces going from the experimental time 2W to 4W (2W ASD1 vs. 4W ASD1 $p < 0.01$; 2W ASD 2 vs. 4W ASD2 $p < 0.01$).

The Calcein Blue - Mineralizing surface vs. Bone surface [CB-MS/BS] was measured in both experimental times 2W and 4W, since the Calcein Blue was administered the second week after surgery in all experimental groups. For the 2W group, no statistically significant differences among materials were came out. For the 4W group, as noted also for the Calcein Green marker, a strong decrease in absolute value for the surfaces ETC and ASD1 carried out (2W ETC vs. 4W ETC $p<0.01$; 2W ASD1 vs. 4W ASD1 $p<0.01$), however a similar situation among surfaces was maintained (Fig. 4).

4. Conclusion

The goal of current implantology on osseointegrated implants aims to design bioactive and biomimetic materials enabling to monitor, pilot and speed up the processes involved in the osteointegration, make possible a more rapid healing. Such strategies were widely considered highly encouraging factors for the development of a better clinical development of an endosseous implant.

The most common cell-binding domain which has beeen used extensively as a candidate peptide to enhance cell adhesion onto biomaterial surface is the RGD-sequence. The exploitation of the RGD sequence for improving cell adhesion has been known since the 1980s; many studies confirmed its suitability as bioactive adhesion peptide (Ruoslahti, 2003) Other non-RGD-containing cell binding domain exist, such as tyrosine-isoleucine-glycine-serine-arginine (-YIGSR-) and isoleucine-lysine-valine-alanine-valine- (IKVAV) in laminin, arginine-glutamate-aspartate-arginine-valine (-REDRV) and leucine-aspartic acid-valine (LDV) in fibronectin aspartate-glycine-glutamate-alanine (DGEA) in collagen I, and various heparin binding domains (Rezania & Healy1999).

Regarding the first study here presented, a higher mineral apposition rate, concomitant with an increase activation in terms of osteogenic surface for the experimental times 4 and 9 days in the HVP functionalized group has contributed to achieved an higer bone-implant contact observed at the experimental times of 9 and 16 days. The surface functionalized with HVP peptide (351-359), improves the osteogenic response in a short time after implant positioning, and therefore stimulates the acceleration of the new bone deposition at the interface. So we can assume a more massive osteoblastic adhesion to the implant surface that produces these effects directly on the interface. Published data indicates that the osteogenic activity of RGD-grafted implants, measured by bone-contact histomorphometric analysis, achieved its highest values within 2 weeks after surgery in mini pigs. In a further in vivo study on rabbits, more than 50% of bone defects are covered using the RGD sequence within 2 weeks. The effects due to components of extracellular matrix (e.g., collagen type I, RGD sequence, and chondroitin sulfate) used for coating titanium implants have been checked in rats, from 4 up to 28 days after surgery. This early stimulation of osteogenic activity improves primary fixation of the implant and, consequently, should lead to a faster osseointegration with the clinical benefits derived.

Different physical treatments of titanium can improve osteointegration through a better mechanical interface but not provide a chemical interaction with bone. To produce a bioactive titanium, biomaterials research has focused on osteoconductive materials, such as hydroxyapatite coatings. The poor long-term performance of plasma-sprayed HA coatings stimulated research for the study of alternative deposition methods of HA coatings (Forsgren et al., 2007) and for the development of new approaches based on the nanoscale modification of the material surface. Among the electrochemical methods, an attractive

technique that can be applied to titanium and titanium alloys to obtain a biomimetic thin and porous surface layer enriched in calcium and phosphate is known as anodic spark deposition (ASD).
Regarding the second study, the biomimetic electrochemical treatments, ASD1 and ASD2, supports the establishment of a greater bone-implant contact after two weeks by implementing primary osteogenic response in vivo. This is not so obvious to the experimental time of four weeks, as the osteogenic response of the control surface ETC is delayed with respect to biomimetic surfaces. All the three tested materials were found to be suitable for their use as prosthetic endosseous implants; however, the ASD biomimetic treatments have shown some benefits in terms of acceleration in the biological response.
In these studies promising results were obtained encouraging to continue deepen the study of these surfaces for the possible development of clinically exploitable endosseous devices. In fact, both the tested biomimetic promoted the primary osseointegration compared to the control surfaces, in short experimental times after implantation.

