



UNESP – Universidade Estadual Paulista
“Júlio de Mesquita Filho”
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.**

Araraquara

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osteogenic differentiation and bone formation**

Tese apresentada à Universidade Estadual Paulista
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área Biociências e Biomateriais.

**Orientador: Prof. Dr. Pedro Paulo Chaves de
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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 espreado foram realizados com células MC3T3-E1 cultivadas sobre essas amostras. O revestimento de PDA associado ao tratamento térmico estimulou a proliferação e espreado celular comparado com 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 quadrangular (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 replacement¹. 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 process², 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 patient³.

In the United States, over 2 million surgeries per year are performed to repair damaged or fractured bones by grafting or implantation⁴. 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 population⁵. 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 Brånemark⁶ 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 today⁷.

Nowadays, dental implant survival rates can reach almost 90% due to several technical breakthroughs on titanium chemistry and manufacture over the time⁸. However, postoperative complications such as periimplantitis, poor implant fixation and components fracture are still relevant in clinical practice⁹. 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 bisphosphonates¹⁰.

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 implant¹¹, changes in the chemistry and structural topography of the material can optimize the cell/substrate interaction, altering its metabolism, migration and growth¹². 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 success¹⁴⁻¹⁶. 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.

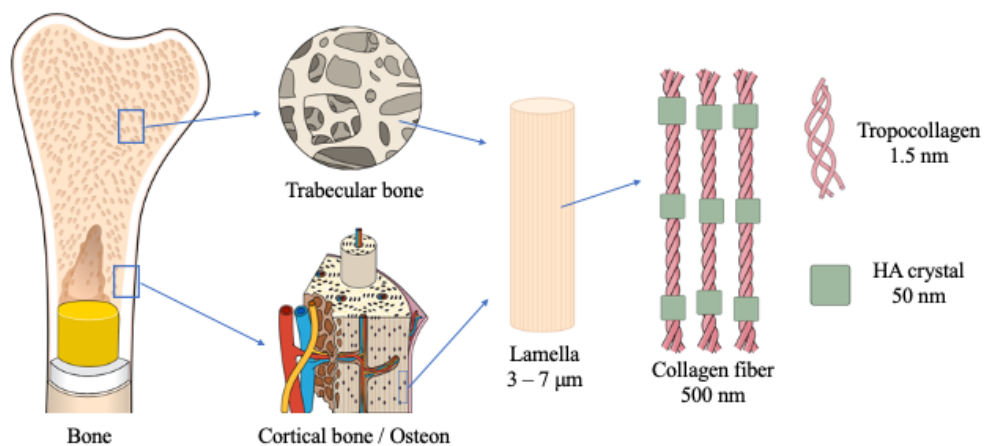
The experimental part of this thesis was divided into two chapters; in the first study we analyzed cellular proliferation and spreading of osteoblast-like cells cultured on polydopamine (PDA) coated Ti samples, with and without heating treatment and room temperature storage period. In the second study 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 (CO_3^{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.



Source: Author personal archive.

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 tissues²⁰.

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 interaction²¹.

Depending on the body reaction they cause, biomaterials can be classified as biotolerable, bioinert and bioactive²². 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 biotolerable 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 bioinert²³. 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 bioactive²⁴.

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 tissue²⁵.

The osseointegration, which is still defined as direct contact between living bone and the implant surface at the level of optical microscopy²⁶, 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 areas²⁷. 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 devices²⁸.

Bone biomaterials can be classified as osteogenic, osteoinductor and osteoconductor. These concepts are interrelated but they are not necessarily the same phenomena²⁹. 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 Formigini, 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 as SUS 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 (TiO₂) layer. Known by passivation process, the formation of this stable 3–10 nm thick TiO₂ film protects the bulk of the Ti implant from the environment and contributes to its high corrosion resistance ³⁹. The TiO₂ 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.

1.1.1 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}.

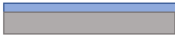
The most common method to infer about a material surface wettability is the sessile drop technique, the contact angle (CA or θ) between the tangent of a drop placed on the sample and the horizontal baseline of the solid surface is measured, and quantifies the wetting of the

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:

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

These interactions can characterize the hydrophilicity and the energy of the surface with WCA ranging from 0 to 180°. θ s close to 0° indicate superhydrophilic or wetting surfaces, below 90° hydrophilic surfaces, θ s above 90° are considered hydrophobic, and surfaces with θ s 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.

CA (θ)	Sessile drop representation	Surface
$\theta = 0^\circ$		Superhydrophilic
$0^\circ \leq \theta \leq 90^\circ$		Hydrophilic
$90^\circ \leq \theta \leq 180^\circ$		Hydrophobic
$\theta = 180^\circ$		Superhydrophobic

Source: Author personal archive.

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 height 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 50° to 60° 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 wound 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 $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ 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.

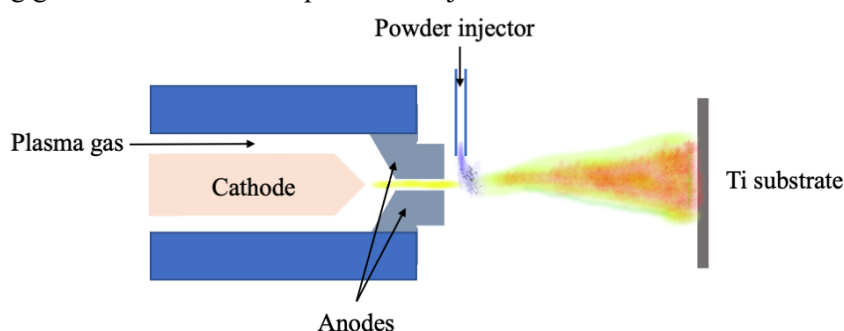
Studies have demonstrated that HA coating can prevent metal-ion release into organic tissues, associated with cell-mediated hypersensitivity reaction ⁷¹. It can also enhance direct physical contact of implants to the hard tissue and attachment of osteoblasts on the coating surface ^{72, 73}. However, 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.

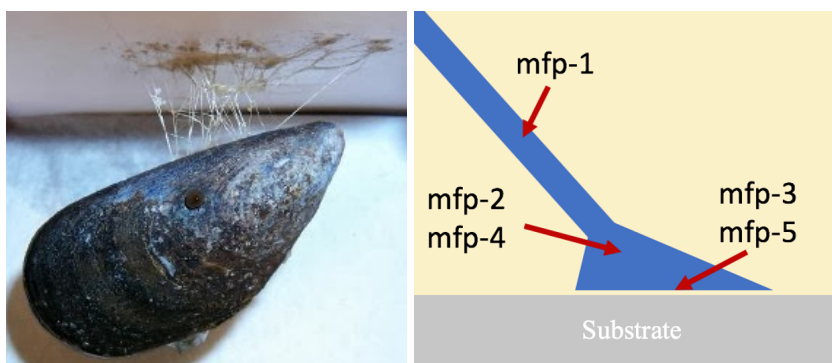
1.2.1.3 Polydopamine (PDA) coating

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).

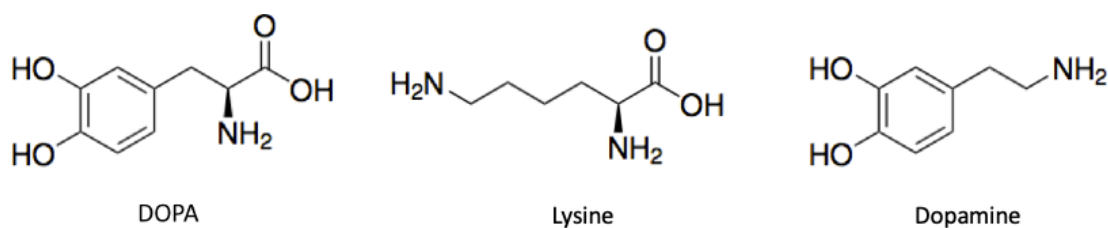
Figure 4 - Mussel adhered to the surface through byssus filaments. Schematic representation of a byssal thread and the different mfps important for substrate adhesion and byssal structure.



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.



Source: Author personal archive.

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 contact¹⁰¹.

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 engineering¹⁰²⁻¹⁰⁴.

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 osteogenesis¹⁰⁵. 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 treatments¹⁰⁶.

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 devices¹⁰⁷⁻¹⁰⁹. It has been reported that laser treatment might increase TiO₂ layer thickness, affecting the quality of bone/implant contact and the subsequent osseointegration¹¹⁰. *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 formation¹¹¹.

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}

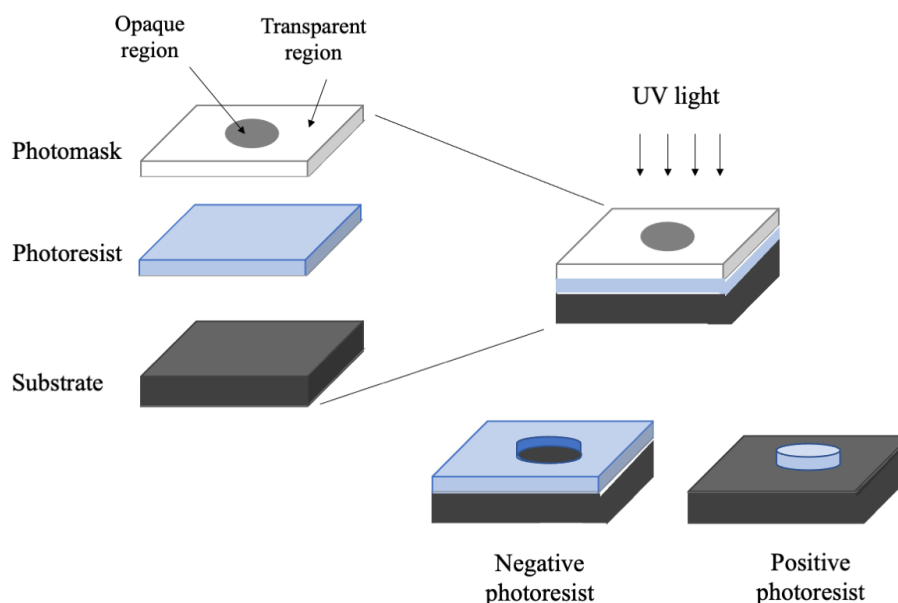
Photolithography is a very precise technique for imprinting desired micro-scaled patterns and features, and it has been widely used for investigation of osteoprogenitor and bone cells response to microtextures. ^{119, 120}. This technique is based on exposure of specific regions of a light-sensitive 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:

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

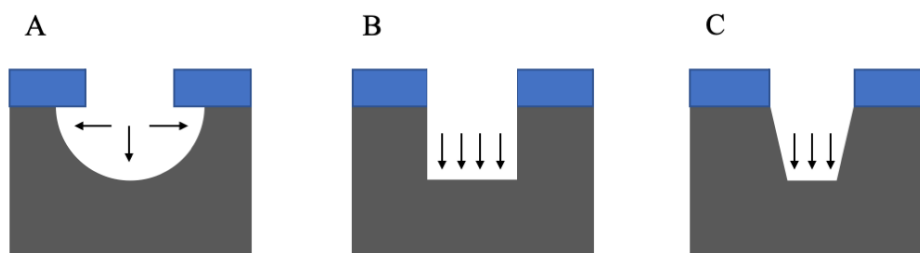
Where CD is the critical dimension or the minimum feature size, the κ_1 (k1 factor) is a coefficient based on the Rayleigh criterion, that defines the minimum separation for preserving the distance between two points in the projected image. The λ is the wavelength of the light used and the NA is the 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 devices¹²⁶.

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 established 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.

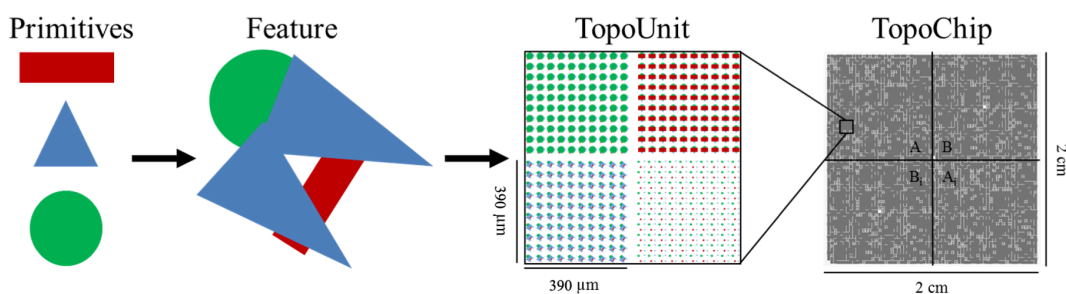
Micro- and nanofabrication techniques have allowed the development of fast, relatively inexpensive cell culture systems assembled with different neighboring topographical constructions. Such devices can probe the same population of cells under a large number of conditions, producing results at a high 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 $390 \times 390 \mu\text{m}$ 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 Kruskal-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

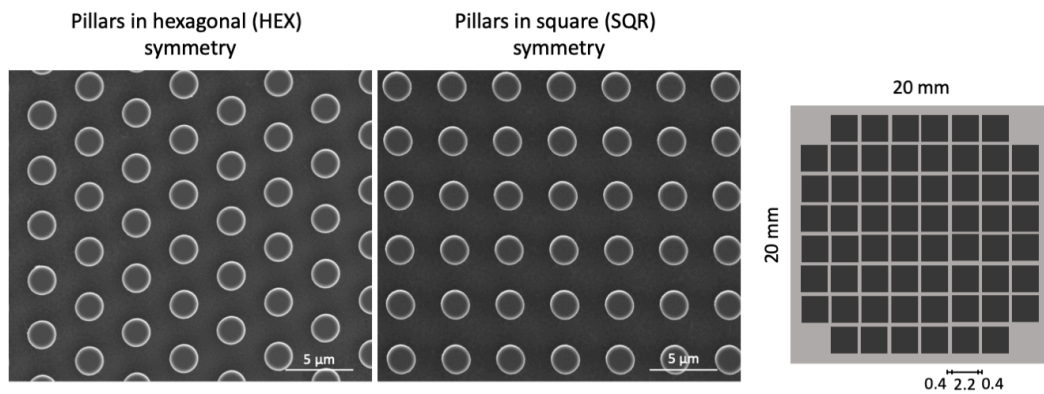
experiments using this device and the exploration of other HTS methods are still needed to unravel specific nanotopographical designs controlling functional cell phenotype.

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 TiO₂ 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}.

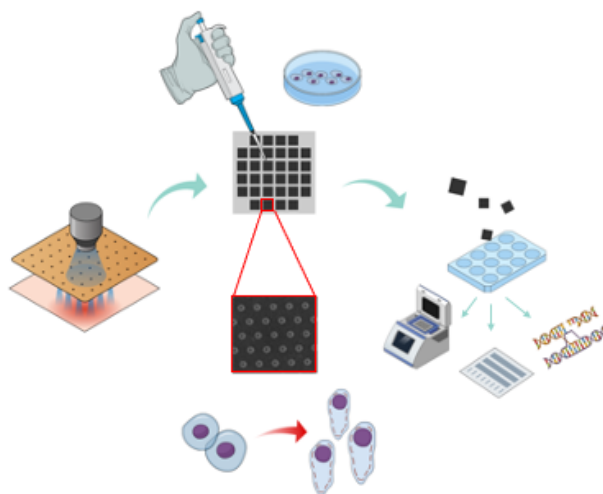
The results obtained in these studies highlighted that some specific topographical parameters, such as pillar height, shape, diameter, lateral spacing and symmetry; can enhance the cellular behavior towards an osteogenic phenotype. With the proposed nanostructured screening platform, we aim to further 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.

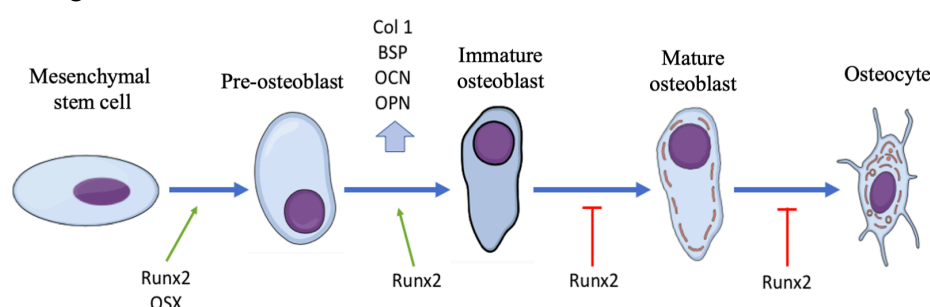


Source: Author personal archive.

