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Faculdade de Odontologia de Araraquara

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Unravelling the role of chemical and mechanical modifications of titanium surfaces in osteogenic differentiation and bone formation.

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Tese apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara para obtenção do título de Doutor em Odontologia, na área Biociências e Biomateriais.

Orientador: Prof. Dr. Pedro Paulo Chaves de Souza
Co-orientador: Dr. Morten Foss

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Unravelling the role of chemical and mechanical modifications of titanium surfaces in osteogenic differentiation and bone formation

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To my parents;

To my sisters;

And to the friends (family) I’ve made in Araraquara and in Aarhus.

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ABSTRACT

To increase osteoblastic differentiation and the production of mineralized tissue, chemical and structural modifications on the surface of prosthetic implants have been proposed. Studies suggest that these modifications modulate and stimulate osteoblastic differentiation and cell response. We investigated chemical and mechanical alternatives for modifying the surface of Titanium (Ti) and to elucidate its biological effect on the adhesion and differentiation of bone cells and formation of mineralized tissue. In the first study, we focused on the chemical biofunctionalization of the Ti surface. Ti samples were coated with polydopamine (PDA) films and received or not post heat treatment due to sterilization or were kept at room temperature for 2 weeks to simulate shelf storage. The physico-chemical characterizations showed changes in the reactivity of the PDA surface after 2 weeks of storage and heat treatment. Adhesion, proliferation and spreading tests were performed with MC3T3-E1 cells cultured in PDA samples submitted or not to post-treatment. The PDA coating associated with heat treatment stimulated cell proliferation and spreading compared to untreated PDA samples. In a second study, a topographical high throughput screening (HTS) device was proposed to study the biological impact of hierarchical surface structural patterns. Human dental pulp stem cells (hDPSCs) and human fibula bone cells (hBDCs) were seeded on the screening arrays containing 60 different nano topographies and 4 control areas, constructed with the nano-pillars arranged in a square (SQR) and hexagonal (HEX) symmetry. They vary in diameter and interpillar distance. Preliminary tests for proliferation, mineralization and immunodetection of Osteocalcin (OCN) showed significant differences in cell response between topographies built with different parameters. Lateral spacing, or interpillar distance, showed a positive correlation with the modulation of proliferation and osteoblastic differentiation. The surface-induced cellular trends were used to identify and individually manufacture key topographies for osteogenic differentiation.

Keywords: Titanium. Dental implants. Cell differentiation. Osteoblasts.Topography.

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RESUMO

Para acelerar a diferenciação osteoblástica e a síntese de tecido mineralizado, modificações químicas e estruturais na superfície de implantes protéticos têm sido propostas. Estudos sugerem que estas modificações modulam e estimulam a diferenciação e resposta osteoblástica. Neste trabalho, investigamos alternativas químicas e mecânicas de modificação da superfície de Titânio (Ti) e elucidar seu efeito biológico na adesão e diferenciação de células ósseas e formação de tecido mineralizado. No estudo 1, foi abordada a biofuncionalização química da superfície de Ti. Amostras de Ti foram revestidas com filmes de Polidopamina (PDA), e receberam ou não pós tratamento térmico que simulou processo de esterilização. As caracterizações físico-químicas demonstraram mudanças de reatividade da superfície do PDA após 2 semanas de armazenamento ou tratamento térmico devido ao processo de esterilização. Testes de adesão, proliferação e espraiamento foram realizados com células MC3T3-E1 cultivadas sobre essas amostras. O revestimento de PDA associado ao tratamento térmico estimulou a proliferação e espraiamento celular comparado com a as amostras PDA não tratadas. No estudo 2, foi proposta uma plataforma de triagem topográfica de alto desempenho (HTS) para estudo do impacto biológico de padrões estruturais de superfície hierarquizados. Células-tronco de polpa dentária humana (hDPSCs) e células ósseas de Fíbula humana (hBDCs), foram semeadas nos *arrays* de triagem contendo 60 nano topografias diferentes e 4 áreas de controle; construídas com nano pilares dispostos em simetria quandrangular (SQR) e hexagonal (HEX) que variam em diâmetro e distância interpilar. Testes preliminares de proliferação, mineralização e imuno detecção de Osteocalcina (OCN) demonstraram diferenças significativas na resposta celular entre topografias contruídas com características diferentes. A lateralidade, ou distancia interpilar, apresentou correlação positiva com a modulação de proliferação e diferenciação osteoblástica. As tendências celulares induzidas pelas topografias foram utilizadas para identificar e fabricar individualmente topografias-chave para diferenciação osteogênica.

Palavras-chave: Titânio. Implantes dentários. Diferenciação celular. Osteoblastos. Topografia.

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1 INTRODUCTION

For many centuries, the treatment of a diseased or damaged tissues was based on the physical removal of the injured region, in many cases without replacement[1]. Evidently, removal of joints, vertebrae, teeth, or organs led to several levels of limitations and impaired quality of life. Nowadays, a medical care revolution towards tissue repair and regeneration has highlighted the importance of the biomaterials field in rehabilitation procedures.

Although bone is a very dynamic tissue and its homeostasis is a self-regulated process[2], large bone defects caused mainly by severe trauma, cancer or congenital diseases might overcome its capacity of self-repair and regeneration. In these cases, bone biomaterials, such as permanent joint replacements, temporary fracture fixation devices and intraosseous implants are needed for an adequate recovery of the patient[3].

In the United States, over 2 million surgeries per year are performed to repair damaged or fractured bones by grafting or implantation[4]. According to the Global Burden of Disease (GBD), in 2010 the global age-standardized prevalence of severe tooth loss was around 2.4% which is expected to increase with the aging of the population[5]. As a result, the bone biomaterials market, projected to reach USD 47.5 billion by 2025, according to the Markets and Markets business report; is constantly on growing demand for new implants substitutes or improving current materials properties and processing techniques. The association between dental implants (DI) and subsequent prosthetic rehabilitation is currently a reliable option for different types of edentulism treatment. The osseointegration described by Brånemark[6] triggered a series of studies that aimed to identify DI materials and geometries capable of accelerating this biological process and decreasing failure rates. Titanium alloys have favorable mechanical properties, corrosion resistance and high biocompatibility, making up the core of the vast majority of DIs today[7].

Nowadays, dental implant survival rates can reach almost 90% due to several technical breakthroughs on titanium chemistry and manufacture over the time[8]. However, postoperative complications such as periimplantitis, poor implant fixation and components fracture are still relevant in clinical practice[9]. In addition, osseointegration can be impaired and treatment is sometimes contraindicated for patients compromised with severe immunosuppressive diseases, diabetes mellitus, osteoporosis and undergoing treatment with bisphosphonates[10].

The macrotopography of a DI refers to its visible structure, like the common conical thread design. The proper shape associated with the ideal surgical preparation of the host bone is the fundamental basis for clinical success in dental implantation. It is known that different

implant designs and variations of surgical techniques lead to different levels of early stability, the mechanical engagement between bone and biomaterial that prevents the formation of a connective tissue layer between implant and bone.

To overcome some Ti implant failures regarding the achievement and maintenance of osseointegration, current studies focus on the biofunctionalization of the surface of the DI with chemical and topographic modifications in micro and nanoscale. Although these modifications don't exert great impact on primary fixation and stability of the implant[11], changes in the chemistry and structural topography of the material can optimize the cell/substrate interaction, altering its metabolism, migration and growth[12]. The upregulation of cytokines and cell growth factors, such as bone morphogenetic proteins (BMPs), osterix (OSX) and runt-related transcription factor 2 (RUNX2), essential factors and modulators of osteogenic differentiation and maturation, can affect the outcome of late Ti implant complications in compromised general health patients or in cases of poor host bone quality and/or quantity [7, 13].

Several titanium surface modification protocols have been developed and tested in the last decade aiming the positive modulation of osteogenic cells behavior and further long-term DI clinical success [14-16]. In this thesis, we aim to review bone tissue properties and their relation with biomaterials; surface modifications techniques, divided into chemical and mechanical modifications; approaches to study topographical surface modifications; and the osteoblastic lineage differentiation, including integrin-mediated mechanotransduction and major cell signaling pathways modulating osteoblast differentiation and activity.