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6. References

Anselme, K.; Linez, P.; Bigerelle, M.; Le Maguer, D.; Le Maguer, A.; Hardouin, P.; Hildebrand, H.F.; Iost, A.; Leroy, J.M. (2000). The relative influence of the topography and chemistry of TiAl6V4 surfaces on osteoblastic cell behaviour. *Biomateriasl,* 21 (15), pp.1567-1577, ISSN: 0142-9612.

Albrektsson, T.; Brånemark, P.I.; Hansson, H.A.; Lindstrom, J. (1981). Osseointegrated titanium implanta. Requirements for ensuring a long-lasting, direct bone anchorage in man. *Acta Orthop Scand,* 52, pp. 155-170, ISSN 1745-3674.

Albrektsson, T.; Wennerberg, A. (2005). The impact of oral implants - past and future, 1966-2042. *J Can Dent Assoc*, May 71 (5), 327, ISSN 1488-2159.

Bagno, A.; Dettin, M.; Gambaretto, R.; Tonin. L.; Di Bello, C. (2003). Strategy to enhance the osseo-integration process: synthetic peptides improving osteoblast adhesion on implant surface. *Acta of Bioengineering and Biomechanics*, 5 (1), pp. 35-42, ISSN 1509-409X.

Brånemark, P.I.; Hansson, B.O.; Adell, R.; Breine, U.; Lindström, J.; Hallén, O.; Ohman A. (1977). Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. *Scand J Plast Reconstr Surg,* Suppl. 16, pp.1-132, ISSN 0284-4311.

Brossa, F.; Cigada, A.; Chiesa, R.; Paracchini, L.; Consonni, C. (1993). Adhesion properties of plasma sprayed hydroxylapatite coatings for orthopaedic prostheses, *Biomed Mater Eng*, 3 (3), pp.127-136, ISSN 0959-2989.

Bucci Sabattini, V.; (2007). Tecniche ricostruttive e rigenerative dei mascellari atrofici. I biomateriali: scelta, indicazioni e metodi di uso. TU.F.OR, ISBN 9788895641003, Italy.

Cacchioli, A., Ravanetti, F., Bagno, A., Dettin, M., Gabbi, C. (2009). Human Vitronectin-Derived Peptide Covalently Grafted onto Titanium Surface Improves Osteogenic Activity: A Pilot In Vivo Study on Rabbits. *Tissue Eng Part A*. Oct, 15, 10, pp. 2917-26, ISSN 1937-3341.

Cardin, A.D.; Weintraub, H.J.R. (1989) Molecular modeling of protein-glycosaminoglycan interaction, *Arteriosclerosis*, 9, pp. 21-32, ISSN: 0021-9150.

Chiesa, R., Mandrini, E., Santin, M., Rondelli, G., Cigada, A. (2003). Osteointegration of titanium and its alloys by anodic spark depositino and other electrochemical techniques: a review. *Journal of Applied Biomaterials & biomechanics,* 1, pp. 91-107, ISSN 1722-6899.

Cooper, L.F. (2000) A role for surface topography in creating and maintaining bone at titanium endosseous implants. *J Prosthet Dent,* 84 (5), pp. 522-534, ISSN: 0022-3913.

Davies J.E. (2003). Understanding peri-implant endosseous healing, *J Dent Educ.* 67 (8), pp. 932-949, ISSN: 0022-0337.

Dee, K.C.; Andersen, T.T.; Bizios, R. (1998) Design and function of novel steoblast-adhesive peptides for chemical modification of biomaterials, *J Biomed Mater Res,* 40, pp. 371-377, ISSN: 1549-3296.

Dettin, M.; Conconi, M.C.; Gambaretto, R.; Pasquato, A.; Folin, M.; Di Bello, C.; Parnigotto, P.P. (2002). Novel osteoblast-adhesive peptides for dental/orthopedic biomaterials, *J Biomed Mater Res,* 5, 60 (3), pp. 466-471, ISSN: 1549-3296.