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 (BSP), 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.



Source: Author personal archive.

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 β -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.

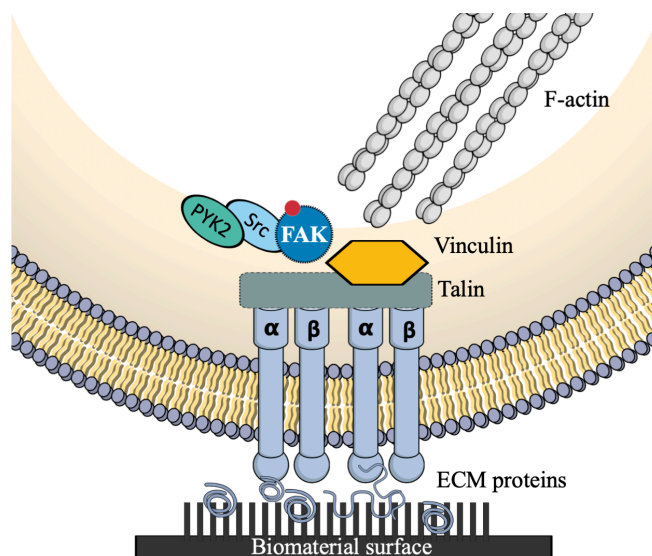
1.3.1 Integrin-mediated mechanotransduction

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 $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αv , $\beta 1$, and $\beta 3$ ¹⁴⁷. Although the exact role of each one of the subunits remains unclear, several studies suggested that $\alpha 5 \beta 1$ regulates osteoblast attachment and growth; while $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ are 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 shear-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- κ B 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 interactions¹⁶¹. 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 (BMPRI) and a lower affinity for the type II receptor (BMPRII). BMPs can bind to BMPRI alone or in combination with the BMPRII. 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/LRP) 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 β -Catenin, leading to a proteasome-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/ β -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 NF κ B 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.

2 OBJECTIVES

Based on the current role of Ti surface modifications in the design and conception of new bioactive implants. The present PhD thesis proposes, as a result of two individual projects, to investigate the biological effect of PDA coating with post heating treatment; and the characterization of a new high throughput screening device for studying the correlation between osteogenic behavior and specific nanostructured topographical specifications.

2.1 Publication 1 Objective

Evaluate the influence of PDA films on MC3T3 osteoblasts adhesion and proliferation after periods of storage or heat treatment at 121°C.

2.2 Publication 2 Objective

Characterize surface screening arrays containing 60 different topographies and 4 control areas, built with nanopillars, with varying size, arranged in a square (SQR) and hexagonal (HEX) symmetry;

Investigate the adhesion, proliferation and differentiation of hDPSCs and hBCDs in the different topographies of the construct, for identification and manufacture of single structures with promising potential to induce an osteogenic cellular response.

3 PUBLICATIONS

3.1 Publication 1

Post-treatments of polydopamine coatings influence cellular response¹

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Keywords: Polydopamine, surface functionalization, post-treatment, cell proliferation

Abstract

Polydopamine (PDA) is the final oxidation product of dopamine or other catecholamines. Since the first reports of PDA coatings starting around 2007, these coatings have been widely studied as a versatile and inexpensive one-step coating option for biomaterial functionalization. The coating attach to a wide range of materials and can subsequently be modified with biomolecules or nanoparticles. However, as a strong candidate for biomaterial research and even clinical use, it is important to unravel the changes in physico-chemical properties and the cell-PDA interaction as a function of heat sterilization procedures and shelf storage periods. Four groups were examined in this study: titanium (Ti), PDA-coated Ti

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samples and PDA-coated Ti samples either stored for up to two weeks at room temperature or heated at 121°C for 24 hours, respectively. We used X-ray Photoelectron Spectroscopy (XPS), Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) and Water contact angle (WCA) to characterize chemical composition and surface properties of the groups. Cell adhesion and proliferation was examined by three different cell types: human primary dermal fibroblasts (hDF), human epidermal keratinocytes (HaCaTs) and a murine preosteoblastic cell line (MC3T3-E1), respectively. Cells were cultured on PDA coated samples for 4 hours, 3 days and 5 days. Both thermal treatment of PDA at 121°C for 24 hours and storage of the samples for 2 weeks increased the amount of quinone groups at the surface and decreased the amount of primary amine groups as detected by XPS and ToF-SIMS. Even though these surface reactions increased the WCA of the PDA coating, we found that the post-treatments increased cell proliferation for both hDFs, HaCaTs and MC3T3-E1s as compared to pristine PDA. This emphasizes the importance of post-treatment and shelf-time for PDA coatings.

Introduction

The cell adhesion to a substrate is largely affected by the physicochemical properties of the surface ¹⁷⁸. It is an intricate process that can determine cellular behavior such as proliferation, migration, and differentiation ¹⁷⁹. Thus, understanding cell-material interaction is crucial for the development of new functional biomaterials that can induce tissue repair ^{178, 180, 181}. This interaction depends on the material surface properties such as surface topography ¹⁸², wettability ¹⁸³, mechanical properties ¹⁸⁴, and surface chemistry ¹⁸⁵, which play key roles in regulating the cellular and tissue response of the material. Tailoring the desired surface properties of biomaterials by surface functionalization is an often used strategy to improve the biological response ¹⁸⁶⁻¹⁸⁸.

Polydopamine (PDA) coatings have been widely studied for more than a decade as a method for modifying surfaces ^{189, 190}. PDA forms in a simple oxidative reaction between dopamine and oxygen in alkaline conditions and can adhere to a substrate surface in a simple dip coating technique ¹⁹⁰. PDA has the ability to stick to almost any kind of substrate and topography, due to its high content of catechol and amine functional groups. ^{189, 190}. The high amount of quinones in the PDA coating is beneficial for secondary reactivity, such as biomolecular attachment, where a nucleophile reacts via Michael addition or a Schiff base reaction at the quinone site ¹⁹¹. Such functionalizing of a surface with biomolecules or chemical groups can optimize the surface properties of a material with respect to biological interactions.

Along with its low cost, low toxicity and production simplicity, PDA applications extend for a variety of biomedical applications.^{190, 192-194}

The exact structure of PDA has not been determined, however most studies agree that dopamine-quinone and 5,6-dihydroxyindole (DHI), formed by air oxidation and intramolecular cyclization, respectively, are key building blocks for the PDA coating¹⁸⁹. The structure of PDA contains trapped un-polymerized dopamine units⁸⁸ leaving it as a heterogeneous material susceptible for chemical changes. For example, thermal treatment of the PDA coating induces the crosslinking between the free amine in dopamine and the PDA structure, increasing the stability of the coating^{195, 196}. Thermal treatment of PDA also increases the amount of intramolecular cyclization and quinone groups at the surface of PDA. Therefore, simple post-treatment of PDA will induce chemical changes in the coating.

In general, literature describes PDA as a biocompatible coating improving both immobilization of biomolecules and proliferation of cells compared to non-wetting surfaces such as polyethylene, PTFE, polycaprolactone (PCL), and PDMS^{90, 91, 190}. However, these studies did not fully elucidate the possible changes in surface properties of PDA upon post-treatment. A thermal treatment is often used for sterilization of biomaterials for clinical use. Thus, it is important to know how the surface change during these processes, as it might affect the biological response to the material. Furthermore, also the length of storage-time at room temperature may affect the surface properties.

In this study, we investigated the effect of thermal treatment and storage of the PDA coating on its chemical structure and hydrophilicity and the biological response to the material measured by cellular attachment and proliferation. Our results show that heat post-treatment decrease the hydrophilicity by increasing the amount of quinone and decreasing the primary amine content at the surface. A significantly increased cell proliferation on post-treated PDA was observed as compared to pristine PDA.

Materials and Methods

Sample preparation

The titanium-coated wafer was produced by E-beam Physical Vapor Deposition (Cryofox Explorer 500, Polyteknik, Denmark). The base-pressure was below 10^{-5} mbar. A 50 nm titanium layer (99.995% pellets, Kurt. J. Lesker) was deposited (0.1 nm/s) on single side polished silicon <001> wafers (Si-Mat, Germany). The final Ti/Si wafer was broken into 6×6

mm samples following pre-cut lines on the non-polished side. For the subsequent PDA coating, the samples were immersed into a dopamine solution (2 mg/mL, 10 mM Tris buffer, pH 8.5; Sigma-Aldrich, USA) as previously described in literature ¹⁹⁰. One control experiment was performed with 10 mM PBS buffer (Sigma-Aldrich) instead of Tris buffer. The PDA-coating was deposited using a 12-well culture plate from VWR with three samples per well for 24 hours at room temperature (RT) and constant stirring on an orbital shaker (130 rpm). Immersion of the titanium coated samples into the dopamine solution yields a thin brown PDA coating on the substrates. Some of these samples were post-treated either by storage for two weeks at RT or by thermal treatment at atmospheric humidity at 121°C for 24 hours (Universal Heating Oven, Memmert GmbH, Germany). These samples are assigned “PDA-2w” and “PDA-th121”, respectively. The pristine coating is named “PDA”, while the uncoated Ti/Si samples are named “Ti”. After PDA coating, the samples were washed three times with Milli-Q water and dried with a jet of N₂.

X-ray Photoelectron Spectroscopy (XPS)

The elemental composition of PDA before and after post-treatment were analyzed by XPS on Axis Ultra DLD (Kratos Analytical, UK) with Al K_α radiation ($h\nu = 1486.69$ eV). Survey scans were performed from 0-1200 eV at 1 eV steps and a pass energy of 160 eV with two sweeps. High-resolution scans were performed for C 1s, N 1s and O 1s with 0.1 eV steps and a pass energy of 20 eV with four sweeps. XPS curve fittings were performed by CasaXPS software version 2.3.17 using a Shirley background subtraction. The spectra were calibrated according to the C 1s C-C bond at 284.8 eV and the Ti-sample was used as control.

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS spectra were acquired using a ToF-SIMS 5 spectrometer (ION-TOF GmbH, Germany) at a pressure of 3×10^{-10} mbar. The scanned area was $300\mu\text{m} \times 300\mu\text{m}$ and the acquisition time was 200 seconds in static mode. The mass spectra were calibrated using three positive masses H⁺, C⁺, C²⁺ (m/z) for positive spectra and H⁻, O⁻ and C⁻ for negative spectra, respectively. The peak intensity was normalized to the total ion intensity. The spectra analysis was performed as described by Ding et al. ¹⁹⁷. However, we focused on the fragments that change intensity significantly after post-treatment (m/z 30⁺ and m/z 66⁻).

Cell culture

Primary human dermal fibroblasts (hDF), isolated from abdominal skin as previously described ¹⁹⁸, were obtained according to the guidelines of the Central Denmark Region Committee on Biomedical Research Ethics for use of anonymous biological tissue. Keratinocyte cell-line (HaCaTs) were obtained from the Department of Dentistry and Oral Health, Aarhus University, and mouse pre-osteoblast cell line MC3T3-E1 (Subclone 4) obtained from São Paulo State University – School of Dentistry in Araraquara, Brazil. The hDFs and the HaCaTs were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), while MC3T3 cells were maintained in minimum essential medium α (α -MEM; Gibco, USA) – all media were supplemented with 10% fetal bovine serum (FBS; Gibco, USA), penicillin (100 μ g/mL) and streptomycin (100 μ g/mL) (Invitrogen, Gibco, USA). Prior to cell seeding, the samples were sterilized in ethanol (70%) for 30 minutes and rinsed twice in PBS buffer. The cells were plated with a cell density of 5,000 cells/cm² (HaCaTs), 2,500 cells/cm² (fibroblasts) and 2,500 cells/cm² (MC3T3-E1) onto the PDA-coated and Ti control samples and incubated for 4 hours, 3 and 5 days, respectively. Cells were placed in a humidified incubator with 95% O₂ and 5% CO₂ at 37°C.

Immunofluorescence staining

The samples were washed with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 10 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, USA) for 5 min, after which the cells were cleaned with PBS and immersed into 2% bovine serum albumin (BSA) for 1 hour at room temperature. Rhodamine- or Alexa Fluor 488-labeled phalloidin (Sigma-Aldrich, USA), diluted to 1:200 in PBS, were incubated at 37°C for 20 min in absence of light. After washing with PBS, the cells nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, D9542 Sigma-Aldrich, USA). In the last step, the stained cells were observed by a Leica DFC 300 FX fluorescence microscope (Leica Microsystems, Germany). The cell number and area was determined with ImageJ v. 17 software.

Statistics

The statistical analysis was performed using Prism GraphPad software v.6.2. The cell data was evaluated using ANOVA one-way followed by unpaired Student's t-test. The number

of samples for each experiment was equal to or more than 5. All the experiments were repeated at least twice. A p-value below 0.05 was considered statistically significant. All are presented with mean $\pm$ standard deviation (SD).

Results and discussion

Surface characterization of pristine and post-treated PDA coatings

The PDA-th121 and PDA-2w was compared to a pristine PDA coating, see Figure 1. Titanium coatings without PDA coating made up the control throughout the experiments in this study and is assigned Ti.

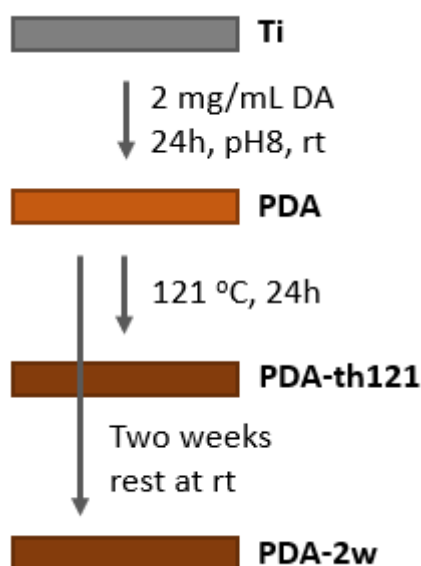


Figure 1. Flowchart of the post-treatments of the pristine PDA coatings on titanium coated samples (Ti). Post-treatment was either annealing at 121 °C for 24 hours (PDA-th121) or storage of the PDA for two weeks (PDA-2w).

X-Ray Photoelectron Spectroscopy

The surface chemistry of the titanium control as well as the pristine or post-treated PDA coatings was examined by XPS. Signals from C 1s, N 1s, and O 1s appear for all three PDA coatings (Table 1) with a similar composition to PDA coatings reported in literature¹⁹⁷ as well as the elemental ratios of N/C and O/C comparable to the theoretical values in PDA (N/C = 0.125 and O/C = 0.250), indicating successful PDA formation. The Ti 2p peak is only observed for the control Ti surface as expected.

	Ti (2p) [At. %]	O (1s) [At. %]	C (1s) [At. %]	N (1s) [At. %]	N/C ratio (1/8=0.125) ^a	O/C ratio (2/8=0.250) ^a	O/Ti ratio (2) ^a
Ti	22.2 ± 0.2	50.9 ± 0.3	25.5 ± 0.3	1.45 ± 0.08	0.057 ± 0.003	1.996 ± 0.026	2.293 ± 0.025
PDA	0	19.03 ± 0.09	72.68 ± 0.19	8.29 ± 0.15	0.114 ± 0.002	0.262 ± 0.0014	-
PDA-2w	0	22.6 ± 0.2	69.0 ± 0.3	8.39 ± 0.12	0.122 ± 0.0018	0.328 ± 0.003	-
PDA-th121	0	22.34 ± 0.05	69.4 ± 0.3	8.3 ± 0.3	0.120 ± 0.004	0.322 ± 0.0016	-

^a Theoretical elemental ratios for PDA and TiO₂, respectively.

Table 1. Atomic composition and ratios in the PDA coatings before and after post-treatment as determined by XPS.