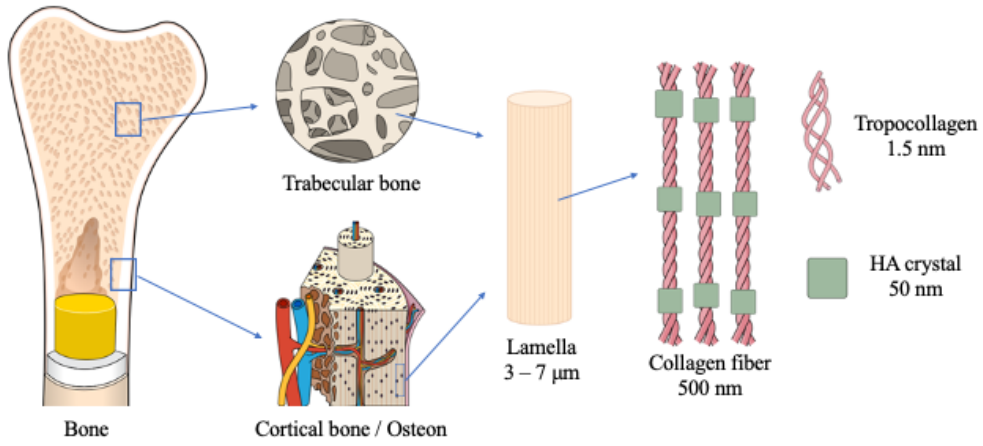
The experimental part of this thesis was divided into two chapters; in the first study we analyzed cellular proliferation and spreading of osteoblast-like cells cultured on polydopamine (PDA) coated Ti samples, with and without heating treatment and room temperature storage period. In the second study we accessed physicochemical and biological properties of a new nanostructured high throughput screening array methodology for studying the surface-induced osteogenic cellular response. Here we report that some of the investigated topographies, even with the same theoretical roughness, were capable of optimizing primary osteoblast-like cells behavior towards different levels of osteoblastic phenotype.

1.1 Bone and Biomaterials

As a rigid organ that constitutes the vertebrae skeleton in animals, the bone is responsible not only for vital support and protection of internal organs but also production of blood cellular components and plasma and mineral storage and regulation, containing 99% of the calcium and 90% of phosphorus present in the human body [17]. It is composed of inorganic elements, mainly calcium (Ca) and phosphorus (P) in the form of hydroxyapatite crystals (HA), but also sodium (Na), potassium (K), magnesium (Mg), fluoride (F), chlorine (Cl) and carbonate ($CO3^{2-}$); and an organic part, predominantly type-1 collagen [18].

This nanocomposite structure, with resilient collagen fibers reinforced by HA crystals, is divided into cortical and cancellous bone. Located at the outer surface, the cortical bone has relatively higher density and strength and it is responsible for 80% of skeleton mass. Cancellous bone is much more porous, collagen fiber bundles called lamella are organized into latticework of bony processes enclosing numerous large spaces filled with hematopoietic cells, adipose tissue and blood vessels (see Figure 1) [19]. These intricate composition and structures are important for bone superior compressive strength and high fracture resistance; however, it has been a challenge to develop biomaterials that meet all the physicochemical and biological requirements for an ideal bone repair treatment.

Figure 1 - Chemical composition and macro to nano structure of natural bone.



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Medical biomaterials must possess the right mechanical, chemical, and biological properties optimized for their specific purpose and biological surroundings. They are defined as engineered materials, from organic and inorganic constitutions, used for replacement,

support, enabling tissue growth and interaction (e. g. drug release) with host organs or tissues [20].

In case of bone tissue, these materials are mainly designed for fracture fixation, permanent joint replacement, and endosteal implants, like dental implants. The essential prerequisite of these bone biomaterials is to be biocompatible, meaning the ability to perform the desired function without causing any adverse response in the host tissue. Biocompatibility is a dynamic process that covers different concepts of material/tissue interaction [21].

Depending on the body reaction they cause, biomaterials can be classified as biotolerable, bioinert and bioactive [22]. A biotolerable substrate can only be tolerated by the body, and usually due to the release of chemical compounds or corrosion products, a fibrous membrane is formed separating the material from the host tissue. Bioinert materials are also tolerated by the body, but in contrast to biotorelable materials, there's no formation of a fibrotic connective tissue encapsulating the implant, and zero or minor chemical reactions between the material and the host tissue are observed. Most types of titanium and titanium alloys-based implants are currently identified as bioinert [23]. Materials that are able to integrate molecularly with the host tissue cells, modulating their response and behavior towards osseointegration and regeneration of the bone tissue, are considered bioactive [24].

For several years, the ideal materials intended for bone repair procedures were bioinert, however, past decade advances in engineering (e. g. nanotechnology) are greatly increasing the development and the sophistication of new generations of smart surfaces for medical devices. Such sophisticated materials are often designed to mimic and integrate a subset of physicochemical properties of natural tissue [25].

The osseointegration, which is still defined as direct contact between living bone and the implant surface at the level of optical microscopy [26], is crucial when it comes to the long-term clinical success rate of implants. Although this definition is well established in the clinical community, the concept of osseointegration has evolved over the years, taking into account the heterogeneity of the bone-implant interface; which consists of mineralized, partially mineralized and non-mineralized areas [27]. The concentration and disposition of mineralized collagen fibrils and non-collagen proteins, such as osteopontin, osteocalcin, bone sialoprotein; dictate the quality of the newly formed bone and the actual anchoring of implanted devices [28].

Bone biomaterials can be classified as osteogenic, osteoinductor and osteoconductor. These concepts are interrelated but they are not necessarily the same phenomena [29]. An osteogenic substrate can carry alive bone cells (osteoprogenitors, osteoblasts and osteocytes) in its interior. Until now, such attribute is basically restricted to autogenous bone grafting. The

osteoinductor material stimulates the recruitment, proliferation and differentiation of osteoprogenitor cells; this type of biomaterial increases the population of osteoblasts at the bone repair site, accelerating and optimizing the osteogenesis process. An osteoconductor material has the ability to serve as an environment for the infiltration of undifferentiated mesenchymal cells, osteoblasts and osteoclasts, acting as a passive framework that is gradually being absorbed and replaced in the bone repair process; a good osteoconductive material must have a structure and porosity similar to the native bone trabecula [30].

At last, a bone biomaterial that complies with all the above-mentioned desirable properties, can only be effectively employed if it can be sterilized. Sterilization commonly involves high temperatures and pressure, gas plasma or vaporized hydrogen peroxide treatment. The sterilization is crucial since the patient safety relies heavily on the removal of all contaminants and microorganisms, and the stability and corrosion resistance to these methods is what makes a material suitable for implantation [31]

Among all the several types and compositions, metal-based biomaterials are largely used and investigated due to their low biological reactivity and structural functions. Generally, all metal implants exhibit excellent strength associated with high fracture and corrosion resistance [32]. In the case on dental implants, these intraosseous structures are constantly subjected to loads, either static or repetitive, and this environment requires an excellent combination of strength and ductility [33]. In this sense, titanium and derived alloys present superior characteristics compared to polymers and ceramics, being therefore the main constituent of dental implants.

1.1.1 Titanium dental implants

The first recorded case of a metallic tooth replacement is dated from approximately 1000 BC. The body of an Egyptian king was found with a copper peg hammered into his upper jawbone. In 1809, J. Maggiolo designed the first metallic implant to replace human teeth in fresh extraction sites [34].

In the 1930's, Drs. Alvin and Moses Strock, discovered the importance of metal biocompatibility for implant success. The Strock brothers experimented with screw fixtures made of chromium-cobalt alloys to restore individual teeth. They are also thought to be the firsts to successfully place an endosteal implant. In the 1940's Formiggini, considered the

“father of modern implantology”, established the spiral design that inspired all the generations of metal dental implants until now [35].

Brånemark's studies with titanium started in 1952; by placing titanium chambers in rabbit femurs, he discovered that the bone actually bonded to the titanium surface and it could not be removed. In 1981 Brånemark presented a two-stage threaded titanium root-form implant to the scientific community in Toronto, after 15 years of clinical follow-up. His concept of osseointegration revolutionized titanium implants for dental rehabilitation [36].

Titanium and its alloys are considered to be excellent biomaterials for manufacturing permanent dental implants. Besides excellent general nontoxicity and biocompatibility, it also combines convenient mechanical properties, like low specific weight and high strength to weight ratio [37]. In addition, titanium and its commercial alloys (110 GPa) have much smaller Young’s moduli compared to other metallic biomaterials, such asSUS 316 L stainless steel and Co-Cr-Mo alloys (210 GPa), preventing higher incidence of bone tissue resorption due to stress shielding and periprosthetic fractures [38].

Titanium oxidizes immediately when exposed to air, forming a thin titanium dioxide (TiO_2) layer. Known by passivation process, the formation of this stable 3–10 nm thick TiO_2 film protects the bulk of the Ti implant from the environment and contributes to its high corrosion resistance [39]. The TiO_2 layer can also induce calcium, phosphate and proteins absorption, changing surface properties towards a more favorable biological interaction [40]. Hence, this metal and its commercial alloys are the most commonly used core material for medical implantation procedures [41].