Forsgren J, Svahn F, Jarmar T, Engqvist H. (2007) Structural change of biomimetic hydroxyapatite coatings due to heat treatment. *J Appl Biomater Biomech*, 5, pp. 23–27, ISSN 1722-6899.

Hanawa, T. (1999). In vivo metallic biomaterials and surface modification. *Materials Science and Engineering: A*, 267 (2), pp.260-266, ISSN 0921-5093.

Huebsch, N.; Mooney, D.J. (2009). Inspiration and application in the evolution of biomaterials. *Nature,* November 26; 462 (7272), pp. 426-432, ISSN 0028-0836.

Ishizawa, H., Ogino, M. (1995a). Formation and characterization of anodic titanium oxide films containing Ca and P. *J Biomed Mater Res,* Jan, 29, 1, pp. 65-72, ISSN 1549-3296.

Ishizawa, H., Fujino, M., Ogino, M. (1995b). Mechanical and histological investigation of hydrothermally treated and untreated anodic titanium oxide films containing Ca and P. *J Biomed Mater Res,* Nov 29, 11, pp.1459-68, ISSN 1549-3296.

Ishizawa, H., Fujino, M., Ogino, M. (1997). Histomorphometric evaluation of the thin hydroxyapatite layer formed through anodization followed by hydrothermal treatment.*J Biomed Mater Res,* May, 35, 2, pp. 199-206, ISSN 1549-3296.

Kim, H.M., Miyaji, F., Kokubo, T., Nakamura, T. (1996). Preparation of bioactive Ti and its alloys via simple chemical surface treatment. *J Biomed Mater Res*, 32, pp. 409–417, ISSN: 1549-3296.

Klein, C.P., Patka, P., Van der Lubbe, H.B., Wolke, J.G., de Groot, K. (1991). Plasma-sprayed coatings of tetracalciumphosphate, hydroxyl-apatite, and alpha-TCP on titanium alloy: an interface study. *J Biomed Mater Res,* 25, pp. 53–65, ISSN: 1549-3296.

Klokkevold, P.R.; Johnson, P.; Dadgostari, S.; Caputo, A.; Davies, J.E.; Nishimura, R.D. (2001). Early endosseous integration enhanced by dual acid etching of titanium: a torque removal study in the rabbit. *Clin Oral Implants Re,* 12 (4), pp.350-357, ISSN 1600-0501.

Macdonald. D.E.; Markovic, B.; Boskey, A.L.; Somasundaran, P. (1998). Physico-chemical properties of human plasma fibronectin binding to well characterized titanium dioxide. *Colloids and Surfaces B: Biointerfaces,* 11 (3), pp. 131-139, ISSN: 0927-7765.

Parfitt, A.M., Drezner, M.K., Glorieux, F.H., Kanis, J.A., Malluche, H., Meunier, P.J. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* , 2, pp. 595–610, ISSN . 0884-0431.

Park, J.B; Lakes, R.S. (Ed II). (1979). *Biomaterials: an introduction*. Plenum Press, ISBN 0-306-43992-1, New York.

Puelo, D.A.; Nanci, A. (1999) Understanding and Controlling the bone-implant interface, *Biomaterials* 20, pp. 2311-2321, ISSN: 0142-9612.

Rahn, B.A. (2003) Fluorochrome labelling of bone dynamics, *European Cell and Materials*, 5, 2, pp.41, ISSN 1473-2262.

Ravanetti, F., Borghetti, P., De Angelis, E., Chiesa, R., Martini, F.M., Gabbi, C., Cacchioli, A. (2010). In vitro cellular response and in vivo primary osteointegration of electrochemically modified titanium. *Acta Biomater,* Mar, 6, 3, pp. 1014-24, ISSN: 1742-7061.

Recker, R. (1983). Bone histomorphometry: techniques and interpretations. Boca Raton, CRC Press; ISBN 0849353734 9780849353734, Florida, USA.

Rezania, A.; Thomas, C.H.; Branger A.B.; Waters, C.M.; Healy, K.E. (1997). The detachment strength and morphology of bone cell contacting materials modified with a peptide sequence found within bone sialoprotein, *J Biomed Mater Res,* 37, pp. 9-19, ISSN: 1549-3296.