From Table 1, it is observed that the atomic composition in PDA-2w and PDA-th121 contain more oxygen than the pristine PDA coating. Luo et al.¹⁹¹ suggested this increase, along with an observed decrease in nitrogen concentration, to be a result of amine oxidation. However, we do not observe a decrease in nitrogen concentration, and therefore attribute the higher oxygen content to be a result of air contamination during post-treatments. The Ti sample contains approximately twice as much oxygen as Ti, confirming the thin native TiO₂ layer that forms on the titanium surface when exposed to air. Signals from C 1s and N 1s on Ti is most likely due to air contamination with adventitious carbon.

Curve fitting of O 1s and N 1s high-resolution scans reveals changes in the functional groups at the surface of PDA after post-treatment. The chemical shifts of the O 1s peak show functional groups such as C-O, C=O, COOH, and N-O (Figure 2a). After post-treatment there is a small increase in the relative abundance of C=O from 27% for PDA to 29% for PDA-2w and 32% for PDA-th121 (Figure 2c). This increase might indicate surface oxidation of catechols into quinone groups after post-treatment (Scheme 1a)¹⁹⁹. The same oxidation takes place in the initial steps of dopamine polymerization, driven by oxygen¹⁸⁹, and seems to continue during post-treatment. Malollari et al.¹⁹⁶ observed the same trend, however, with a 25% increase in quinone content after thermal treatment at 130°C. The higher content of quinone in PDA improves the attachment of nucleophiles as these react via Michael addition or the Schiff base reaction at the quinone¹⁸⁹. Luo et al.¹⁹¹ demonstrated this by thermally treating PDA for one hour at 150°C, which showed a 15% increase from 33.5% to 48.2% in quinone concentration with an enhanced immobilization of biomolecules to the surface. We do not observe as large increase in quinone concentration after thermal treatment at 121°C. In addition, Proks et al.¹⁹⁵

found an almost constant quinone concentration after thermal treatment at 110°C, suggesting that the temperature for thermal annealing makes a big difference on the quinone content at the surface of PDA.

Fitting the N1s peak involve functional groups such as N-O, primary amine (C-NH₂), and secondary amine (C₂-NH), see Figure 2b. There is a notable decrease in the concentration of primary amine at the surface of PDA after post-treatment, especially obvious for the PDA-th121: after two weeks of storage the concentration of primary amine decreases from 19% to 17% however thermal treatment lead to only 2% primary amine at the surface (Figure 2d), which is comparable to earlier published data in literature^{195, 196}. The fact that there is a small change in concentration of primary amine after two weeks of storage suggests that the primary amines in the PDA coating reacts over time and that this should be taken into account e.g. when performing biological studies on PDA coatings.

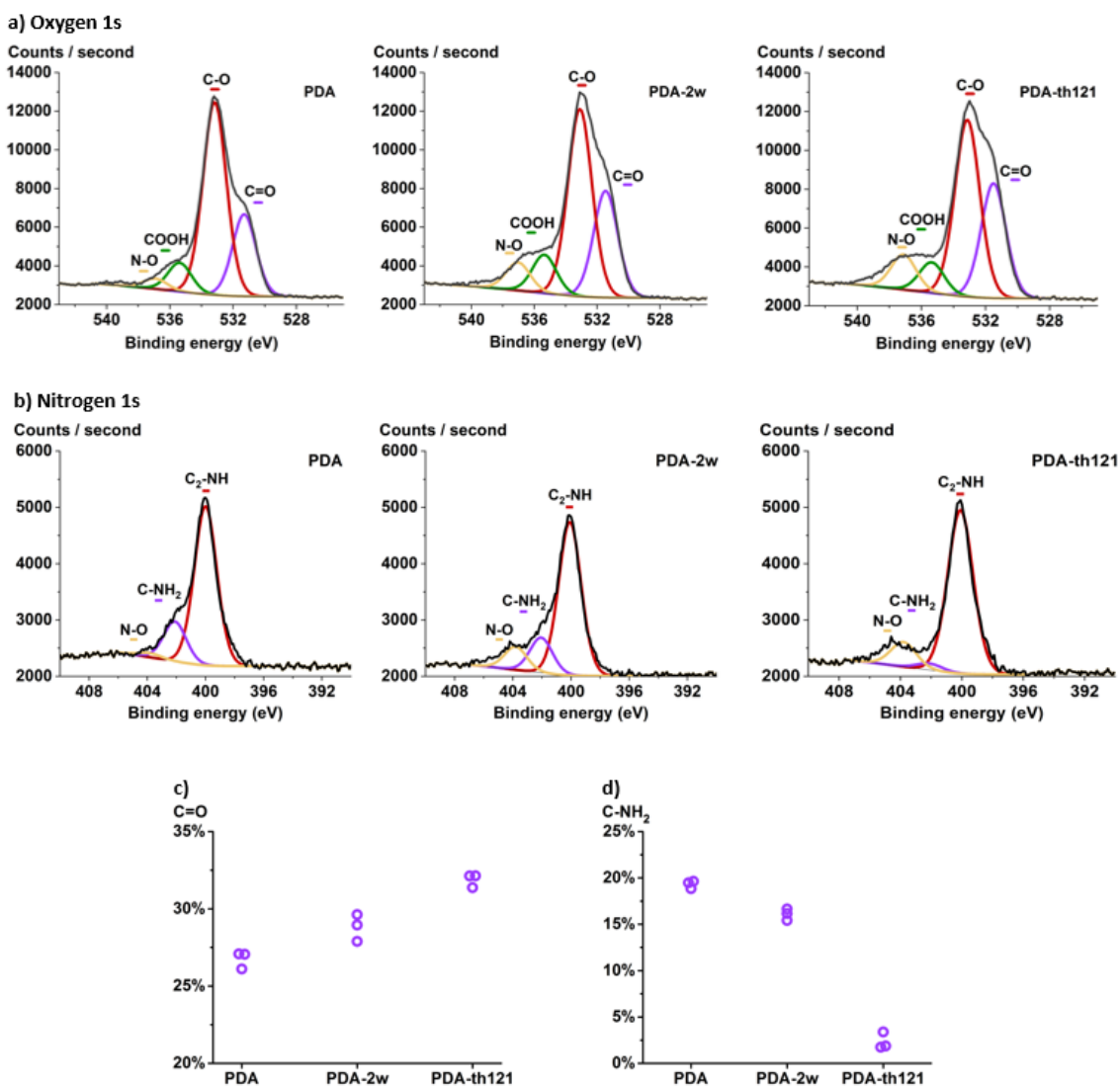
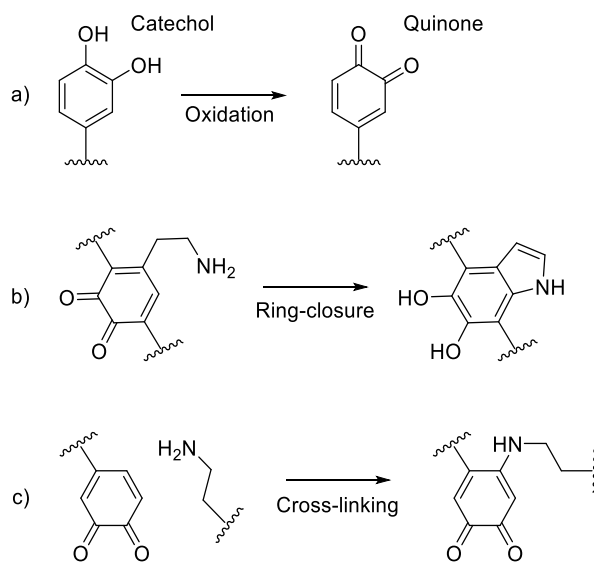


Figure 2. High resolution XPS of a) O 1s and b) N 1s on PDA (left), PDA-2w (middle) and PDA-th121 (right). The curve-fits represent the different functional groups. For O 1s these groups were attributed to; Purple: C=O, red: C-O, green: COOH, and yellow: N-O. For N 1s; Red: C2-NH, purple: C-NH₂, and yellow: N-O. c) and d) show the relative concentration of C=O and C-NH₂, respectively, before and after post-treatment, suggesting conversion of catechol into quinone and reaction of free primary amines into secondary amines. ($n=3$)



Scheme 1. Suggested surface reactions at PDA surface during post-treatment with a) oxidation of catechol into quinone, b) ring-closure of free primary amine, and c) cross-linking of free amine in between PDA chains.

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

The decrease of the primary amine identified by XPS was also observed by ToF-SIMS analysis. For PDA-th121, the positive fragment m/z 30⁺, related to a primary amine, decreased significantly, see Figure 3a, as compared to PDA. A smaller ion intensity decrease was observed for PDA-2w. Proks et al.¹⁹⁵ suggested, based on NMR data, that the decrease in primary amine after thermal treatment was a result of intramolecular cyclization and intermolecular crosslinking of the primary amine (Scheme 1b and 1c), which previously has been attributed take place between PDA and dopamine monomers trapped in the PDA structure^{88, 196}. This agree well with the ToF-SIMS data, as an increase in the ion intensity at m/z 66⁻ is observed for PDA-th121, corresponding to the pyrrole fragment (cyclized secondary amine), figure 3a. This behavior was consistent when changing to PDA formation in PBS buffer (Figure 3b). However, no increase in m/z 66⁻ ion intensity appears for PDA-2w. Even after eleven weeks,

the intensity of this pyrrole fragment stays constant (Figure 3c). The decrease in primary amine for PDA-2w therefore might be a result of cross-linking in the coating and not intramolecular cyclization.

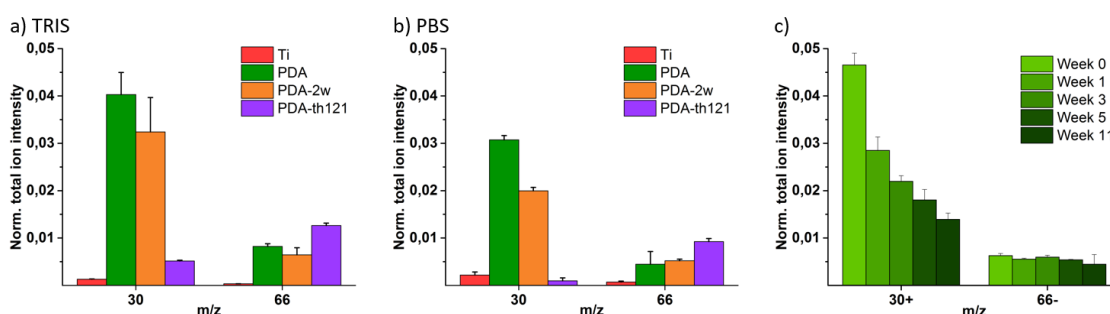


Figure 3. ToF-SIMS data of PDA, PDA-2w, PDA-th121 and Ti, respectively. The fragments m/z 30⁺ and 66⁻ correspond to a free primary amine fragment and a ring-closed secondary amine, respectively. The data are obtained from a) PDA formation in TRIS buffer, b) PDA formation in PBS buffer, and c) a time dependent experiment where the surface chemistry of a PDA sample is followed for 11 weeks.

Accordingly, there is a difference in the surface chemistry of PDA, PDA-2w, and PDA-th121. PDA-th121 contains a minimum amount of free primary amines as these most likely convert into the cross-linked and cyclized amine products. PDA-2w, on the other hand, contains a higher amount of free primary amines as well as some cross-linked amine products, and, finally, the pristine PDA coating contains a high concentration of free primary amines.

Water contact angle

The decrease in primary amine after post-treatment along with the increasing concentration of quinone, observed from XPS and ToF-SIMS shows that the surface chemistry of PDA, PDA-2w and PDA-th121 differ from each another. Water contact angle (WCA) measurements confirm this difference (Figure 4). The contact angle, theta, of a freshly synthesized PDA coating is 20°-30°, slightly higher than the Ti surface (< 20°), however lower than the 56°-70° observed in literature^{91, 191, 197}. The high contact angle reported for PDA in literature resembles more the 60° found for PDA-2w in this study. The contact angle for PDA-th121 is slightly hydrophobic, reaching a value of around 95°. The contact angle increase is

associated with decreasing/increasing amount of amine/quinone at the surface, which agrees with the fact that conversion from primary to secondary amine and from catechol to quinone go from polar groups to less polar groups thus decreasing the WCA after post-treatment.

The change in surface properties after post-treatment may have an impact on biological studies for PDA, as cellular response also depends on contact angle/surface energy ²⁰⁰. Since factors such as storage-time after synthesis and heat-treatment (steam-sterilization) can change the surface properties of the PDA coating, this has to be taken into account when performing e.g. biological experiments.

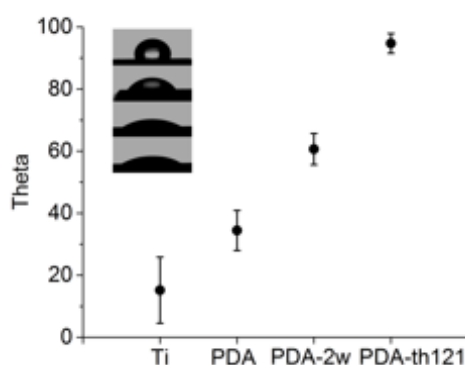


Figure 4. WCA of Ti, PDA, PDA-2w and PDA-th121, showing that the water contact angle, theta, increases after post-treatment. Five samples were measured (n=5). Photographs top left from bottom and upwards: Ti, PDA, PDA-2w, PDA-th121.

Adhesion and proliferation of cells

The physico-chemical properties of biomaterial surfaces guide the cellular behavior and is thus important for applications where cells/tissue interact with such surfaces ²⁰¹. Since its discovery, PDA coatings have been a well-studied alternative for improving the adsorption of serum proteins to a substrate, which positively affects cell adhesion and proliferation at otherwise bio-inert materials ⁹¹. Our chemical surface analysis of the PDA coating showed that its functional groups chemically change after post-treatment (Figure 1).

To test whether the post-treatments influences the biological response to the PDA coatings, we tested the adhesion and proliferation of three different cell types: murine pre-osteoblasts (MC3T3-E1), primary human dermal fibroblasts (hDF), and human keratinocytes (HaCaTs). All three cell types were cultured on PDA samples with and without post-treatment, with Ti as positive control.