1.1.2 Titanium surface properties

The material surface characteristics and chemistry are responsible for Ti implants to be uncommonly rejected by the body and for its inherent capacity to osseointegrate [42]. The surface wettability of a biomaterial combined with other surface properties, such as topography, surface energy, charge, mechanical properties and presence of functional groups, directly impact the biological cascade of events at the biomaterial/host interface from proteins adsorption to cellular adhesion, proliferation and differentiation [43, 44].

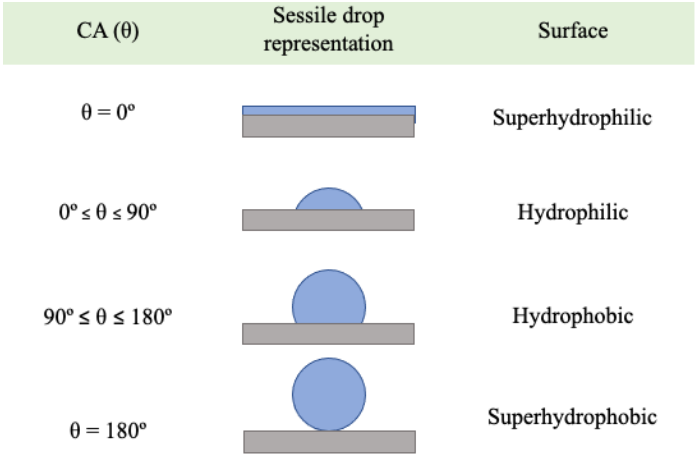
The most common method to infer about a material surface wettability is the sessile drop technique, the contact angle (CA or θ) between the tangent of a drop placed on the sample and the horizontal baseline of the solid surface is measured, and quantifies the wetting of the

surface by the specific liquid used [45]. Considering an ideal flat and homogenous surface, the CA is determined by the interactions of the liquid/vapor (γ_{lv}), solid/liquid (γ_{sl}), and solid/vapor (γ_{sv}) interfacial tensions; according to the Young's equation [46]:

$$\cos \theta_{Young} \gamma_{lv} = \gamma_{sv} - \gamma_{sl}$$

These interactions can characterize the hydrophilicity and the energy of the surface with WCA ranging from 0 to 180º. θs close to 0º indicate superhydrophilic or wetting surfaces, bellow 90° hydrophilic surfaces, θs above 90° are considered hydrophobic, and surfaces with θs above 150°, are often defined as superhydrophobic surfaces (Figure 2). Although it has been reported that WCA measurements are not the ideal parameter to explain cellular behavior [47], several studies have also associated moderate hydrophilic Ti implant surfaces with favorable biochemical conditions for cellular adhesion and further proliferation and differentiation [48, 49].

Figure 2 - Water contact angle (θ) diagram. Schematic representation of contact angle (CA) for a water drop placed on surfaces of different wetting behavior.



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The wettability which is intimately related to the surface energy is increased by combining high energy solid surfaces and wetting liquids with low surface tension [50]. Both properties are affected by interactions and the unfulfilled bonding potential of molecules at a surface; therefore, they can be modulated by the surface topography and roughness [51]. The liquid droplet interaction with a micro- or nanostructured substrate is dictated by its wetting state. When a liquid drop sits on the top of the solid surface features and air is trapped

underneath, the liquid/surface interface is considered a Cassie-Baxter state. When the drop impregnates the topographical textures, the interaction consists the Wenzel state [52].

Machined Ti surface, as any other fabricated substrate, embody a complex shape made of a series of micro and nano peaks and troughs of varying heights, depths, and spacing. Surface roughness is determined by calculating the amplitude parameters. There are many roughness parameters in use but the arithmetical mean roughness, Ra, is by far the most common. Ra gives the average of the absolute heigh value. Typically, the value is expressed in micrometers (μm). Roughness is often correlated with cellular behavior; several studies have demonstrated that different surface roughness can induce varying levels of bone cells response during the healing process of dental implants. [53-55].

Although aluminum and vanadium could cause minor negative immunological responses on the host tissue; the Ti6Al4V is still the most frequently used Ti alloy for biomedical implant devices [56]. The polished surface of Ti6Al4V has a WCA ranging from 50º to 60º and average roughness Ra of 0.2 μm [57]. Despite the fact that this Ti alloy has acceptable surface properties and biocompatibility levels allowing the initial interactions between the implant and the host tissue fluids, this material is not capable of improving biological performance for a faster would healing or a complete osseointegration in compromised patients. Hence, surface modifications are required. Blood compatibility, bone conductivity and the capacity of directly modulate cellular components are some of the desirable attributes biomedical engineers have emphasized over the last decade with chemical and mechanical surface modification techniques.

1.2 Surface Modifications

After the implantation surgery, the implant surface act as a medium for the contact and interactions of blood, immune, bone cells and its surrounding tissues. Thus, the surface quality of dental implants is one of the keys for clinical success of these procedures [58]. For many years, it was believed that changes at the surface of the implant couldn't be recognized by eukaryotic cells. To date, it is well known that cells are able to respond to chemical and topographical variations at a nanoscale on the implant substrate. Several ways of modifying implant surfaces have been proposed, chemical treatments and nanotechnologies have been developed, aiming the biological improvement of implant devices [59].

1.2.1 Chemical modifications

1.2.1.1 Functional peptides coating

The cell–protein–surface interaction is a critical process that must be taken into account for the engineering of biomedical implant substrates [60]. After the implant placement, proteins from the plasma adhere immediately to the DI surface through ionic bridges and a fibrin clot is formed. During the first hours, ECM proteins such as fibronectin and vitronectin will gradually replace the plasma proteins on the implant surface. These adhesion proteins can be recognized by transmembrane receptors of osteogenic cells [61]. The cellular interaction with ECM proteins can trigger and modulate molecular activities towards bone healing [62]. Considering the role of the adhesion proteins on initial cellular response and the osseointegration result, coating of titanium implants with ECM proteins or core amino acid sequences (derived from the original adhesion proteins) has been widely investigated. Due to low antigenicity and simpler chemical binding of smaller peptides sequences, core functional peptides are considered more promising candidates for implant surface biofunctionalization [63].

The peptide sequences can be immobilized through covalent grafting. However, usually the peptide sequence is physically adsorbed to the oppositely charged solid surface through electrostatic interactions, and when this spontaneous binding happens, a single layer, known as Self-Assembled-Monolayer (SAM), is formed [64].

Arginyl-glycyl-aspartic acid (RGD) sequence is one the most investigated peptide motifs for surface modifications aiming the improvement of cell/implant interaction. Studies have demonstrated that RGD-coated Ti samples exhibited better bone histomorphometry results

and higher attachment and proliferation of epithelial cells compared to uncoated surface [63, 65, 66].

Coatings of Ti implants with growth factors and bone morphogenetic proteins (BMPs) have also been well explored in the last few years [65]. BMP-2 is known to have a direct effect on modulating cellular response to promote osteogenesis [67]. Although human recombinant BMP-2 (rhBMP-2) is currently available in the dental implantology market for bone regeneration and healing; this protein has different biologic effects depending on its concentrations and surroundings, including bone resorption [68, 69]. Besides that, growth factors are usually active in free form, not in bound forms, possibly limiting their use when bound or attached to implant surfaces [67]. That could explain why, to the present day, no BMP or growth factor-coated implant has been used clinically, or even in clinical trials.

1.2.1.2 Hydroxyapatite coating

Hydroxyapatite (HA) coatings for implants and medical devices have become very popular over the last couple of decades. 50% of the human bone total volume is composed by HA $Ca_{10}(PO4)_6(OH)_2$ crystals. In theory, coating implant devices with the same material of the host tissue would enhance its biocompatibility increasing not only the bone-forming cells interactions in the microenvironment but also physical and mechanical integration of the implant substrate and the surrounding bone [70].

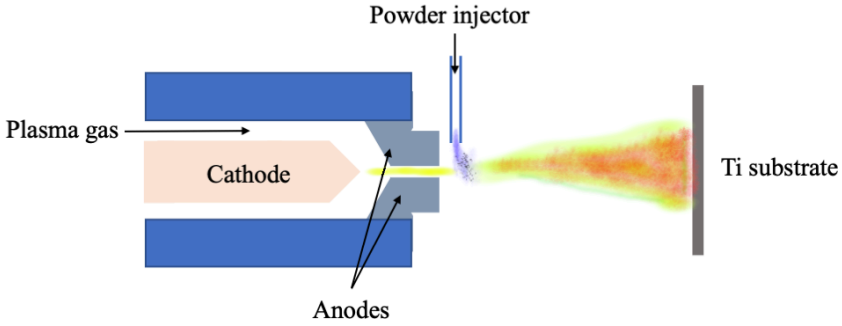
Studies have demonstrated that HA coating can prevent metal-ion release into organic tissues, associated with cell-mediated hypersensitivity reaction [71]. It can also enhance direct physical contact of implants to the hard tissue and attachment of osteoblasts on the coating surface [72, 73]. However, issues regarding adhesion failure and local inflammation reaction due to wear debris of the hydroxyapatite layer, have been reported [74].