Rezania, A.; Healy, K.E. (1999). Biomimetic peptide surface that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteoblast-like cells, *Biotechnol Prog,* 15, pp. ISSN: 19-32, 8756-7938.

Ruoslahti, E. (2003) The RGD story: a personal account. Matrix Biol, 22, pp. 459, ISSN 0945-053X.

Sandrini, E., Chiesa, R., Rondelli, G., Santin, M., Cigada, A. (2003). A novel biomimetic treatment for an improved osteointegration of titanium. *J Appl Biomater Biomech*, 1, pp. 33–42, ISSN 1722-6899.

Sandrini, E., Morris, C., Chiesa, R., Cigada, A., Santin, M. (2005). In vitro assessment of the osteointegrative potential of a novel multiphase anodic spark deposition coating for orthopaedic and dental implants. *J Biomed Mater Res B Appl Biomater*, 73, pp. 392–399, ISSN 1552-4973.

Schnitzler, C.M., Mesquita, J.M. (2006) Cortical bone histomorphometry of the iliac crest in normal black and white South African adults. *Calcif Tissue Int,* 79, pp. 373–382, ISSN 0171-967X.

Schreckenbach, J.P., Marx, G., Schlottig, F., Textor, M., Spencer, N.D. (1999). Characterization of anodic spark-converted titanium surfaces for biomedical applications. *J Mater Sci Mater Med*, 10, pp. 453–457, ISSN: 0957-4530.

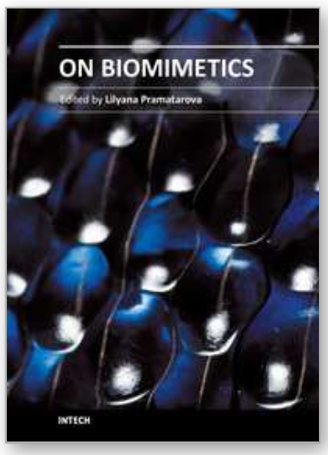
Sun, T.; Qing, G. (2011) Biomimetic smart interface materials for biological applications, *Adv Mater*, Mar 25, 23 (12), pp.57-77, ISSN 1521-4095.

Thomas, K.A.; Cook, S.D. (1985). An evaluation of variables influencing implant fixation by direct bone apposition. *J Biomed Mat Res,* 19 (8), pp. 875-901, ISSN: 1549-3296.

van Gaalen, S.M., Kruyt, M.C., Geuze, R.E., de Bruijn, J.D., Alblas, J., Dhert, W.J. (2010). Use of fluorochrome labels in in vivo bone tissue engineering research. *Tissue Eng Part B Rev*, Apr, 16, 2, pp. 209-17, ISSN 1937-3368.

Wilson-Hench, J. (1987). Osteoinduction. In: *Progress in biomedical engineerin,* Williams DF (ed), vol 4, p 29. Definitions in biomaterials. Elsevier, ISBN 9780444428585, Amsterdam.

Zhu, X., Kim, K.H., Jeong, Y. (2001). Anodic oxide films containing Ca and P of titanium biomaterial. *Biomaterials,* Aug, 22, 16, pp. 2199-206, ISSN: 0142-9612.



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Bio-mimicry is fundamental idea â€˜How to mimic the Natureâ€™ by various methodologies as well as new ideas or suggestions on the creation of novel materials and functions. This book comprises seven sections on various perspectives of bio-mimicry in our life; Section 1 gives an overview of modeling of biomimetic materials; Section 2 presents a processing and design of biomaterials; Section 3 presents various aspects of design and application of biomimetic polymers and composites are discussed; Section 4 presents a general characterization of biomaterials; Section 5 proposes new examples for biomimetic systems; Section 6 summarizes chapters, concerning cells behavior through mimicry; Section 7 presents various applications of biomimetic materials are presented. Aimed at physicists, chemists and biologists interested in biomineralization, biochemistry, kinetics, solution chemistry. This book is also relevant to engineers and doctors interested in research and construction of biomimetic systems.

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