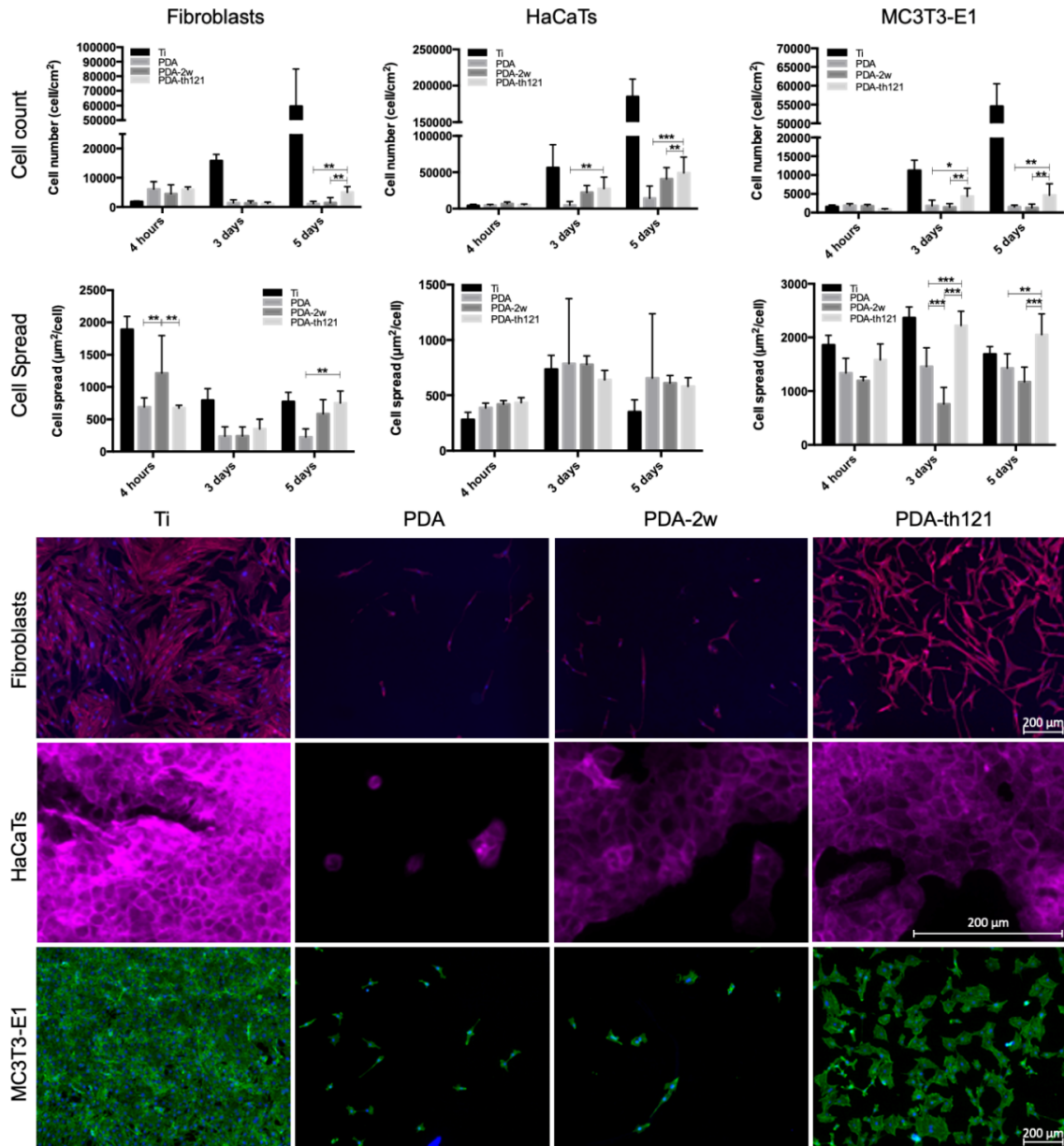


Figure 5. (top) quantification of cell number for hFBs, HaCaTs and MC3T3-E1 on PDA coated substrates. (middle) quantification of cell area for hFBs, HaCaTs and MC3T3-E1 on PDA coated substrates ($n = 5$, mean $\pm$ SD, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (bottom) representative images after 5 days of culture. Blue color is cell nucleus and green and red colors are actin staining for MC3T3-E1 and HaCaTs/Fibroblasts, respectively. Scale bars are 200 μ m.

The number of adherent cells were counted as well as cell spreading after 4 hours, 3 days, and 5 days, respectively. After 4 hours, fibroblasts, HaCaTs, and MC3T3-E1 show no difference in adhesion (Figure 5). After three days, post-treatment affects HaCaTs, which show an increase in the cell count for PDA-2w and PDA-th121, compared to pristine PDA. There is also a slightly higher proliferation for MC3T3-E1 at PDA-th121 after three days. However, at

day five PDA-th121 shows enhanced cell proliferation, for all three cell types as compared to PDA, where the cell count for PDA-2w only increases for HaCaTs. This suggest that thermal treatment mainly increases cell proliferation.

It should be noted, that the cells in general proliferate significantly better on the Ti controls as seen in Figure 5. Interestingly, hDF and MC3T3-E1 cultured on PDA-th121 surfaces have higher cell spreading after 5 days, showing similar behavior to the cells seeded on bare Ti samples (Fig. 5 middle). Representative fluorescence images are shown after 5 days. Lee *et al* reported a decreased attachment capacity of a human megakaryocytic cell line to PDA films as compared to glass and tissue-culture polystyrene ¹⁹⁰. Since cells attach well to Ti, see Figure 5, this finding may explain why less cells are found on all the treated surfaces. One possible explanation, apart from an actual difference in proliferation, is that the PDA-th121 group may still have slightly higher attachment capacity as compared to the other PDA groups, even though similar attachment at 4 hours is found. This is supported by the cell area measurements, where cell area for 5 days is in general high for PDA-th121 as compared to PDA and PDA-2w, especially for the MC3T3-E1s. Also, it has previously been demonstrated, that thermal annealing of PDA coatings increases quinone concentration, enhancing immobilization of biomolecules to the surface and furthermore cellular attachment and proliferation ¹⁹¹. Lee *et al* also reported fibroblastic cell adhesion at a level similar to the bare glass/TC-PS ¹⁹⁰, which is in contrast to our observations for the hDFs. Lastly, the reason for the different cell densities could also be a cytotoxic effect of the coating. It is known that unpolymerized dopamine can have cytotoxic effects ²⁰², so even minor contributions from monomers in the non-treated coating can imply such an effect ¹⁹⁶. It should be noted, that the PDA-th121 treatment was performed similar to a “dry” sterilization, since initial experiments showed that the mechanical stability of the PDA-coating improved significantly when performing heating under dry conditions (data not shown).

Although the WCA of an artificial material is not necessarily a good predictor of biological responses, e.g. cellular interactions ⁴⁷, it is still an often used measure. Contact angles between 50 and 60 are often stated as beneficial for cellular attachment and growth ²⁰³. Our findings suggest that the decreased surface hydrophilicity after thermal treatment (Fig. 4) has a positive association with cell adhesion and proliferation compared to the pristine PDA sample, even though this most likely correlate with the detailed surface chemistry.

Conclusion

In this study, we demonstrate that simple post-treatments of polydopamine coatings (PDA) improve the cellular response. Thermal treatment of PDA at 121°C for 24 hours increase the amount of quinone groups in an oxidation reaction at the surface and decrease the amount of primary amine groups. The reaction of the primary amine is a result of intermolecular cross-linking and/or intramolecular cyclization within the PDA coating. These reactions also take place, however at a lower rate, in a simple storage of the PDA coating for two weeks at room temperature. This shows the importance of keeping track of time after polymerization (shelf-life) when using PDA coatings for further studies. Even though these surface reactions increase the water contact angle of the PDA coating, we found that formation of more quinone groups and removal of primary amines favor cell proliferation of both MC3T3-E1, HaCaTs and hDFs, all relevant cell types for biomedical implant surfaces. The increased cell proliferation on post-treated PDA as compared to pristine PDA, is still much lower as compared to titanium.

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3.2 Publication 2

Title: Unravelling cellular mechanisms by combinatorial screening: a new investigational approach aimed at nano-topography induced osteogenic differentiation. ²

Abstract

The interface between a biomaterial and cells plays a vital role in bone repair. It can modulate cell commitment and function, influencing cellular adhesion, proliferation and differentiation. It is well known that different surface topographical architectures and parameters can induce distinct levels of cellular response. Nevertheless, due to the infinity of possible nanostructured topographies, the standardization and understanding of these specific parameters triggering desired cell fate, remains unclear. Herein, the biological impact of combinatorial systematic pillar-based surfaces was evaluated through a nanostructured high throughput screening (HTS) topographical platform, constructed by Deep-UV Lithography (DUVL). The surface was coated with Titanium by Atomic Layer Deposition (ALD). Human dental pulp stem cells (hDPSCs) and human fibula bone cells (hBDCs) were seeded at screening array devices containing 64 squares: 60 different nano-topographies and 4 flat control areas, constructed with pillars arranged in either square (SQR) or hexagonal (HEX) symmetry. The features varied in pillar diameter and interpillar distance. Surface wettability and higher lateral interpillar distance of the topographical cues seemed to have a greater impact on osteogenic differentiation. The size of the pillars influenced cell adhesion and proliferation in a direct correlation. However, some topographies with smallest diameter presented relatively enhanced signs of osteogenic differentiation as compared to similar neighboring topographies. The observed surface-induced proliferation and differentiation trends, determined the identification of key topographies for single surface samples fabrication. Preliminary experiments on alkaline phosphatase activity and expression pattern of osteoblastic markers showed that differences in pillar diameter and lateral spacing can change osteoblast activity. However, further investigation with the single structures are still needed for understanding the underlying molecular mechanisms.

² According to the FOAr Academic Works Guide. Available on the Library website: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

Introduction

The sequence of biological events that occur from the implantation of a biomaterial until its complete osseointegration in a bone site involves damage to intact bone, activation of the immune response, angiogenesis, recruitment and differentiation of progenitor cells. Modification on implant surface parameters have a well-established impact in all these events, triggering an osteogenic cellular response ^{96, 204, 205}.

Differentiation of osteoblasts, deposition of extracellular matrix (ECM) and its subsequent mineralization are crucial for a successful implant osseointegration ²⁰⁶. These bone forming cells are differentiated from bone marrow mesenchymal stem cells through stimulation of extracellular growth factors and cytokines, such as transforming growth factor-beta (TGF- β) and bone morphogenetic proteins (BMPs) ²⁰⁷. Osteoblasts can also respond to mechanical changes in the environment by the activation of heterodimeric integrin adhesion receptors. In particular, the activation of integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$, mostly associated with osteoblast differentiation, activates downstream RAS/Raf and MAPKs signaling proteins, resulting in activation of bone-related transcription factors (e.g. Runx2, Osterix) and subsequent transcription of genes encoding osteoblast-related proteins, such as osteocalcin (OCN), osteopontin (OPN) and alkaline phosphatase (ALP) ^{148, 154, 156}.

Due to the cell capacity to recognize and respond to mechanical changes in the environment, tailoring surface topographies can directly affect osteogenic differentiation ^{208, 209}. Thus, cellular behavior on microstructured topographies has been widely investigated ^{96, 137, 138}. Adhesion and the modification on the cell cytoskeleton on micro- and nanostructured surfaces, mediated via integrin signaling, may play a determining role in modulating the osteogenic phenotype ^{209, 210}. Understanding the mechanisms by which cells interact with the surfaces at the nanometric level is particularly important, since integrin clustering and proteins that cross-link these receptors into focal adhesion complexes are sized within this scale ²¹¹.

The application of nanotechnology to the surface of dental implants involves a three-dimensional association of surface characteristics, which lead to new physical-chemical behaviors and changes in surface roughness that would interfere with cellular behavior ²¹². These structural modifications on Ti surface influence cytoskeletal organization and consequently the morphology of the cells that adhere to the substrate ^{99, 213}. Besides directly interfering with cell cytoplasmic stress, it is suggested that nanoconstructions facilitate the adsorption and extracellular accumulation of non-collagen proteins, such as OCN and OPN, that contribute to cell proliferation and ECM mineralization ^{214, 215}. Moreover, the mechanical

stress caused in the cell cytoskeleton can affect gene transcription, inducing osteoblastic differentiation *in vitro* and osseointegration *in vivo* ²¹⁶⁻²²⁰.

Current nanofabrication techniques have the potential to produce miniature devices for high throughput screening (HTS) that can be used for probing cell cultures under large numbers of neighboring surface configurations ¹²². In the past decade, topographical screening approaches have been often employed to study the relationship of surface cues and cellular response, whereby cell parameters are investigated on a large number of random surface designs and specifications ^{133, 137, 221, 222}. In spite of current technological breakthroughs of nanofabrication techniques used in medical devices, there is a lack of evidence on how specific topographic patterns and dimensions stimulate osteoblastic cell differentiation and consequent osseointegration ²²³.

In this study, we propose a nano topographical screening array (NTSA), constructed with systematic nano pillar-based Ti topographies, with well-defined topography variables, for investigating surface-induced cellular behavior of human dental pulp stem-cells (hDPSCs) and human bone-derived cells (hBDCs). Here, we also introduce a further step on the process of understanding cell-nanosurface interaction by crafting single structure devices based on the NTSA results. These single-structure tailored surface will allow a deeper probing on cellular mechanisms involved in the recognition of nanostructures, by removing the possibility of paracrine signaling and allowing the extraction of RNA and protein for further analysis of intracellular mechanisms. Such approach might contribute to the conception and design of a new generation of smart surfaces for implant devices, or substrates that control and modulate desired cellular fates.

Materials and methods

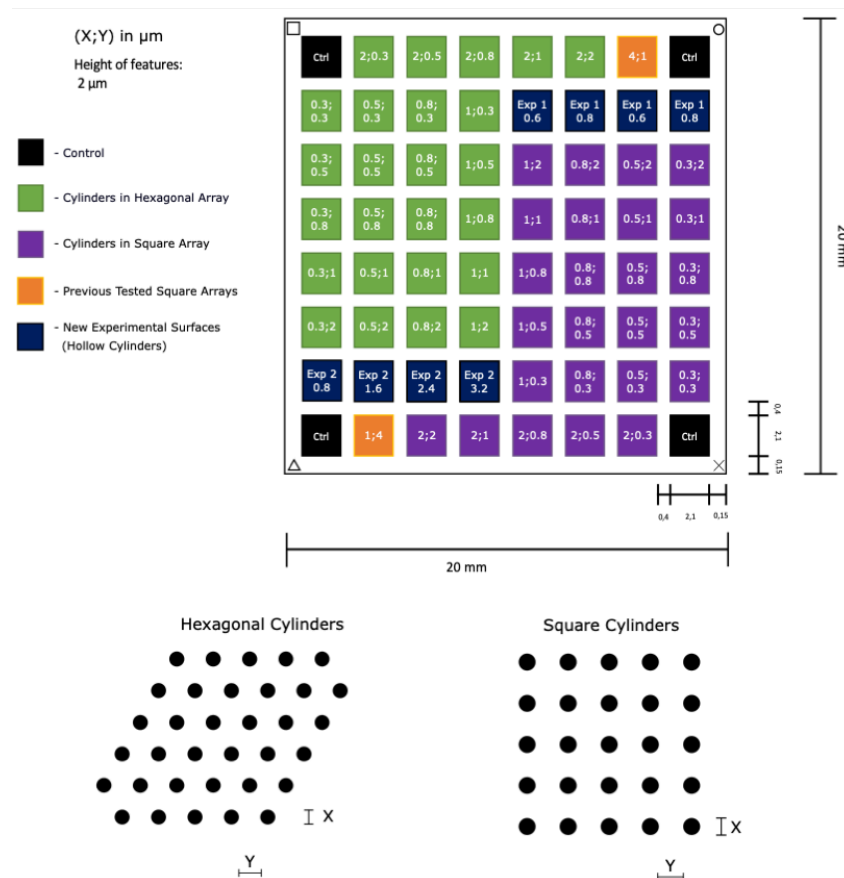
Fabrication of the Ti screening array platform

The fabrication of the 8 x 8 arrays has previously been described ¹³⁷. The screening arrays were constructed by deep ultraviolet lithography (DUVL) on silica wafers and followed by Ti coating through atomic layer deposition (ALD). A 10 nm thick TiO₂ layer was deposited using TiCl₄ and H₂O as precursors at 150°C. Pulse- and purge time for TiCl₄ was 0.1 s and 4 s respectively while the pulse- and purge time for H₂O was 0.1 s and 5 s. The purging step was performed with a 150 and 200 sccm nitrogen flow rate for TiCl₄ and H₂O, respectively. Total

cycle amount was set to 250. They are composed of 64 surfaces in a mesh of 8 x 8 topographic areas with 60 exclusive topographies and 4 control areas (Figure 1). Each surface is a 2.2 x 2.2 mm² square, and labeled “X;Y” equivalent to the diameter (X) and interpillar distance (Y) in μm , the height of the pillars is 2 μm .

In a total of 60 surfaces, 2 areas were dedicated to previously tested surfaces (orange in Figure 1)¹³⁷, with pillars in quadrangular orientation. 8 of the 60 areas were used for experimental topographies, built with hollow cylinders (dark blue areas in Figure 4), which will not be explored in this study. The last 50 topographies (green and purple in Figure 1) are pillars with diameters and interpillar distances varying from 0.3, 0.5, 0.8, 1 to 2 μm . Half of the 50 pillars are arranged in a HEX symmetry, while the other half are SQR patterned.

Figure 1 – Ti coated screening array design. The color of each area describes the pattern of the pillars and the structural tendency. Each area is labeled X; Y, describing the column diameter and the interpillar distance, respectively.



Source: Biomedical Surface Group (Aarhus University) archive.

Surface characterization

The fabrication process and general quality of the nanotopographies were analyzed by scanning electron microscopy (SEM Magellan TM 400 XHR, FEI, Oregon, EUA). The chemical composition and the presence of possible contaminants on the array surface was verified by X-Ray Photoelectron Spectroscopy (XPS Kratos, Axis UltraDLD). Survey spectra were obtained with pass energy of 160 eV. The software used was CasaXPS.