The thickness of the HA coating, its calcium/phosphorus ratio and crystallinity seem to directly influence the material surface properties, including its biodegradation and the subsequent foreign body reaction [75, 76]. Although a few techniques have been tested for decreasing the HA layer thickness, enhancing its adaptation to the irregular implant surface and reduce the wear debris formation. The plasma spraying is still the most used method for HA coatings and it is the only one with long term clinical results [76].

In this method, HA particles are contained and heated in a plasma flame, that can go up to 20,000 Kelvin, and sprayed on the Ti surface. The thermal spraying process takes place under

normal atmospheric conditions and is based on a gas stream to carry HA powders, which are then passed through an electrical plasma produced by a low-voltage, high-current electrical discharge [77]. The spray temperature, flow rate, and composition can affect the chemical and physical features of the coating material. The semi-molten HA particles are deposited onto the implant substrate and after solidification, a 50–100μm thickness HA layer is formed [78] (Figure 3).

Figure 3 - Thermal HA plasma spraying system. Consisted of an anode and a cathode, usually cooled by water. Plasma gas (argon, nitrogen, hydrogen, helium) surrounds the cathode and exits the torch by passing through the anode nozzle. A high-voltage discharge between the cathode and anode ionizes the plasma-forming gas into which the HA powder is injected.



Source: Author personal archive.

HA coatings have reasonable low cost, straightforward manufacturing process and a fast deposition rate, allowing large scale production. Notwithstanding the fact that this technique has been widely investigated *in vitro* and clinically [79-82].

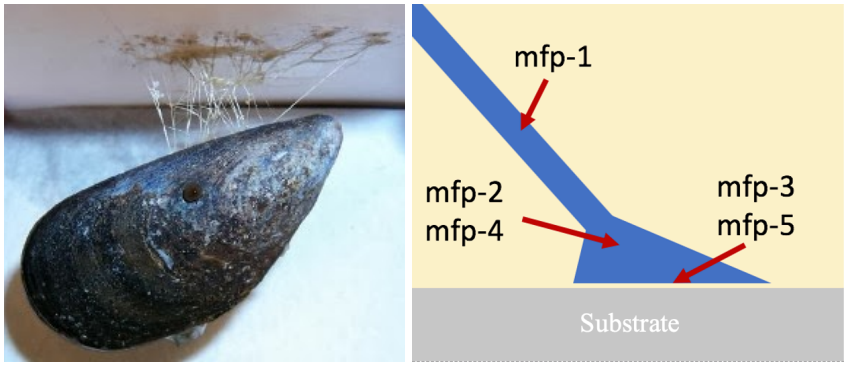
1.2.1.3 Polydopamine (PDA) coating

Polymeric coatings stand out as an alternative for chemical surface modification; controlling their functional groups and the structural chain of monomers we can easily dictate their properties [83]. The deposition of films with controllable surface properties has already been proven effective for altering substrates functionalities, such as increased corrosion and wear resistance, better biocompatibility, electrical conductivity and surface chemical reactivity [84].

During the last decade, adhesive systems inspired by the mussel bioadhesion have been widely studied due to their versatility regarding biomaterials coating [85]. These crustaceans are able to bind to organic and inorganic surfaces through byssus filaments, small bundles of

threads composed of mussel foot proteins (mfps), mainly the mfp-3 and mfp-5 proteins (Figure 4).

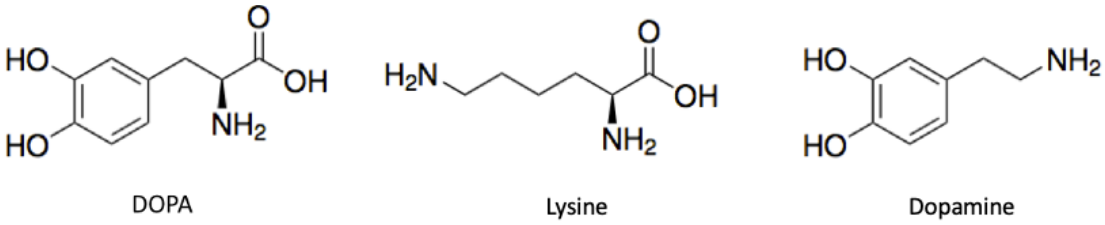
Figure 4 - Mussel adhered to the surface through byssus filaments. Schematic representation of a byssal thread and the different mfps important for substrate adhesion and byssal structure.



Source: Wilker Group, Purdue University. And author personal archive.

These proteins are rich in DOPA amino acids (3,4-dihydroxyphenylalanine), with a catechol functional group; and lysine, with amine functional group. The adhesion properties depend on the bonding, by hydrogen bonds, of the catechol and amine groups of the two amino acids, forming the dopamine molecule (Figure 5) [86].

Figure 5 - Chemical structure of the DOPA, lysine and dopamine molecule that contains the catechol and amine functional groups.



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Polydopamine films are formed through an oxidative reaction between dopamine and oxygen in alkaline conditions. The dopamine self-curing product has low cost, low toxicity and it is easy to manufacture. PDA films have the ability to adhere to almost any type of substrate and topography; and due to the high amount of catechol groups and free amines, the PDA increases the biocompatibility of materials and allows their subsequent functionalization with biomolecules or chemical groups, contributing to better biological interactions of the material [86].

The exact structure of the PDA has not yet been determined; however, most studies agree that the molecules of dopaminequinone and dihydroxyindole (DHI), formed by oxidation

of dopamine by air and intramolecular cyclization, respectively, are the molecular constituents of PDA films [86, 87]. These coatings generally have unpolymerized dopamine units, trapped in the chemical structure of the film, which can make the PDA a heterogeneous material and susceptible to chemical changes [88, 89]

In general, the literature describes the PDA as a biocompatible coating, improving the immobilization of biomolecules and the proliferation of cells, compared to surfaces with low wettability [85, 90, 91]. However, these studies do not take into account this heterogeneity and chemical plasticity of the PDA. To be a viable option for implant surface modification it needs to remain stable after post-heat sterilization procedures and long periods of storage.

1.2.2 Micro and nano-roughened topographical modifications

1.2.2.1 Sandblasted, Large-grit, Acid-etched (SLA)

The SLA surface is commercially available and it is one of the most investigated modifications employed in dental implants. This modification on Ti surface is made by sandblasting the polished substrate with large-grit particles (usually from 250 μm to 500 μm), and by etching the blasted surface with strong acids, in most cases hydrochloric, sulfuric or nitric acids. This technique changes the Ti surface topography, creating random micro-scaled irregularities, such as large dips, small micropits, sharp edges, and pointed tips in a random and uneven conformation [92]. With average roughness ranging from 18 to 23 μm, it has been reported that SLA surfaces show high rates of bone integration of titanium implants [93].

It is well known that surface topography plays a critical role, at the molecular level, in the implant-to-bone response and therefore, the successful osseointegration of a dental implant [94]. After the surgical procedure and implant insertion, a fibrin membrane is formed at the peri-implant site [95]. This adsorbed protein cloth will facilitate the adhesion and migration of osteogenic cells, that seem to recognize the irregularities of the SLA surface and increase the production of several bone-forming factors, like osteocalcin, osteoprotegerin, alkaline phosphatase, TGF-β and BMPs [96-98].

As previously discussed, the wetting property of a surface might play a critical role on the initial cellular interaction to a substrate. Even though SLA surfaces present good cellular response and osseointegration rates. The SLA technique was modified for enhancing the Ti surface hydrophilicity; an attempt to accelerate the bone healing process [99, 100].

The dental implant with increased hydrophilicity SLA surface, is commercially available as SLActive (Institute Straumann AG, Basel, Switzerland). The final surface was developed with a water rinse in a nitrogen chamber, as last step, and maintained with the implant kept in an isotonic sodium chloride buffer with no atmospheric contact[101].

Although both the original SLA and the SLActive implants have been extensively tested in clinical trials, with excellent long-term results, the characterization of well-defined topographical properties and features are crucial for the design of a new generation of smart surfaces that can manipulate the cell fate and the tissue engineering[102-104].

1.2.2.2 Laser ablation

Most part of current methods for chemical and mechanical surface modifications of Ti substrates are complex processes that involve several chemicals and particles treatment, adding potential contaminants to the surface; which may influence osteogenesis [105]. Laser micromachining of Ti surfaces can generate micro to nano-scale channels and patterns by controlling the laser processing parameters. Described as highly controllable, this approach produces substrates with increased surface roughness and generally higher degree of purity compared with materials modified by other surface treatments [106].

Studies using laser-microtextured Ti samples have demonstrated not only faster osseointegration, but also increased connective tissue perpendicular attachment to the implant surface, differing of the usual parallel orientation of the peri-implant fibers seen in recently implanted devices [107-109]. It has been reported that laser treatment might increase TiO_2 layer thickness, affecting the quality of bone/implant contact and the subsequent osseointegration [110]. *In vitro*, laser-treated Ti surfaces influenced osteoblasts morphology and enhanced expression of key osteoblastic transcription factors and ECM proteins, leading to increased mineralized nodule formation [111].

Commercially available for almost 10 years now, the laser-modified Ti implants Laser-Lok® has showed favorable responses to hard and soft tissues compared to turned Ti surfaces. Long-term case studies have also demonstrated excellent survival rates for this laser-machined implant, evaluated to be around 96% [112, 113].

Although *in vitro, in vivo* and clinical investigations have showed laser ablation as reliable alternative for Ti implants surface modification, it is not easy, due to many possible

laser parameters, to standardize this process and specific topographic patterns modulating cellular response [109].

1.2.2.3 Micro- and nanofabrication

Great part of the micro- and nanofabrication techniques were developed and are still mostly used by companies engaged in the design and fabrication of semiconductors [114]. However, these technologies have been revolutionizing the biomedical field. They offer the possibility of construction of micro- and nano-scale structures and substrates, with different geometries and functionalities, that can interact with biological systems at these levels; and could be used for unraveling cellular and molecular processes, drug delivery systems, biosensors [115].

These material fabrication methods have been widely investigated recently in the implantology field. It is well known that micro- and nanotexturized implant surfaces are capable of affecting specific protein interactions and modulating cellular adhesion, proliferation and differentiation towards an osteogenic phenotype (37, 38). Nevertheless, using very precise and reproducible sequential techniques to produce desired structures, built within the bulk of a substrate material or on the surface of the substrate, could optimize and standardize mechanical-induced cellular behavior. This precise fabrication might improve the design of a new generation of implant devices with surfaces targeting specific cell and tissue function.

1.2.2.3.1 Photolithography

In the natural bone tissue microenvironment, cells are subjected to complex chemical and topographical cues, from macro, such as resorption areas or ligament shape, micro, like the morphology of other cells, to nano, such as protein folding and collagen fibers [116]

The effects of micro- and nanotopographies on molecular and cellular response have been well documented, including impact on cell adhesion, contact guidance, cytoskeletal organization, apoptosis, macrophage activation and gene expression [117, 118]

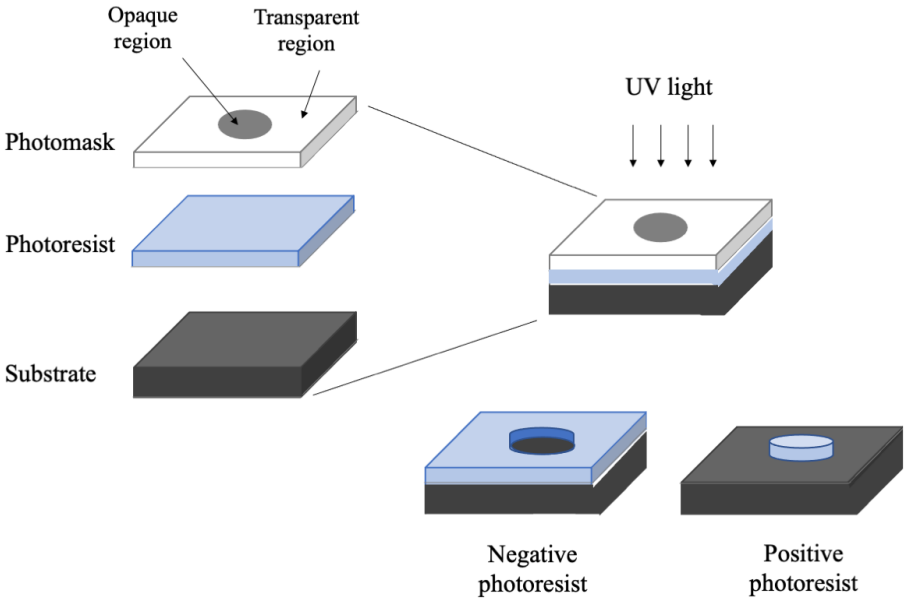
Photolithography is a very precise technique for imprinting desired micro-scaled patterns and features, and it has been widely used for investigation of osteoprogenitor and bone cells response to microtextures. [119, 120]. This technique is based on exposure of specific regions of a light-sensitive substrate to ultraviolet (UV) light.

First the material surface is coated with a layer of a photoresist, usually a light-sensitive polymer. A photomask, constructed with an opaque and transparent material is placed on top of the substrate and photoresist. The desired shape and pattern of the features to be constructed on the substrate is imprinted on the photomask by a laser beam (laser writer) or a beam of electrons (e-beam writer) [121].

The projection system, consisted by the photomask, photoresist and the substrate (wafer) is irradiated with UV light, exposing parts of the photoresist not covered by the opaque sections of the photomask. When exposed to light the photoresist can undergo two different chemical reactions. The exposed areas of the photoresist can break down and become more soluble to a developing solution (negative); or become crosslinked upon light exposure and becoming insoluble in the developing solution (positive). Depending on the case, the parts exposed to the light can be washed out by the developing solution or be intact after this process (Figure 6) [122].

The resulting pattern on the photoresist is used to protect the covered substrate from the subsequent etching process, where the selective removal of the substrate material leaves the pattern design on the surface [123].

Figure 6 - Photolithography process. Photoresist coated onto a flat substrate, usually a silicon or glass wafer. This photosensitive layer is exposed to UV light through a photomask. The regions of the photoresist exposed to the light undergo a chemical modification. Depending on the type of photoresist utilized, the photoresist polymer will undergo one of two possible transformations upon exposure to light (positive and negative photoresist.



Source: Author personal archive.

The resolution and the size of the projected image of the desired feature on the wafer is limited by the wavelength of the light that is used. The feature size that a photolithography projection system can print is approximately determine by the equation:

$$CD = \kappa_1 \cdot \frac{\lambda}{NA}$$

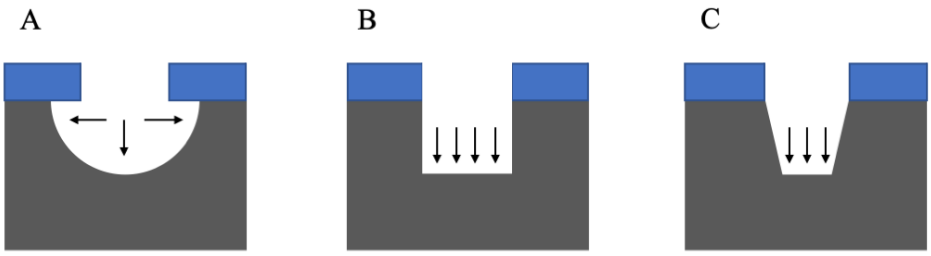
Where *CD* is the critical dimension or the minimum feature size, the κ_1 (k1 factor) is a coefficient based on the Rayleigh criterion, that defines the minimum separation for preserving the distance between two points in the projected image. The λ is the wavelength of the light used and the NA is the numerical aperture of the lens. According to this equation, the size of the features can be reduced by decreasing the wavelength and increasing the NA (tighter focused beam). Modern photolithography systems use deep ultraviolet (DUV) light from excimer lasers with wavelengths ranging from 190 to 248 nm which allow minimum feature sizes down to 50 nm [124, 125].

Photolithography is well accepted in the microfabrication field due to its high resolution and variety of possible surface pattern designs. Nonetheless, further technique adaptation and pre-clinical investigations are necessary before the employment of this method for surface modification of metallic implant devices [126].

1.2.2.3.2 Etching

As the name suggests, the etching technique is based on the controlled and precise removal of parts of a substrate material through physical or chemical processes. It can be classified as isotropic, when the etching occurs in all directions within the substrate, both in the direction of depth but also laterally; and anisotropic, when the etching occurs only in the direction of depth (Figure 7)[123].

Figure 7 - Etching profiles generated with (A) isotropic etching, (B) dry anisotropic etching, and (C) wet anisotropic etching.



Source: Author personal archive.

The material removal can be done by liquid chemicals, wet etching; or gaseous physico-chemical reactions, dry etching. The dry anisotropic etching usually results in a flat tunnel-like pattern, while the wet anisotropic etching, produces uneven inclined side-walls. This effect is the result of the interaction of etching reagent with the crystalline structure of the substrate. The chemical structure of the material also determines the rate of etching and the flatness of the etching profile [121].