Contact Angle Measurement

The wettability of different topographies on the screening array was characterized by contact angle (CA) measurements. Static CA measurements were performed using the sessile drop method on an FTA1000 goniometer (First Ten Angstroms, USA) under ambient conditions at room temperature. Milli-Q water (0,5 μ L) was dropped on the surfaces and pictures were taken after water drop stabilization. Three samples of each array were used for the measurements.

Primary Cell Culture

hBDCs were isolated from a human fibula sample, collected from a patient undergoing orthopedic bone grafting surgery (single donor). hDPSCs were isolated from impacted fully developed healthy third molars, surgically removed by clinical indication of ectopia (single donor). Tissue specimens were obtained under informed consent according to the guidelines of the Central Denmark Region Committee on Biomedical Research Ethics. The isolation and culturing of both hBDC and hDPSC cell lines followed the protocols previously described^{224, 225}. The cells were maintained inside cellular incubator (37 °C, 5% CO₂), in 75 cm² culture flasks in MEM supplemented with 10% fetal bovine serum (FBS), 25.000 IU ml⁻¹ penicillin and 25 mg ml⁻¹ streptomycin (complete medium). Culture medium was changed twice a week. Right before the cell experiments, the arrays were sterilized by rinsing once in PBS (pH 7.4 at 37 °C), immersion into 70% ethanol for 30 min and rinsing three times in PBS.

Cell proliferation, osteocalcin immunostaining, mineralization analysis, Alkaline Phosphatase activity assay and RT-qPCR for osteoblast markers, were carried on with established hDPSC and hBDC populations, following the experimental periods selected for each of the experiments.

Cell proliferation

hBDCs from the fibula were seeded at a density of 2500 cells/cm² on the screening arrays placed in 6-well plates (n=3), and maintained in complete culture medium. After 4 hours, 4 and 7 days of culture, the cells were fixed with 4% buffered paraformaldehyde for 30 min and washed thoroughly with PBS. The cells were stained with DAPI diluted 1:1000 (Sigma-Aldrich, Missouri, USA) and rhodamine-labeled phalloidin diluted 1:400 (Thermo-Fisher, Massachusetts, USA) in T-PBS (0.1% Triton X-100 in PBS) for one hour. After washed for three times with PBS, cell morphology was evaluated and compared between experimental times, and cells were counted using a fluorescence microscope (Olympus IX73, Tokyo, Japan) and the Olympus Cellsens v1.17 software.

Mineralization

For analyzing the formation of mineralized nodules, hBDCs were seeded on the arrays (n=3) at a density of 15000 cells/cm². The cells were maintained in osteogenic medium, complete medium supplemented with ascorbic acid 50 µg/ml (Sigma-Aldrich, Missouri, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, Missouri, USA) and 0.1µM dexamethasone (Sigma-Aldrich, Missouri, USA). After 14 and 18 days, on regular medium changes every 2 days, the cells were fixed with 70% ethanol and stained for 10 min, with Alizarin Red 1% w/v solution in distilled water (Sigma-Aldrich, Missouri, USA). The samples were washed thoroughly with distilled water, dried and photographed with a fluorescence microscope (Olympus IX73, Tokyo, Japan). The positive area for Alizarin staining was quantified using Cavalieri's histological point-counting technique²²⁶.

Osteocalcin immunodetection

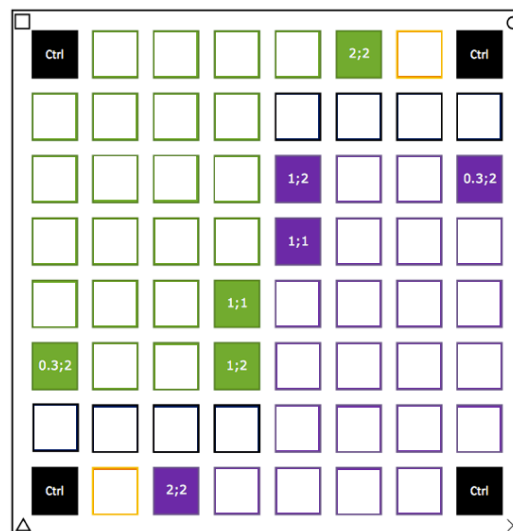
The expression of non-collagenous bone protein Osteocalcin (OCN) was detected by immunofluorescence staining. hDPSCs were seeded onto the screening platforms (n=3) for 14 and 18 days in complete culture medium. After the experimental periods, the cells were fixed in 4% paraformaldehyde, washed gently in PBS and submerged for 15 min in a solution of 0.1% Triton X-100 in PBS (T-PBS) for permeabilization. The samples were then transferred to a 2% BSA solution in T-PBS for 2 h, before being subjected to the primary antibody, anti-osteocalcin (rabbit polyclonal) (FL-100, Sigma) diluted 1:400 in T- PBS, for 1.5 h at room

temperature. The cells were then washed three times with T-PBS and stained with the secondary antibody, Alexa Flour 555 (anti-rabbit) (711-025-152, Jackson ImmunoResearch) diluted 1:200 in T-PBS, while 40.6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 was added simultaneously for nuclear fluorescence.

Single surfaces fabrication

Based on the results of the physiochemical and biological characterization of the screening arrays, four HEX and four SQR topographies with the same surface configurations, were selected to be individually manufactured (Figure 2). The Ti coated 3 x 3 mm single surface samples were fabricated by the same DUVL and ALD techniques used for the screening platforms. The topographies 0.3;2, 1;2, and 2;2 (HEX and SQR) were selected for showing some sort of distinct cellular behavior. While the topography 1;1 was selected because it has the same surface roughness as surface 2;2, although they showed a different cellular response.

Figure 2 - Selected topographies for single surface samples fabrication. HEX 0.3;2, 1;2, 2;2 and 1; 1 (green). SQR 0.3;2, 1;2, 2;2 and 1;1 (purple).



Source: Author personal archive.

Alkaline phosphatase activity assay

To evaluate how different pillar-based topographies would affect osteoblast response and activity, a pilot experiment comparing the ALP activity of hBDCs cultured on flat control samples and the SQR 1;2 surface (n=4) was performed. The cells were seeded at 15000

cells/cm² on the samples placed in 48-well plates. The cells were maintained in osteogenic medium and after 14 days, the single structures were washed with PBS and lysed with alkaline buffer containing 1.5 M 2-amino-2-methyl-1-propanol (100 µl/well; Sigma- Aldrich, St. Louis, USA), pH 10.3, for 10 min at 37°C. Then, 100 µl pNPP substrate solution (1 mg/ml pNPP substrate in 0.1 M Glycin buffer; Sigma-Aldrich, St. Louis, USA) was added to the cell lysate for 2.5 min at 37°C, followed by addition of 100 µl 2 M NaOH for 10 min to stop the enzymatic ALP conversion of pNPP into pNP. The enzymatic activity was analyzed through photometrical quantification of pNP, using an absorbance microplate reader (BioTek, Winooski, USA) at 405 nm. To calculate cell-specific ALP activity, expressed as mM pNP/ minute/cell, a standard curve was prepared diluting 1-mM stock pNP from 0.05 to 0.4 mM (Sigma-Aldrich, St. Louis, USA).

Osteoblast markers gene expression

A preliminary real-time polymerase chain reaction (RT-qPCR) was performed to evaluate the mRNA expression of the osteoblastic markers ALP, OCN, and OPN of hBDCs cultured on flat control single surfaces and HEX 0.3;2 and 1;2 surfaces. The cells were seeded at 15000 cells/cm² on the selected single structures placed in 48-well plates (n=4), and maintained in osteogenic medium. After 3 and 14 days of culture, hBDCs were harvested and total RNA was extracted and purified using GenElute Mammalian total RNA Miniprep kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. RNA concentration and purity were spectrophotometrically determined using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). The cDNA was synthesized using 40 ng of total extracted RNA with cDNA synthesis kit (Thermo-Fisher, Massachusetts, USA). RT-PCR was performed on a Stratagene Mx3000P system (Stratagene, San Diego, USA) using TaqMan universal PCR master mix (Applied Biosystems, Waltham, USA) and TaqMan gene expression assays (Applied Biosystems, Waltham, USA) with the following primers: secreted phosphoprotein 1 (SPP1) Hs00959010_m1 (OPN), bone gamma-carboxyglutamate (gla) protein (BGLAP) Hs01587814_g1 (OCN), and ALP-liver/bone/kidney (ALPL) Hs00758162_m1 (ALP; Waltham, USA). Data analysis was performed using Stratagene Mx3000P RT-PCR system sequence detection software version 1.3 (Stratagene, San Diego, USA). Expression levels were normalized to the threshold cycles (Ct) from ribosomal protein beta-glucuronidase (GUSB).

Statistics

At least two independent experiments were carried out with three or four technical replicates. Statistical analysis was performed using the Prism GraphPad v.8 software (GraphPad Software Inc., La Jolla, USA), and described as means $\pm$ standard deviations (SD). For all screening arrays data graphs, the surfaces were separated according to the pillars geometrical position, HEX (green) and SQR (purple). They were further subdivided according to the interpillar distance (Y), where in each subgroup only the column diameter (X) varies from 0.3 to 2 μm . Normality of the data was tested and confirmed with the Kolgomorov–Smirnov test. The data were subjected to parametric one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Comparations with groups of topographies and the control were accessed by one-way ANOVA followed by Dunnet’s multiple comparisons post hoc test. Differences between two groups were calculated based on the Student’s t-test. $p < 0.05$ was considered statistically significant.

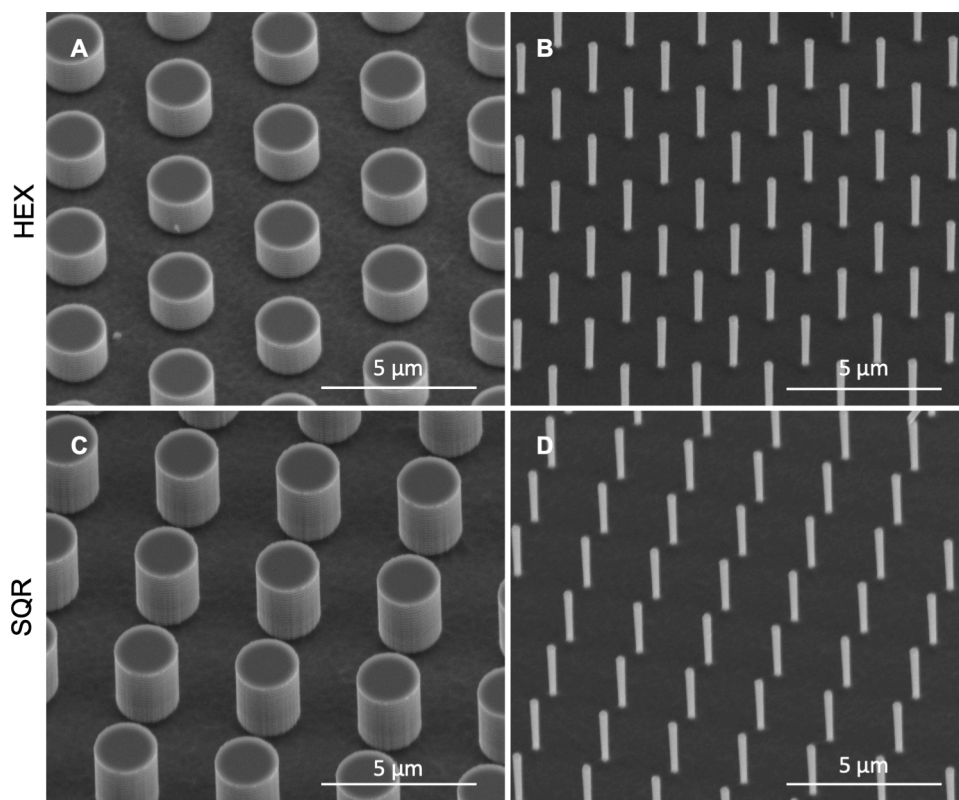
Results

hBDCs and hDPSCs were cultivated on the topographical screening platforms, to investigate the osteogenic cellular response to different topography cues and surface properties in a high throughput screening. Key topographies were identified based on the results obtained with the screening arrays. These surfaces were individually manufactured for further understanding of surface-induced behavior of osteogenic lineage cells, through molecular tools that couldn’t be used on the screening device.

Surface characterization and wetting property

The fabrication of the topographies was verified by SEM imaging. The DUVL and the ALD Ti coating process was confirmed to be precise and reproducible. The topographies showed the desired ordered pattern, and no pillar distortion or displacement was observed in the measurements for both symmetries (Figure 3).

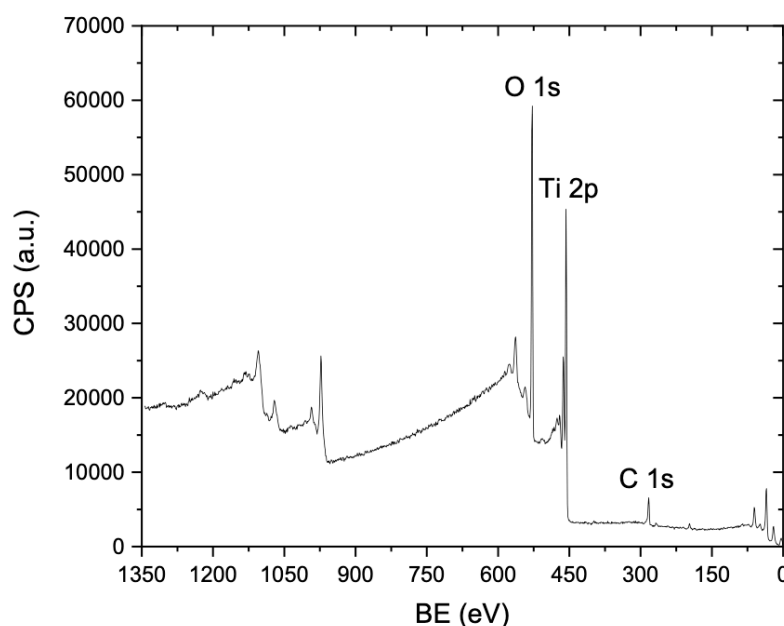
Figure 3 - High resolution SEM images of the topographies 2;2 and 0.3;2 both in HEX (A and B) and SQR (C and D) symmetries. A 45° angled view of the pillars is shown.



Source: Author unpublished data, 2021.

To check possible contaminants and the deposition of TiO_2 layer, the chemical composition of the Ti coated silica wafers was observed using XPS. The measurements showed peaks for oxygen, titanium, carbon and traces of chlorine (less than 1%); in order of concentration (Figure 4).

Figure 4 - Atomic percentage computed from XPS measurements of a single point of the array surface ($n = 3$).

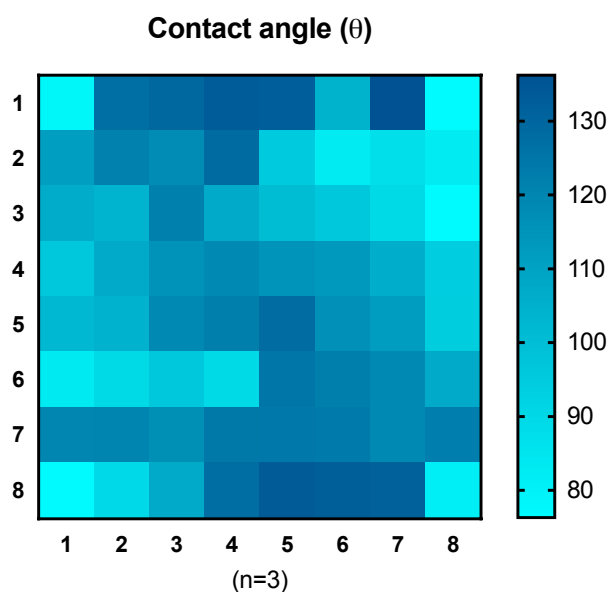


	Ti	O	C	Cl
At. %	29.2	56.5	14.3	Traces
StDev	0.3	0.5	0.2	

Source: Author unpublished data, 2021.