This method is usually associated with lithography processes, and it has been used for engineering micro-scaled topographies to modulate and access cellular response [122, 127]

1.2.3 Approaches to study topographical surface modifications

Topography, defined as the structural aspect of a surface, is often investigated within the implantology field, as a positive modulator of osteogenic cellular response [128]. It is well established that rough surfaces with micro- and nanostructured features are able to accelerate osteogenesis on orthopedic and dental implants compared to smooth machined controls [129].

Most of these topographies are usually constructed by sand blasting techniques resulting in random structured surfaces with divergent scale sized features. Although random structured surfaces are well stablished in terms of application, the current investigational trend is focused on hierarchically designed topographies, composed of ordered, semi-disordered or disordered structures with well-defined feature sizes and surface physicochemical properties. Such systems can help to elucidate the correlation between specific topographical parameters and cellular response, for the design of a new generation of smart substrates and medical implants that might control cell fate.

However, testing an infinity of topographical constructions and parameters by usual cell culture methodology, would require a large population of cells and at least one cell culture surface for each condition to be investigated. Considering the production of the culture surfaces itself, large medium volumes, bulky incubators and expensive human labor, the investigation of cohesive surface-induced cellular responses needs excessive experimentation time and massive financial investments [122].

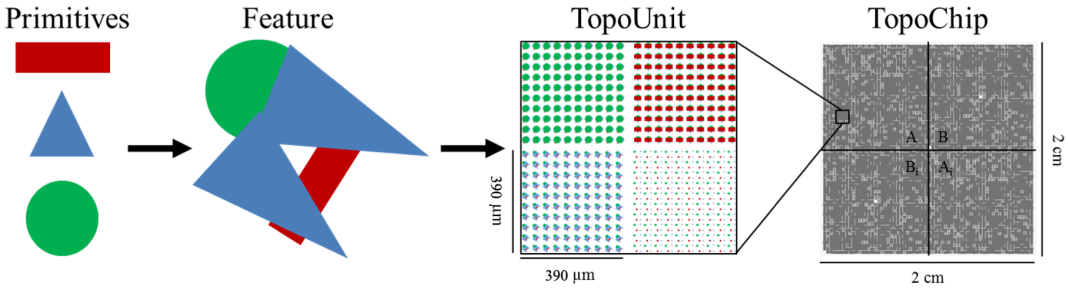
Micro- and nanofabrication techniques have allowed the development of fast, relatively inexpensive cell culture systems assembled with different neighboring topographical constructions. Such devices can probe the same population of cells under a large number of conditions, producing results at a high throughput screening (HTS) method. In this section, two approaches for investigation of surface-induced cell behavior by HTS will be reviewed.

1.2.3.1 Nano-TopoChip

Nanotechnology has gained wide attention in biomaterials field and can be defined as science engaged in conception, synthesis and characterization of materials and devices with functional organization at the nanometer scale [130]. As nanostructured topographies are in the same size range of filopodia, focal adhesion complexes and extracellular matrix fibers, it has been demonstrated that these surfaces are capable of enhancing bone cell adhesion, growth and differentiation, through specific protein interactions [131, 132]. However, the exact mechanism by which topographical cues manipulate cellular behavior remains unclear.

Proposed by Hulshof et al. in 2017, the Nano-TopoChip is a high-throughput screening system with nanostructured features, that allows the study of morphology and proliferation of cells cultured onto a library of different nanoscale topographies [133]. The system design was created by a C++ script that randomly selected 1246 unique topographies from a theoretical library of millions of topographies generated by combining and overlapping basic shapes, such as triangle, rectangle and circle. After the computational pattern generation, the script creates an image file that can be applied on a photomask. The authors used a combination of conventional UV lithography, Deep-UV lithography and nano imprint lithography (NIL), to fabricate a final polystyrene Nano-TopoChip device of 2 x 2 cm surface, containing 1246 topography landscapes. They grouped these topographies in individual test surfaces of 390 by 390 μm called TopoUnits, each separated by 30 μm high, 10 μm wide walls (Figure 8).

Figure 8 - The Nano-TopoChip. Combined and overlapped primitive shapes (circles, triangles, and rectangles) are used to design topographical features. TopoUnit comprising a 390 × 390 µm array of a topographical feature.



Source: BiS-Biointerface Science in Regenerative Medicine group webpage. Eindhoven University of Technology.

This screening approach was used to study the effect of the nanostructured topographies on cell morphology and proliferation. Considering the large number of topographies and the extensive dataset, they used bioinformatics tools to identify which pre-defined cellular morphological parameters (such as cell integrity, alignment, number, size, cell spreading and extent) were being most affected by the topographies. A Kruskall-Wallis test was performed to identify the cell parameters with highest variation within the TopoUnits.

With this preliminary investigation using the Nano-TopoChip, the authors could infer about important surface/cell morphology relationships. They reported not only that differences on the topographical landscapes mostly affect cell area and orientation, but also that topographies inducing small, round shaped cells, were constructed with relatively larger features with large spacing between them; and the topographies with higher variation of cell spreading were assembled with smaller features covering a relatively smaller area [133].

It is unquestionable that the Nano-TopoChip device has been demonstrated as an excellent tool to elucidate the biological effect of nanostructured topographies. The library design is reproducible, and covers an impressive number of topographical feature shapes and possible landscapes. The bioinformatics method to analyze the dataset can automate the investigation of cellular parameters, and theoretically, predict cell morphological response to specific topographical constructs.

However, we believe that the large number of topographies constructed with such high level of randomness in so many possible feature shapes, add too many variables to the understanding of how specific and defined surface parameters manipulate both cell morphology and differentiation. As can be observed in this particular study, the correlation of the topographical specifications with further cellular behavior remains imprecise. Future

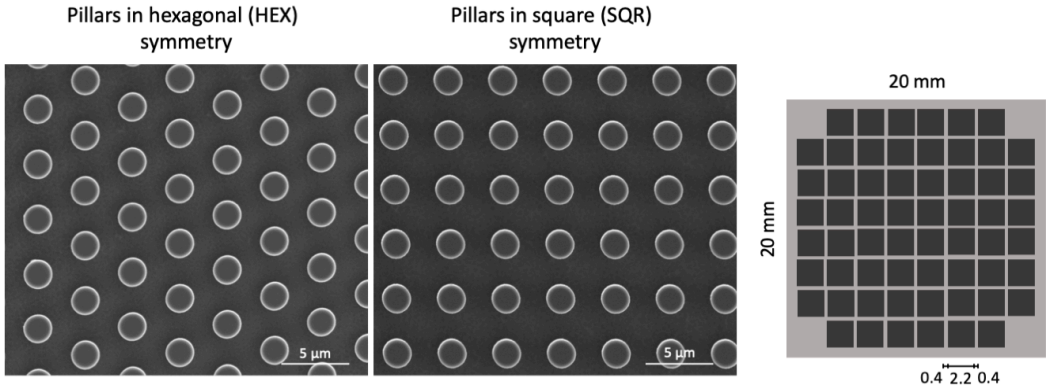
experiments using this device and the exploration of other HTS methods are still needed to unravel specific nanotopographical designs controlling functional cell phenotype.

1.2.3.2 Nano Topographical Screening Array (NTSA)

Research has shown that nanostructures can be imprinted on an implant surface. However, these topographical structures can also appear spontaneously on titanium surfaces due to machining process or deposition of TiO_2 particles [134]. *In vitro* and *in vivo* studies have demonstrated higher bone cells proliferation and a more effective osseointegration when implants contain such nanostructures [135, 136]. Despite this, there are still controversies in the literature regarding the standardization of a large number of nanostructures specifications and their role on *in vitro* cell fate, and further clinical relevance.

To study well defined topographical parameters, at the nano scale, governing cellular proliferation and osteogenic differentiation, we propose an HTS combinatorial library consisting of 8 x 8 (64) distinct topographies, which 4 of them are unstructured flat controls. Each topography corresponds to a 2.2 x 2.2 mm square that has a pillar-based landscape. They are labeled "X;Y", where the X stands for the pillar diameter (varying from 0.3, 0.5, 0.8, 1 to 2 μm); and Y is equivalent to the distance between the pillars (varying from 0.3, 0.5, 0.8, 1 to 2 μm). All the pillars have the same height (2 μm) and they were placed in two symmetries: hexagonal and square (Figure 9).

Figure 9 - Scanning electron microscopy images of the nano-pillars placed according two symmetries: hexagonal (HEX) and square (SQR). Representative image of the NTSA containing 4 flat control areas and 60 different topographies.