The wetting behavior of all the neighboring topographies on the 8x8 screening platform was investigated by water contact angle (WCA) measurements. The topographies showed hydrophilic and hydrophobic surfaces, with the WCA ranging from 77° to 136°. The control flat areas and the topographies designed with interpillar distance of 2 μm , both HEX and SQR (0.3,2, 0.5;2, 0.8;2, 1;2 and 2;2) showed the most hydrophilic behavior among the topographical library (Figure 5).

Figure 5 – Contact angle (θ) of each surface of the screening array. Heatmap of the WCA measured individually on the center of each topography. 1 to 8 rows and columns were designated to describe all the 64 surfaces present in the screening platform, starting with first flat control at the upper left corner (surface 1,1) to the last flat control (surface 8,8). (n = 3, mean).

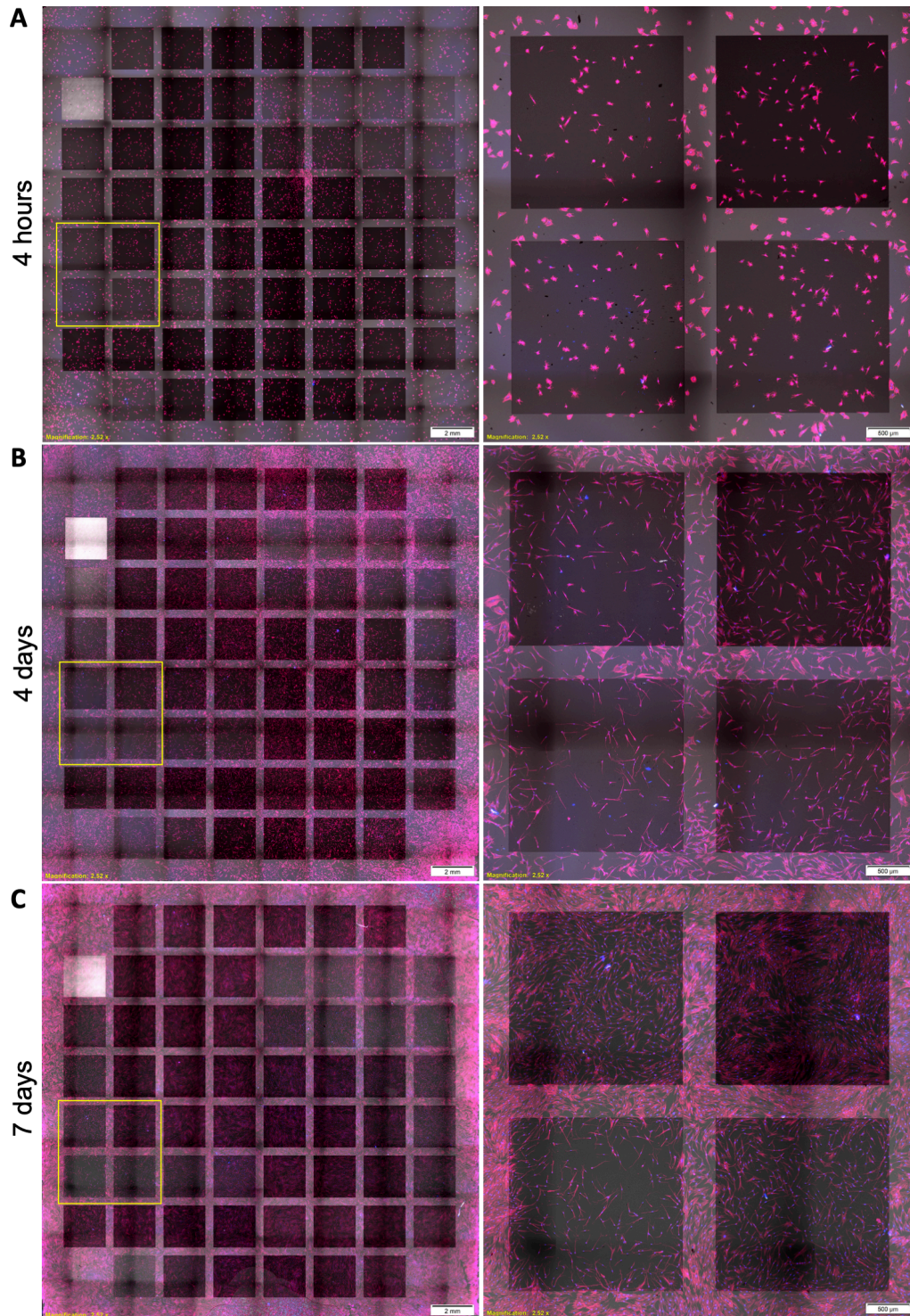


Source: Author unpublished data, 2020.

Influence of pillar-based surfaces on cell adhesion and proliferation

To investigate the different topographical cues on cell adhesion and proliferation, the fibula osteoblast-like cells were cultivated on the screening platforms for 4 hours, 4 days and 7 days. After 4 hours, the cells were equally attached to the entire area of the array, without showing cytoplasmic extensions with rounded morphology (Figure 6 A). After 4 days, it is distinguishable that the cells begin to increase proliferation in specific regions of the array (Figure 6 B). From 4 days it's also possible to observe cell growth through contact guidance. After 7 days of culture, the samples show almost 100% cell confluence. However, in some surfaces it is possible to observe a lower adhered cell density, following the geometric orientation of the constructed pillars (Figure 6 C).

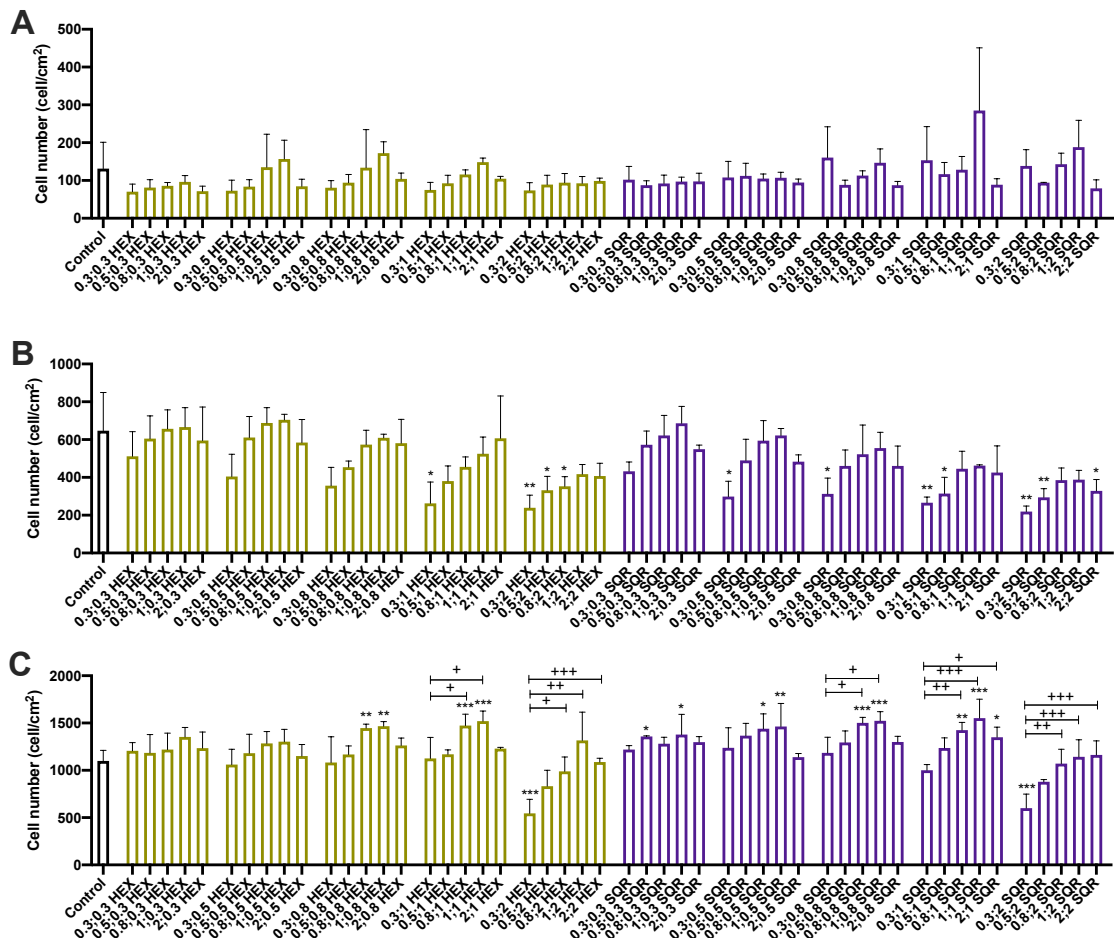
Figure 6 – Cell adhesion and morphology after 4 h, 4 days and 7 days. (A), (B) and (C) Fluorescence images indicating the presence of viable hBDCs on the screening arrays after 4 hours, 4 days and 7 days, respectively (Scale bar size 2 mm). Zoom in pictures of the surfaces HEX 0.3;1, 0.5;1, 0.3;2 and 0.5;2, showing different cell density and spreading by contact guidance (Scale bar size 500 μm).



Source: Author unpublished data, 2020.

The cell number was also individually measured for each surface within the array. After 4 hours, it is possible to confirm the uniformity of the cell seeding procedure (Figure 7 A). The 4- and 7-days data (Figure 7 B and C) suggested a proliferation pattern, which showed higher cell number by increasing the diameter of the pillars, both in the SQR and HEX pattern; according to these measurements, the variation on the interpillar distance only seemed to interfere with cell proliferation, on some topographies grouped by the interpillar distance of 2 μm . These surfaces (0.3;2, 0.5;2 HEX and SQR) showed slightly lower cell number compared to the same topographies grouped by the interpillar distances 0.3, 0.5, 0.8 and 1 μm .

Figure 7 – Cell adhesion and proliferation after 4 h, 4 days and 7 days. Quantification of hBDCs adhered cell number on the individual surfaces of the screening array, after 4 hours (A), 4 days (B) and 7 days (C). (n = 3, mean $\pm$ SD *** p < 0.001, ** p < 0.01, * p < 0.05, compared to control. +++ p < 0.001, ++ p < 0.01, + p < 0.05, compared within the subgroup).



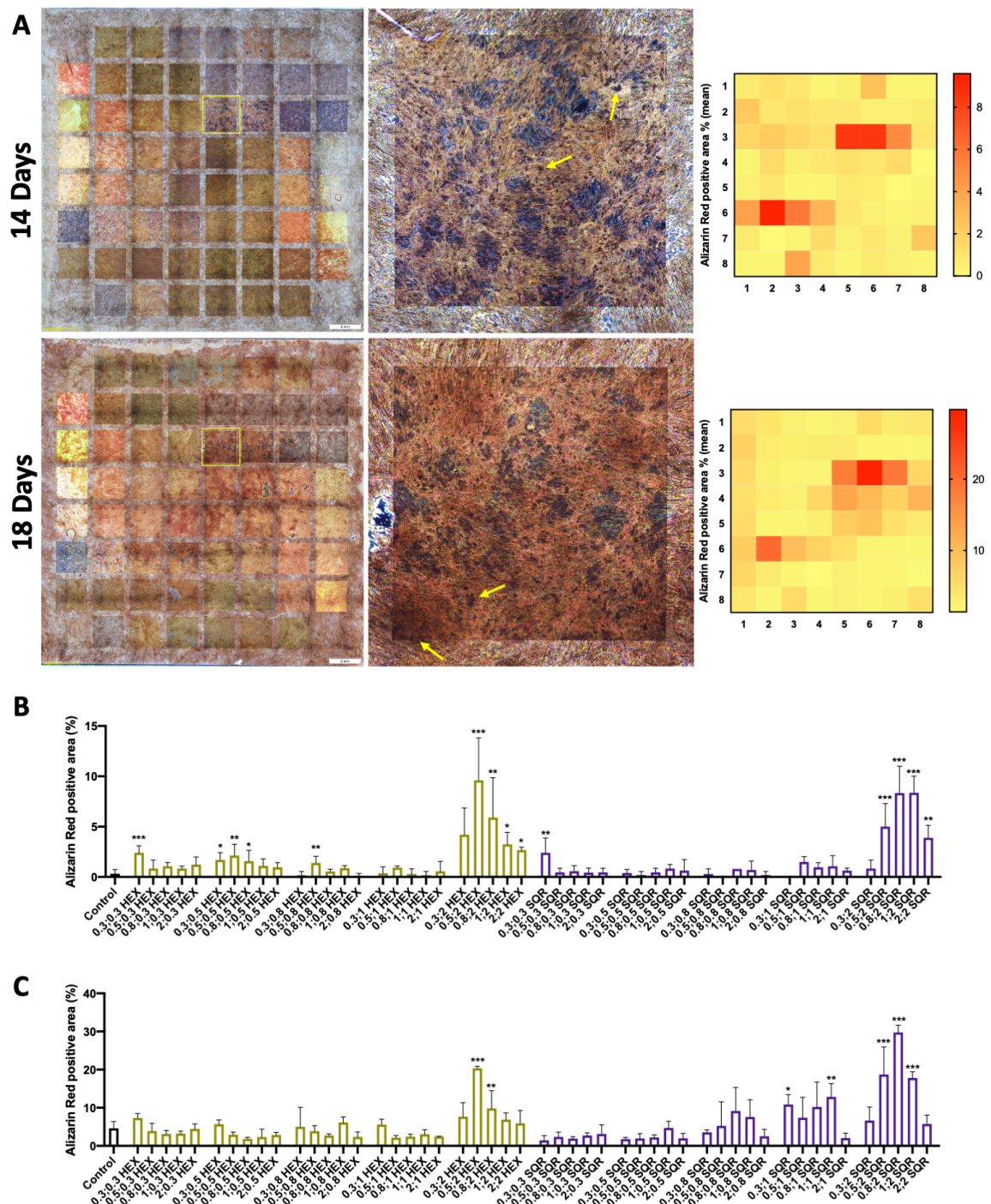
Source: Author unpublished data, 2020.

Influence of pillar-based surfaces on osteogenic differentiation

In order to unravel the surface-induced osteogenic differentiation using the proposed screening platform, the mineralization/calcium deposition levels of hBDCs cultivated on the screening arrays for 14 and 18 days, were measured by Alizarin Red staining. Moreover, we measured the level of OCN, an important marker for mature osteoblasts, on hDPSCs after 14 and 18 days by immunostaining. To confirm the reproducibility of the NTSA-induced cellular response, all the experiments were performed using two cell lines with potential to osteogenic differentiation (hDPSCs and hBDCs), thus both showed similar results. Due to technical issues during the OCN immunodetection with the hBDCs, we decided to present the result obtained with the hDPSCs to illustrate a continuity of the proliferation and differentiation cellular trends observed within all data set.

The human fibula bone cells cultured on the arrays and maintained in osteogenic culture medium for 14 and 18 days, showed independent levels of mineralized nodules formation on different topographical patterns (Figure 8 A). The Alizarin Red positive areas were quantified individually on the 4 control areas and the 60 surfaces of the array by an adaptation of Cavalieri's point-counting histological technique ²²⁶. These data suggest an increased cell differentiation trend in the topographies 0.3;2, 0.5;2, 0.8;2, 1;2 and 2;2 in both SQR and HEX (Figure 8 B). The same group of surfaces, with interpillar distance of 2 μm , that showed hydrophilic behavior and lower cell number, compared to the other topographies grouped by the interpillar distance.

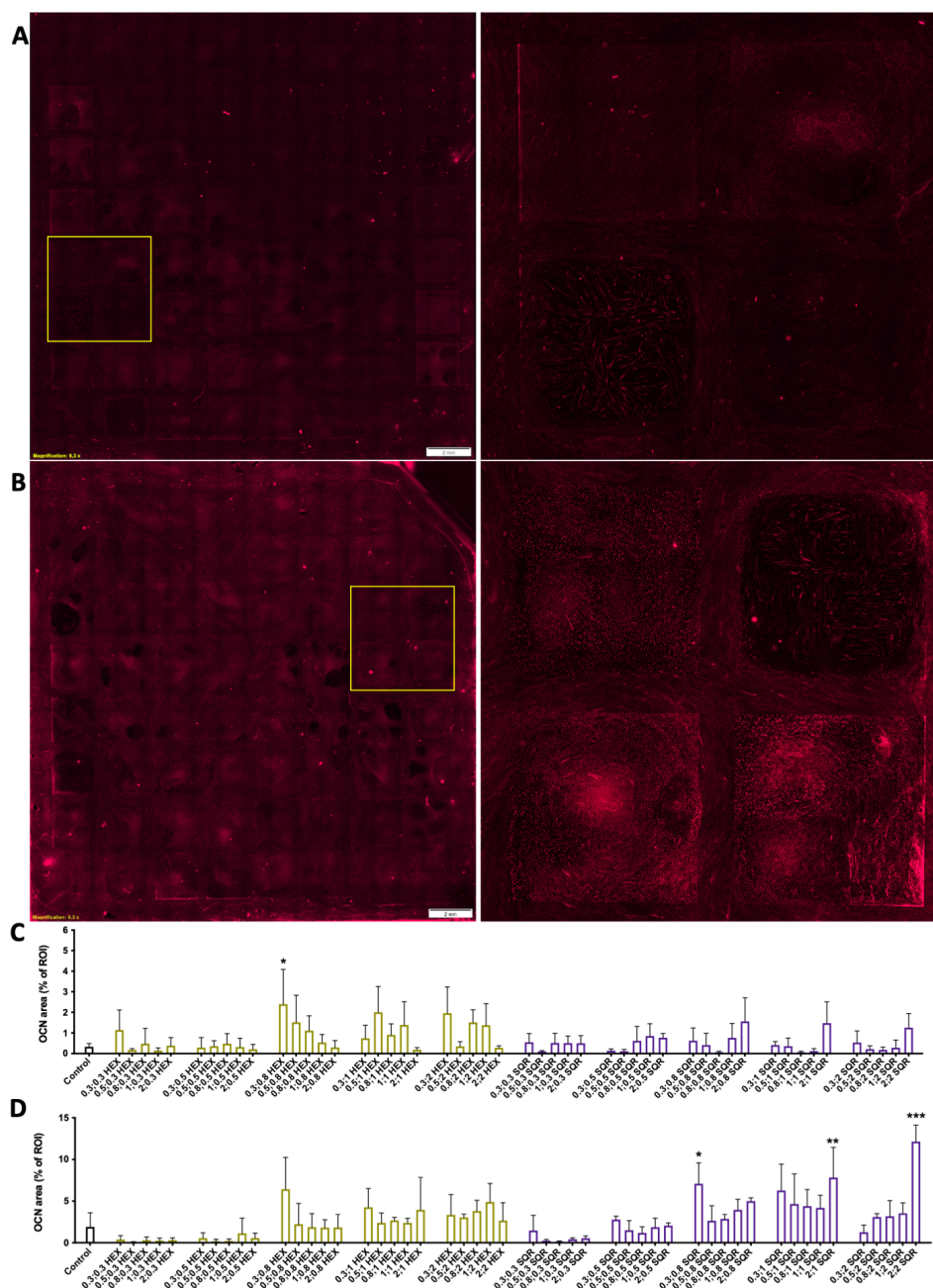
Figure 8 – Evaluation of mineralized nodules formation. (A) Micrographs of hBDCs cultured in osteogenic culture medium and stained with Alizarin Red after 14 and 18 days (Scale bar size 2 mm). Zoom image of the topography SQR 1;2 showing mineralized nodules formation (center panel). Heat-map of the mineralized nodules quantification on each surface after 14 and 18 days ($n = 3$, mean of positive Alizarin Red % area) (right panel). (B) and (C) measurement of Alizarin Red positive areas after 14 and 18 days, respectively ($n = 3$, mean $\pm$ SD, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).



Source: Author unpublished data, 2020.

OCN expression of hDPSCs detected by immunofluorescence, showed extracellular deposition of the protein limited within the topographic constructions, in SQR and HEX pattern, both after 14 (Figure 9 A) and 18 days (Figure 9 B). The fluorescent signal for OCN was measured on each surface; it is clearly observed that the interpillar distance seems to influence the expression of OCN. Some of the HEX and SQR topographies grouped with the interpillar distances of 0.8, 1 and 2 μm clearly presented higher deposition of OCN than the topographies grouped by interpillar distance of 0.3 and 0.5 μm , both after 14 (Figure 9 C) and 18 days (Figure 9 D).

Figure 9 – Analysis of OCN expression. (A) Fluorescent images that highlight osteocalcin expression in hDPSC cultured on the screening array for 14 days and magnified area corresponding to surfaces HEX 0.3;1 and 0.5;1 on the top and HEX 0.3;2 and 0.5;2 on the bottom. (B) Fluorescent images after 18 days and magnified area corresponding to surfaces SQR 0.3;2 and 0.5;2 on the top and SQR 0.3;1 and 0.5;1 on the bottom. (C) and (D) Measurement of fluorescence OCN area after 14 and 18 days, respectively. Using Olympus cellSens v1.17 software (n = 3, mean ± SD, *** p < 0.001, ** p < 0.01, * p < 0.05).



Source: Author unpublished data, 2019.

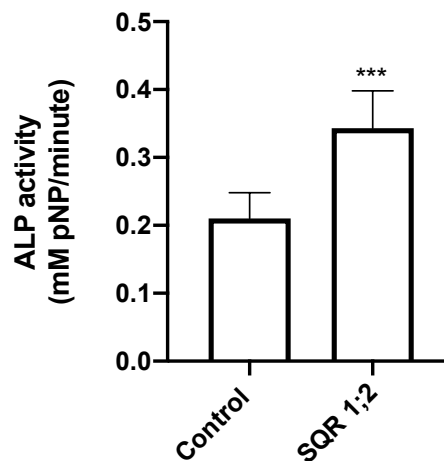
Influence of pillar-based single surfaces on cellular osteogenic behavior

Based on the physicochemical and biological characterization of the screening arrays, we have identified and selected the 0.3;2, 1;2 and 2;2 HEX and SQR topographies (Figure 2), that showed proliferation and differentiation distinct cellular responses. The surface 1;1 was selected because it has the same surface roughness as topography 2;2, although, it demonstrated different surface-induced cellular behavior.

Alkaline phosphatase activity

The alkaline phosphatase activity of hBDCs was accessed, in a preliminary experiment, comparing the flat control and the SQR 1;2 surface after 14 days. The SQR 1;2 topography significantly stimulated the initial rate of p-NPP hydrolysis over time in the fibula osteoblast-like cells culture as it can be seen in Figure 10.

Figure 10 - Evaluation of alkaline phosphatase (ALP) activity. The expression level of intracellular ALP activity of hBDCs grown on Ti flat control surface and SQR 1;2 at 14 days, maintained in osteogenic medium. (n = 4, mean $\pm$ SD, *** p < 0.001).



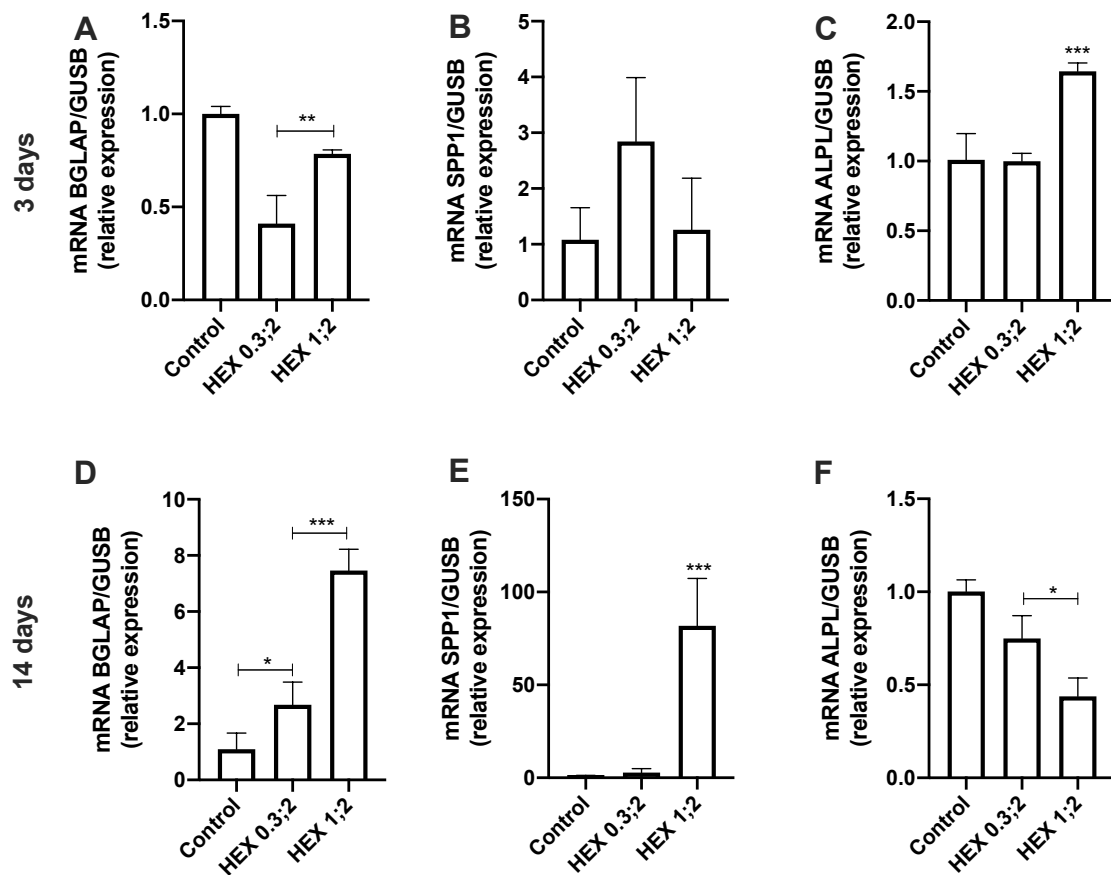
Source: Author unpublished data, 2020.

Osteoblast markers gene expression

To investigate the effect of the single pillar-based surfaces on the expression of osteocalcin, osteopontin, and alkaline phosphatase (encoded by the genes BGLAP, SPP1 and ALPL respectively), hBDCs were cultured on flat control Ti surfaces and on 0.3;2 and 1;2 HEX

surfaces, for 3 and 14 days. This preliminary pilot experiment showed that after 3 days, as expected, the expression of late differentiation markers *BGLAP* and *SPPI* was not increased compared to the control. The expression of *ALPL*, a gene associated with active osteoblasts, is already enhanced for the topography 1;2 (1.6-fold increase) after 3 days. After 14 days, the topographies, specially the HEX surface 1;2, showed a remarkable increase on the mRNA expression of *BGLAP* (6.8-fold) and *SPPI* (78-fold), compared to the control (Figure 11 D and E). Contrastingly, the *ALPL* expression decreased on both surfaces, compared to the flat samples, suggesting that by 14 days the osteoblasts are fully differentiated (Figure 11 F).

Figure 11 - Evaluation of mRNA expression of osteoblast phenotypical markers. (A) Osteocalcin mRNA expression of hBDCs grown on flat control surfaces, 0.3;2 and 1;2 HEX in the presence of osteogenic medium for 3 days. (B) *BGLAP* mRNA expression after 3 days. (C) Alkaline phosphatase mRNA expression after 3 days. (D) Osteocalcin mRNA expression after 14 days. (E) *BGLAP* mRNA expression after 14 days. (F) Alkaline phosphatase mRNA expression after 14 days. The mRNA expression for the genes of interest was normalized to that of beta-glucuronidase (n = 4, mean $\pm$ SD, *** p < 0.001, ** p < 0.01, * p < 0.05).



Source: Author unpublished data, 2020.

Discussion

We have proposed a high throughput screening device for optimizing the characterization of surface-induced osteogenic cellular behavior. Using deep UV projection lithography, the neighboring surfaces were constructed with a range of micro- and nanopillars, varying in size, interpillar distance and geometrical arrangement. Surface roughness has been widely described as a major modulator of cellular proliferation and differentiation^{227, 228}. Topographical organization of surface cues has also been reported to have a key impact on cell fate²²⁹. Here we focused on a systematic approach for nanostructured surface features where defined variables, such as feature diameter and lateral spacing, were established to better correlate topographical specifications and cell fate. Another nano topographical HTS device has recently been proposed, where bioinformatics and overlapped basic shapes were used to create a library of 1249 random nano topographies¹³³. Although the bioinformatics approach associated with different topographies was shown to be a valid tool to predict cellular response, the identification of topographical parameters and their impact on cell behavior remained unclear, due to the large number of topographical variables and randomness of surface constructs¹³³. In this study, we report that topographies with the same theoretical surface roughness but different specific structural organization, may have different effect on osteogenic differentiation, mineralization as well as cell proliferation.

Each surface of this topographical library has a 4.8 mm² area, which might facilitate the tested cell population to develop separated culture environments, that dictate different and distinguishable cellular responses. Another major advantage of our screening device, compared to previous investigated topographical libraries^{133, 222, 230}, is that once we identify the topographical cues parameters triggering the cells to the desirable behavior; these topographies can be manufactured as single samples for deeper understanding of the involved molecular mechanisms.

The deep UV lithography is a gold standard technique for addressing nanofabrications^{133, 231}. The screening arrays processing was precise and reproducible. SEM images of the libraries showed no damaged or displaced pillar constructions (Figure 3). The chemical analysis didn't show any unexpected contamination. The atomic proportions of oxygen/titanium and the absence of a silica detection peak suggested the successful deposition and integrity of the TiO₂ layer. The chlorine residue on the surface is probably caused by the ALD process²³². Due to its low theoretical concentration (less than 1%), we believe that the chlorine trace did not affect the cytotoxicity of the arrays.

The wettability of implant surfaces depends on the surface energy of the substrate which is strongly dictated by its nanoscale roughness ²³³. Although topography has a major role in defining the implant surface/blood proteins interactions, and consequently cell adhesion, proliferation and differentiation; micro- and nanostructured surfaces can also affect the physics of liquid/substrate reactions ²³⁴.

Our contact angle measurements demonstrated that increasing the distance between the pillars (up to 2 μm), increases hydrophilicity. We hypothesize that on the topographies with 0.3 μm interpillar distance, the surface has a Cassie-Baxter state, and the drop stays on the nanostructured protrusions. As long as the interpillar distance goes up, we drive the surfaces towards a Wenzel state, where the liquid drop penetrates the interpillar sites and increase the hydrophilic interaction ^{52, 235}.

Contrastingly, hydrophobic surfaces are considered to be more protein-absorbent than hydrophilic ones, due to the increase in the entropy of water in solution ²³⁶. It has been reported that more hydrophobic substrates can present increased cell adhesion; however, surfaces with moderate hydrophilicity behavior, display improved cell response towards biocompatibility ^{48, 49, 51}. Despite the fact that WCA measurements are not the most adequate criteria to explain cell/material interactions ⁴⁷, our findings suggest that the topographies grouped by higher interpillar distance (2 μm), improved the modulation of cellular osteogenic differentiation, nevertheless showing slightly lower initial adhered cell number.

Although cells on flat surfaces are more inclined to migrate randomly and at a slower speed compared to patterned topographies ^{237, 238}; since a higher cell clustering on the flat control areas is not observed, it is unlikely that the cells are migrating away from the nanostructured surfaces. We reported a direct correlation between the pillar diameter and the adhered cell number. This proliferation trend on the nanostructured topographies is corroborated by the findings of Koo et al. 2002 ²³⁹, showing that the smallest nanostructures are still capable of inducing the organization of integrins and formation of limited sized focal points, contrasting with larger nanostructures showing a greater number of adhered cells and adhesion strength. The transmembrane agglomeration of integrins form focal adhesion complexes that extracellularly bind the cell to matrix proteins, while, on the other side, anchor to the cytoskeleton through adapter proteins ²⁴⁰, suggesting that the size of focal adhesion points formed in topographies with a larger pillar diameter affects cell adhesion and proliferation.

Cell binding to serum proteins of the ECM, such as fibronectin, might act as important regulator of cellular lineage commitment ²⁴¹. We believe that the pillar-based topographies, constructed with defined surface parameters, influence the spatial organization of ECM that

might influence the adsorption of some of these proteins and adhesion molecules, such as integrins, and indirectly modulate primary cells commitment and differentiation¹⁶¹. It is well known that nanostructured topographies can increase production and influence the architecture of the ECM; and affect cell migration and differentiation, possibly through increased activation of integrin-linked kinases (ILK) and subsequent signaling via β -catenin^{218, 242, 243}.