Source: Author personal archive. Unpublished data 2021.

The topographies were created on Si wafers using deep UV lithography. Considering that titanium is the most used core material for dental implants and medical devices, the screening arrays were coated with a 10 nm thick layer of TiO_2 by atomic layer deposition (ALD).

The design of the topographies used in this screening device was inspired by a previous study where another combinatorial library consisting of 13 x 13 (169) distinct microstructured topographies, was used to investigate the osteogenic behavior of MC3T3s and DPSCs, cultured onto 16 combinations of different pillar shapes, symmetries, heights, size and interpillar distances [127, 137, 138].

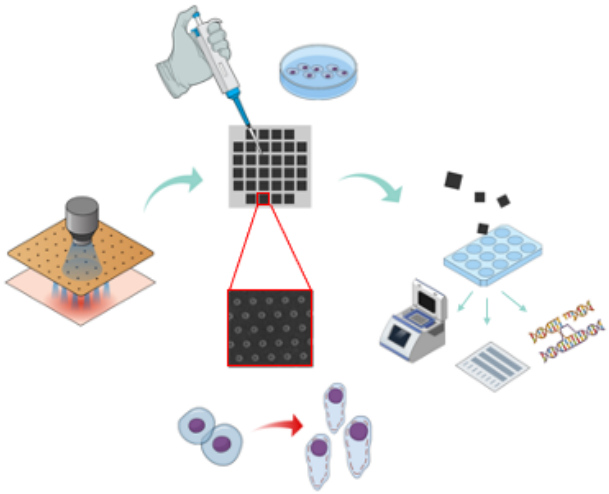
The results obtained in these studies highlighted that some specific topographical parameters, such as pillar height, shape, diameter, lateral spacing and symmetry; can enhance the cellular behavior towards an osteogenic phenotype. With the proposed nanostructured screening platform, we aim to further investigate the effect of these topographical trends on cell response, at the nano scale level. The highly ordered and hierarchical approach, with reduced topographical variables, might facilitate the connection of nanostructured surfaces specifications and their associated cell fate.

Notwithstanding the fact that the nano screening arrays are a worthy investigational tool for studying the role of surface topographical trends on cellular behavior, it has a limitation regarding the underlying molecular mechanisms of surface-induced biological response. With this HTS approach, it is not possible to quantify individually on each surface, the expression of osteoblastic proteins, gene markers or enzyme activity.

In this thesis, the adhesion, proliferation and differentiation of hBDCs and DPSCs were investigated on the topographies within the array. The data was analyzed and compared for each one of the surfaces. A smaller set of key topographies inducing an increased cell response was identified.

These key topographies were manufactured as single structure samples, by the same DUVL and ALD processes, for a more systematic investigation of the topographical parameters and the mechanisms dictating the osteogenic behavior (Figure 10).

Figure 10 - Schematic representation of the research flow. From NTSA fabrication, biological characterization and biomolecular investigation with single structure samples.

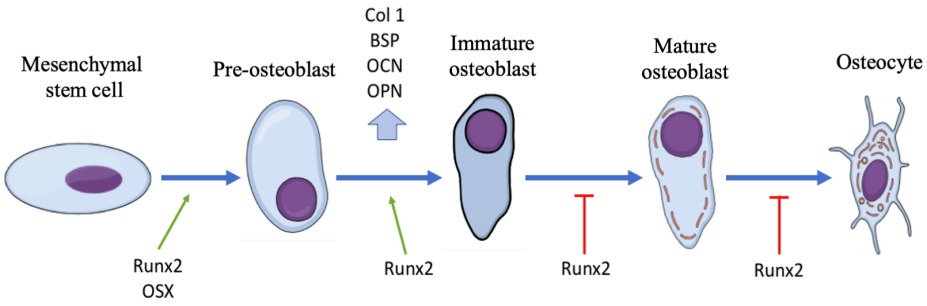


Source: Author personal archive.

1.3 Osteoblast Differentiation and Activity

Osteoblasts are cells capable of forming mineralized tissue and together with osteoclasts, the bone resorbing cells, they regulate the bone turnover, maintaining the shape, quality, and size of the skeleton [139]. They are derived from bone marrow mesenchymal stem cells through processes involving commitment, osteoprogenitor expansion and terminal differentiation. The Runx2 transcription factor is known as the main regulator of differentiation, function and gene expression of osteoblast markers. Together with the transcription factor Osterix, they are necessary and sufficient for the commitment and differentiation of mesenchymal progenitor cells into osteoblastic lineage [140]. Runx2 expression increases in mesenchymal cells after commitment, reaches its expression peak in immature osteoblasts and is down-regulated again in mature osteoblasts (Figure 11). This transcription factor binds to the OSE2 cis-acting element on the promoter region of the main osteoblast genes, such as osteocalcin, bone sialoprotein (BSPII), osteopontin and type I collagen and regulates their expression [141, 142].

Figure 11 - Representation of mesenchymal precursor cells commitment and differentiation into the osteoblastic lineage.



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Besides Runx2 and Osterix, it has been demonstrated that a few other transcription factors play critical roles in osteoblast turnover. Such as the AP-1 family (composed of the members Fos and Jun), Atf4 (activating transcription factor 4), and ß-Catenin [143].

Mesenchymal precursors and osteoblasts can sense external mechanical stimuli and transduce these signals through various cellular signaling pathways into the nucleus. This process, called mechanotransduction, regulate the expression of osteoblastic transcription factors, genes and cytokines; and has a key role on determining osteoblastogenesis and subsequent bone formation [140].

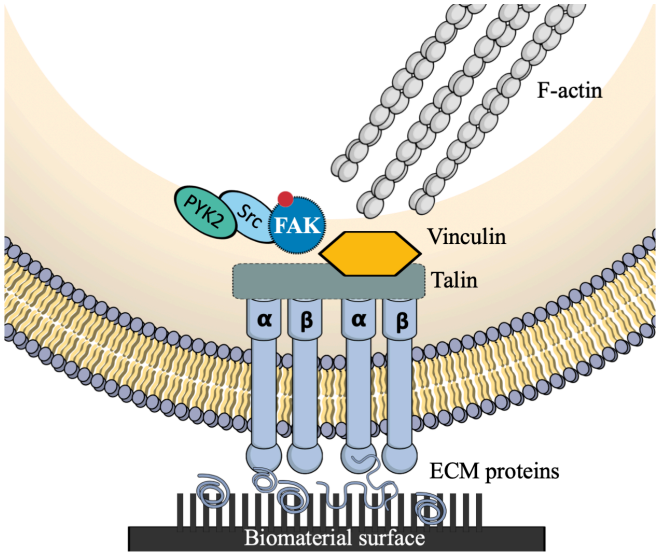
1.3.1 Integrin-mediated mechanotransduction

Osteoprogenitor cells, osteoblasts and osteoblast derived osteocytic cells primarily interact with an implanted material through integrin binding to ECM proteins. They bind specifically to motifs located on fibronectin and collagen type I, such as arginine-glycine-aspartic acid sequences (RGD) and the GFOGER motif [144, 145]. Integrins are heterodimeric transmembrane highly glycosylated receptor proteins, consisting of an α and β non-covalently linked subunits. These proteins are responsible for sensing and attaching the cytoplasm to the extracellular environment [146].

Osteoblasts express several integrin α and β subunits including αl, α2, α3, α4, α5, α6, αv, β1, and β3 [147]. Although the exact role of each one of the subunits remains unclear, several studies suggested that α5β1 regulates osteoblast attachment and growth; while α1β1 and α2β1are involved in osteoblast differentiation [148-150].

The integrin-mediated osteogenic phenotype is triggered when the ECM-integrin initial interaction forces overcome a critical threshold, in the nanonewton range, required to support further cell adhesion [151]. These receptor proteins will cluster and form complexes with structural adaptor proteins, such as vinculin, talin, and paxillin, that will mechanically couple the ECM to the cytoskeleton by actin filaments (F-actin). This requires focal adhesion kinases (FAK) phosphorylation, as a modulator of protein recruitment, and PYK2 and Src activation (Figure 12). It has been reported that the adaptor protein Shc1 is coupled by FAK to mediate sheer-stress-induced osteoblastic response [152, 153]. These protein complexes, called focal adhesions, lead to activation of RAS/Raf and MAPKs, resulting in ERK1/2 and p38 activation with subsequent activation of bone-related transcription factors (e.g. Runx2, Osterix, Jun/Fos). Integrin function also induces NF-κB activation, leading to cyclooxygenase 2 (COX2) and OPN production, required for osteoblast proliferation [154-156].

Figure 12 - Schematic overview of the molecular composition of the focal adhesion. The complex attach ECM proteins to the cytoskeletal F-actin through the dynamic interactions between the adapter proteins, in particular talin and vinculin. FAK acts as a modulator of protein recruitment to the focal adhesion.



Source: Author personal archive.

The impact of micro- and nanostructured surfaces on mechanotransduction can be classified as direct and indirect. The topographical cues of a surface can exert different levels of cytoplasmatic stress and cytoskeleton conformations that are capable of directly change gene expression by modifying nuclear components organization [157, 158].

The surface properties of a substrate, including its topographical landscape, can dictate the adsorption pattern of biological molecules, such as proteins, ions, sugars and lipids present in the surrounding blood [159, 160]. It has been demonstrated that surface features can induce and change the adsorption and alignment of ECM proteins, affecting cell/material adhesion interactions [161]. The indirect changes on integrin clustering and focal adhesion complex formation might alter the downstream cell signaling leading to expression of osteoblastic genes and proteins.

1.3.2 Cell signaling pathways governing osteoblastic cells function

For many years, the understanding of how surface modifications of biomaterials could influence the bone homeostasis, from mesenchymal precursors commitment to achievement of proper bone mass and architecture, remained unclear. With the improvement of molecular and genetic tools, our comprehension of the intracellular signaling pathways governing bone cells activity has evolved significantly. It is known today that changes in the cellular mechanosensing

by surface modifications might alter the sophisticated network of receptors, ligands, intracellular kinases/phosphatases, transcription factors, and expression of cell-specific genes. In this section, major cell signaling pathways modulating osteoblast function will be briefly described.

1.3.2.1 Bone morphogenetic proteins (BMPs) pathway

Bone morphogenetic proteins (BMPs) are growth factors members of the transforming growth factor β (TGF-β) superfamily of proteins. They were originally identified as paracrine inducers of bone formation. Now, they are known to exert direct osteogenic effect by acting on osteoblast differentiation and maturation [162].

These cytokines have been widely investigated in the past decades and, currently, recombinant human BMPs (rhBMPs) are used in several tissue-engineering products aiming to increase bone regeneration, such as polymer-based rhBMPs carriers, nanoparticles and surface biofunctionalized devices [163].

BMPs have a high affinity for BMP receptor type I (BMPR1) and a lower affinity for the type II receptor (BMPR2). BMPs can bind to BMPR1 alone or in combination with the BMPR2. The two receptors have different cellular expression, but they share the same downstream kinase activity. The activation of BMPs receptors phosphorylate Smad proteins. Smad1, Smad5 and Smad8, are known as receptor-regulated Smads or R-Smads. After activated, R-Smads bind to Smad4 and this complex is translocated to the nucleus, whereby it modulates osteoblast-related transcription factors [164]. One of the transcription factors regulated by Smad-mediated BMP action is Runx2. The activation of this signaling pathway results in the transcription of proteins involved in osteoblast differentiation and function, such as osteocalcin, collagen type I and alkaline phosphatase [165].

TGF-β binds to TGFBR2 and TGFBR1, also promoting phosphorylation of Smad proteins. However, its downstream signaling has an intricate effect on bone formation. TGF-β can induce proliferation, chemotaxis, and early differentiation of osteoprogenitor cells, yet it inhibits osteoblast maturation, mineralization, and participates in osteoclastogenesis via directly binding with receptors on the osteoclast [166].

1.3.2.2 Wnt-induced signaling

Wnts and the Wnt/β-catenin signaling pathway are mostly associated with osteogenic differentiation. Wnt protein was first described in Drosophila, as the Wingless (Wg) gene that is involved in wing development [167]. They comprise a series of 19 members of secretory glycoproteins, and can be classified as classical Wnt proteins (Wnt1, Wnt2, Wnt3 and Wnt3a) and non-classical (Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, and Wnt11, among others). Wnts induce intracellular signaling by binding to the Frizzled/LRP (Fzd/LPR) receptor [168].

Through binding to Fzd/LRP, classical Wnt proteins activate the called canonical Wnt/β-catenin pathway. Under resting conditions, the glycogen synthase kinase 3 (GSK3) phosphorylate ß-Catenin, leading to a proteosome-dependent degradation of this protein. Following Wnt stimulation, the receptor activates Dishevelled (Dvl), Axin and Frat-1, involved in the inactivation of GSK3. The disruption of GSK3/ß-Catenin complex causes the accumulation of cytoplasmic β-catenin, that is eventually translocated to the nucleus and bind to the promoters of Runx2 and Osx. While non-classical Wnt proteins are responsible for activating heterotrimeric G protein and regulating the intracellular level of Ca^{2+} ions [169].

Activation of canonical Wnt signaling leads to increased transcription of Runx2, Osx and subsequent target genes. In osteoblastic cells, one of the most relevant target genes is osteoprotegerin (OPG). It is a key factor on bone turnover, acting as inhibitor of receptor activator of NFκB ligand (RANKL), an osteoclastogenesis modulator. Studies have showed that deletion of the gene encoding β-catenin in osteoblasts and osteocytes results in low OPG levels and reduced bone mass [170].

1.3.2.3 Insulin-like growth factors (IGFs)

IGF I and II share structural and properties similarity with insulin. They're mainly produced in the liver, bone and adipose tissue. IGFs activate two receptors, IGF-IR and IGF-IIR; with lower affinity, they can also stimulate insulin receptor [171].

The IGF receptor is formed by two extracellular ligand binding α subunits and two transmembrane β subunits. The tetramer activation is tyrosine kinase dependent and results in the phosphorylation of adaptor protein insulin receptor substrate (IRS), followed by activation of the growth factor receptor binding protein 2 (Grb2)/Sos/Ras/Raf/MAPK signaling complex.

It leads to activation of ERK and PI3K that are responsible for phosphorylation and activation of Akt, mTOR, eIF4E and finally p70S6 kinase [172].

It has been demonstrated that in osteoblastic cells some members of IGF family activate PI3K/Akt and Ras/Raf/MAPK signaling pathways, both triggering the activation of p70S6, which modulate proliferation, survival and anti-apoptotic actions [173].

1.3.2.4 Fibroblast growth factors (FGFs)

FGFs are a family of proteins that regulate a range of paracrine and endocrine biological functions, including cellular proliferation, survival, migration, and differentiation [174].

With their biological potential, recombinant human FGFs have been targeted as therapy for the regeneration of damaged tissues, such as skin, ligament, cartilage, bone, tooth. Its association with bone biomaterials has been widely studied, to secure the molecule biological activity since free-FGFs are highly degradable *in vivo* [175, 176].

With similar mechanism to IGFs, except for the downstream activation of IRS, FGFs activate their tyrosine kinase receptors FGFRs that phosphorylate adapter protein Grb2 triggering the Sos/Ras/Raf/MAPK pathway. This allow MEK1/2 and ERK1/2 to phosphorylate transcription factors such as c-Jun, c-Fos, AP-1, Runx2 regulating transcription of osteoblastic genes in the nucleus. FGFs are also responsible for the up-regulation of TGF-β, IGF-I and VEGF. They indirectly affect osteoblastic cells activity through FGFRs stimulation [177].

5 CONCLUSION

Herein, we address chemical and topographical alternatives for titanium surface modification and the investigation of their biological impact on osteoblastic cells behavior. We report the potential of PDA biofunctionalization for modulating different levels of cellular response. A new HTS topographical platform for investigation and optimizing surface specifications aiming the surface-mediated control of cell fate. Furthermore, a laser ablation technique to modify titanium surface and its impact on paracrine osteogenesis.

5.1 Conclusion Publication 1

We demonstrate that storage and post heat treatment are capable of altering some physiochemical properties of polydopamine coatings. The thermal post-treatment at 121 ºC, showed a considerable increase in hydrophobicity and better adhesion and proliferation of MC3T3-E1 compared to storage period. Although the PDA coating and post-treatments interfere with the cell response in different levels, it falls short compared to the adhesion and proliferation results obtained in the Ti control samples. Our findings highlight the need for future studies aiming the improvement of the surface properties of these coatings.

5.2 Conclusion Publication 2

In this phase of the project, we proposed a physio-chemical and biological characterization of a new topographical screening device for sorting and studying surfaces with different characteristics and organizational symmetries of nanostructured pillars. We demonstrate that the manipulation of these topographic characteristics, by varying the size and distance between the pillars, directly interferes with the cellular response. We show differences in proliferation, formation of mineralized nodules and OCN expression between surfaces; and a topographic trend where the lateral spacing and the size of the pillars seem to have a greater influence on the biological response than their symmetry. After comparing obtained results, we selected the topographies 0.3;2, 1;2, and 2;2 HEX and SQR for individual manufacture. Topography 1;1 was selected because although it has the same theoretical surface roughness as topography 2;2, it induced a divergent cellular behavior.

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