Notwithstanding the fact that further biomechanical investigations are still needed for a complete understanding of how different aspects of the surface topographical architecture can impact the protein adsorption and subsequent mechanotransduction²⁴⁴, it is possible to observe that the pillar lateral spacing seems to have pronounced influence on cellular osteogenic behavior. The topographies with higher interpillar distance (2 μm) are the ones inducing more formation of mineralized matrix nodules. The topographies grouped with the interpillar distances of 0.8, 1 and 2 μm showed greater deposition of OCN than the topographies grouped by 0.3 and 0.5 μm , both in in HEX (green) and SQR (purple) arrangement. On the other hand, we also observed that topographies with smallest pillar diameter (0.3 μm), which in the proliferation experiments had a lower cell count (Fig. 10 C), showed Alizarin Red positive area and increased OCN deposition. These findings suggest that although topographies constructed with smaller pillars diameter present low proliferation, they can still stimulate cell differentiation. These findings are consistent with the fact that proliferation and differentiation have an inverse relationship, directly dependent on the stage of the cell cycle; where the terminal differentiation of precursor cells generally coincides with the stop of proliferation and the exit of the cell cycle division phase^{245, 246}.

ALP is a membrane-bound homodimeric metalloenzyme that has the physiological role of catalyzing the dephosphorylation of phospho-monoesters, releasing inorganic phosphate (Pi) and alcohol, and is one of the most important biochemical markers to assess osteoblast activity²⁴⁷. Nanostructured surfaces are well known for their capacity of increasing ALP activity^{248, 249}. Not surprisingly, the surface 1;2 SQR exhibited higher level of ALP activity in comparison with the flat Ti surface.

Alkaline phosphatase is considered an early differentiation marker, its regulation of Pi is crucial prior the biomineralization phase²⁴⁷. Nevertheless, osteopontin and osteocalcin are often studied as late differentiation markers; where OPN strongly binds to extracellular non-collagen proteins and modulate matrix mineralization; and OCN seems to have a role in bone mineralization and turnover, as the most abundant non-collagenous bone matrix protein^{250, 251}. Our preliminary Real-time PCR analysis showed that after 3 days, the surface 1;2 HEX

enhanced the gene expression of ALP. After 14 days, both 0.3;2 and 1;2 HEX surfaces stimulated higher expression of osteocalcin and osteopontin.

These findings demonstrate that the proposed topographical constructions are not only useful as substrates for inducing an osteogenic cellular behavior, but they are also important tools for standardizing the relation between surface architecture and cell fate. We do not exclude the surface-mediated cell signaling pathways that could be responsible for the regulation of the osteogenic cellular behavior by the different topographical specifications, showed in this study. Further investigation will be necessary to identify and elucidate the underlying mechanisms.

Conclusions

In this work an innovative approach for pillar-based hierarchical surface library was developed for unraveling the role of specific topographical parameters and surface properties on osteogenic cellular behavior. It is found that surfaces with different pillar sizes, lateral spacing and geometric positioning; can induce divergent levels of osteogenic differentiation. Even though in some cases they exhibit similar surface properties, such as roughness and wettability, the data obtained suggest that the lateral spacing between the topographical features has great impact on cell response. The cells cultured on surfaces with higher interpillar distance (2 μ m) showed enhanced osteoblastic activity. We further demonstrate that differences in cell initial adhesion and proliferation might be regulated by the diameter of the nanostructured pillars. An increasing cellular proliferation trend was observed as the pillars diameter systematically escalated. Overall, the study provides a new HTS topographical platform worthy of investigation for optimizing surface specifications, in the design of titanium-based substrates for bone regeneration, stem cell therapy or tissue engineering.

4 FUTURE PERSPECTIVES

Regarding the nano topographical screening array (NTSA) manuscript draft, our initial intention was to conclude a two-phase study to unravel not only ideal nano topographical specifications for surface-induced osteogenic differentiation, but also the underlying molecular mechanisms.

In a first moment we used the combinatorial topography library to address specific surface architecture parameters, such as feature size, lateral spacing and construct symmetry, to identify how the systematic changing of these variables would affect the response of osteoblastic cells populations. Not surprisingly, different nanostructured topographical properties resulted in different cellular behavior. We demonstrated that lateral spacing and size of surface features might play a more crucial role on osteoblastic lineage proliferation and differentiation, compared to topographical symmetry.

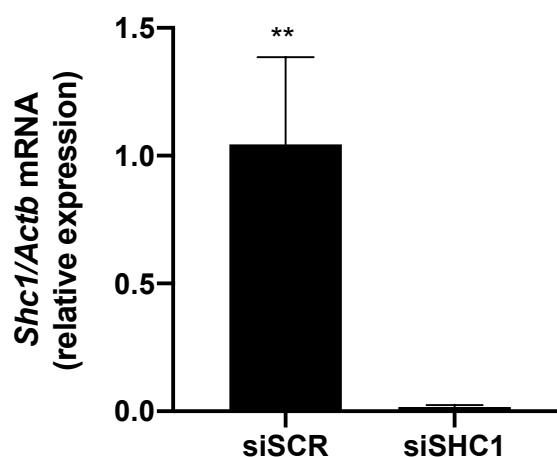
Although the NTSA has been proven as an excellent device for investigating surface-induced cell response with high throughput results, neighboring surfaces are limited when biomolecular tools (e. g. RT-qPCR, ELISA) need to be employed. For the second experimental phase we manufactured single structure samples of the topographies identified as inducing distinct cellular proliferation and differentiation, narrowing down the topographical variables to be associated with the desired osteogenic phenotype. At the moment, we intend to use the selected topographies to investigate the expression pattern of the early and late osteoblast phenotypical markers such as Runx2, Sp7, Colla1, Alpl, Bglap and Spp1. The single structure devices will also allow the investigation of Alkaline Phosphatase enzyme, mineralization and bone-related protein immunodetection experiments. Our preliminary data presented in this thesis demonstrate that this new platform is adequate for this kind of analysis. At last, small-interference RNA and Western blotting techniques will be employed to investigate the role of the adaptor protein Shc1 on the integrin-mediated topography-induced osteogenic differentiation, and illustrate how systematic variations of topographical parameters can influence its downstream cellular signaling pathway.

It is well known that integrin-mediated signaling can trigger the activation of the MAPK pathway through recruitment and phosphorylation of adaptor proteins and protein complexes. It has been reported that Shc1 is a pivotal factor in proliferation of osteoblast-like cells induced by shear stress. Where mainly $\alpha_v\beta_3$ associated with FAK couples Shc1, that is necessary for the downstream activation of Akt and ERK^{152, 153}. Recently, the potent effect of Oncostatin-M to

induce osteoclastogenesis and bone resorption was reported to be dependent on the recruitment of Shc1 to the OSMR subunit of the OSMR:gp130 heterodimer receptor ²⁵². Suggesting the importance of this adaptor protein to the osteoblastic lineage. However, the participation of Shc1 in nanotopography-induced osteoblast differentiation remains unclear.

All the proposed experiments and techniques have been extensively performed by our group, including the short-term silencing of Shc1 coding gene by siRNA (Figure 13).

Figure 13 - Small interfering RNA was used to silence the adaptor protein Shc1 gene on mouse calvarial osteoblasts. Analysis of Shc1 mRNA expression by RT-qPCR after 24 hours. A scrambled sequence (siSCR) served as control. ***P< 0.001; analyzed by unpaired t test with Welch's correction.



Source: Innovation on Biomaterials Group unpublished data, 2017.

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 °C, 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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³ According to the FOAR Academic Works Guide, adapted from the Vancouver Standards. Available on the Library website: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

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APPENDIX – Supplementary Publication

Laser-modified Ti surface improves paracrine osteogenesis by affecting Wnt signaling modulator expression.⁴

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⁴ According to the FOAR Academic Works Guide. Available on the Library website: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>

ABSTRACT

OBJECTIVES: The aim of this study was to evaluate the influence of laser-modified-Ti conditioned medium on bone marrow cells (BMCs) osteogenic differentiation in a paracrine manner. **MATERIAL AND METHODS:** Calvarial osteoblasts isolated from neonatal mice were seeded on polished (P) and Yb:YAG laser-irradiated (L) surfaces (n=8). The culture media was collected and filtered in alternate days to stimulate bone marrow cells (BMCs) cultures, isolated from the tibia and femur of 4 weeks old mice. The osteoblast-conditioned media was replaced every 48 hours. The modified disks were characterized by Scanning Electron Microscopy (SEM). To access the BMCs viability and proliferation, the AlamarBlue® assay was performed every other day for 20 days. After 7 and 14 days, the bone marrow cells were submitted to Alkaline Phosphatase activity test, Alizarin Red staining, real-time PCR to quantify cell differentiation and osteoblasts phenotypic markers. ELISA of the osteoblasts cultured on P and L surfaces was carried out to assess the expression of bone related proteins. **RESULTS:** SEM images of polished and laser-modified discs showed micro and nano-topography variations after Yb:YAG laser irradiation. BMCs cultured with conditioned media showed increased mineralized nodules formation and alkaline phosphatase activity. The L-conditioned media also enhanced the BMCs expression of transcription factor *Sp7* and the osteoblastic markers *Bglap* and *Alpl*. Calvarial osteoblasts cultured on L surfaces exhibited decreased expression of Wnt cell signalling pathway inhibitor Dkk-1. **CONCLUSION:** The modification of Ti surface with Yb:YAG laser may improve osteogenic differentiation in a paracrine way decreasing the expression of Dkk1 and stimulating osteogenic mediators expression.

Keywords: Laser ablation. Titanium. Nanotopography. Osteoblast. Osseointegration.

INTRODUCTION

Osseointegrated implants are widely used with high clinical success rates and predictability in oral rehabilitation ^{253, 254}. Since the mid 60s, Titanium (Ti) has been considered a reliable material for bone implantations due to its excellent biocompatibility, corrosion resistance and mechanical properties, providing stable implant anchorage in most cases ^{217, 255, 256}.

The clinical success of a dental implant depends on many factors, including surgical procedure, bone quality and how fast and uniform the osseointegration occurs ^{257, 258}. Faster osseointegration allows an earlier loading and increases success rates even in patients with poor bone quality or systemic conditions ²⁵⁹.

Disordered microstructures are common cues that influence cell morphology and spreading ^{111, 260, 261}. Thus, modification of titanium surfaces is commonly performed by the manufacturers to increase osseointegration ¹²⁸. Implants surface modification usually involves chemical treatment or particles incrustation, which raise the product final cost and add potential contaminants to its surface ^{105, 262}.

High-power laser irradiation is a cheaper, faster and reproducible alternative for Ti surface modifications that can eliminate chemicals and residual particles adhered to the substrate. This method creates different roughness and topographical patterns with a superficial oxide layer of nanometer thickness that improves corrosion resistance, biocompatibility and plays a critical role in osseointegration ²⁶³⁻²⁶⁶. Laser-modified Ti surfaces exhibit high wettability, increasing cell spreading and adhesion to the material ^{111, 267, 268}. Preliminary *in vivo* studies have demonstrated that high-power laser Ti modifications produced nano and microstructures capable of accelerating the implant osseointegration ^{264, 266}; which is a complex process that includes the recruitment and differentiation of osteoblasts, controlled mostly by the early maturation runt-related transcription factor 2 (Runx2), and the downstream transcriptional regulator Osterix (Sp7). Differentiated osteoblasts secrete bone matrix proteins, including collagen type 1 alpha 1 (Colla1), osteocalcin (OC), and alkaline phosphatase (Alp) that will regulate mineralized matrix deposition and new bone formation ^{269, 270}.

For effective integration of the implant, osteogenesis must take place in two distinct sites; in contact with the Ti implant surface, and at distance, on the host bone surface ^{255, 271}. The distance osteogenesis will connect the host bone to the implant surface while the contact osteogenesis will adequate bone apposition to the implant. Although contact osteogenesis seems to require other factors to be triggered, histological evidence demonstrates that implant

surface modifications play a critical role on paracrine osteogenesis and might accelerate the final implant osseointegration²⁷¹⁻²⁷³.

To date, *in vitro* studies using the contact osteogenesis approach showed that modifications on the Ti surface can accelerate and improve the implant/bone interface consolidation^{111, 217, 256, 269}. Although it is well known that micro and nano patterns on surface topography influence the cells tendency of assuming an elongated and round shape, increasing the chemotactic signal of cell adhesion and integrin receptors and their secretion of cytokines that may modulate an osteogenic differentiation²⁷⁴, but how this microenvironment may influence bone formation in a paracrine manner has not been well described.

The Wnt/ β -catenin signalling pathway is essential to embryonic skeleton development, it modulates bone formation and resorption by promoting osteoblasts and osteocytes differentiation, maturation, survival and inhibiting osteoclastogenesis²⁷⁵. Its activation by binding of Wnt proteins to a Frizzled (Fzd) receptor and a co-receptor (LDL receptor-related protein [Lrp5 or Lrp6]) results in accumulation of non-phosphorylated β -catenin in the cytoplasm that will eventually be translocated into the nucleus and regulates gene transcription²⁷⁶. The Dickkopf (Dkk) family and the Sclerostin (SOST) family are well known groups of proteins that modulate the Wnt signalling pathway; they prevent the formation of the Wnt-Fzd-Lrp complex binding to co-receptor Lrp5/6²⁷⁷. Despite the fact that Wnt proteins and its downstream signalling components have been widely studied and targeted as bone formation modulators on modified surfaces^{278, 279}; the role of Wnt signalling inhibitors on distance bone formation within the implant/bone interface microenvironment remains unclear. Here we report that conditioned medium for laser-modified-Ti surface can modulate murine bone marrow cells (BMC) metabolism and influence the osteogenesis by increasing expression of osteoblastic markers and decreasing Wnt signalling inhibitor Dkk-1 expression.

MATERIAL AND METHODS

The *in vitro* study was approved by the local Ethical Committee for Animal Experimentation from the Faculty of Dentistry of Araraquara and conducted according to the ARRIVE guidelines. The experiments were carried out in triplicate with three independent experiments being performed.

Ti discs preparation and characterization

Commercially pure (grade 4) Ti discs (175 mm² of area and 2 mm thickness) were sequentially polished under water cooling with 320- to 1200-grit grinding paper. They were immersed in acetone PA ACS (Qhemis, Indaiatuba, SP, Brazil) and ultrasonically cleaned for 15 min, washed with deionized water and 100% ethanol (Qhemis, Indaiatuba, SP, Brazil). The disks were finally rinsed with distilled water for 15 min and dried for 90 min in incubator at 60°C (Orion 515, Fanem, São Paulo, SP, Brazil). The polished samples were divided in two experimental groups, polished (P) and laser (L). The L surface was obtained by laser ablation with a pulsed Yb:YAG laser (Omnimark 20F, Ominitek Tecnologia Ltda, São Paulo Brazil) as previously described (Mariscal-Munoz et al., 2015). To access the surface topography, the specimens were characterized by scanning electron microscopy (SEM) (JMS-T33A, JEOL, Tokyo, Japan).

Isolation and culture of mouse calvarial osteoblasts

Primary osteoblasts were isolated from neonatal calvarial bones obtained from 2 to 3 days old mice (*Mus musculus* C57Bl/6) using sequential enzymatic digestion²⁸⁰. The cells obtained were seeded in plain Minimum Essential Medium α (α -MEM, Invitrogen, Carlsbad, CA) supplemented with penicillin/streptomycin (Pen-Strep, Sigma Aldrich, St. Louis, MO) and 10% fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO). After three days of incubation, 20.000 cells were seeded on Ti discs with L or P surface (24-well plates) in osteogenic differentiation media, α -MEM supplemented with 10 mmol/L β -glycerophosphate and 2 mmol/L ascorbic acid (Sigma Aldrich, Carlsbad, CA). The α -MEM+ β -Gly+Aa was collected and filtered every other day to stimulate the bone marrow cells culture and analyse bone metabolism, as described below.

Isolation and culture of mouse bone marrow cells

BMCs were isolated from the tibia and femur of 4-week-old mice as previously described²⁸¹. 5×10^5 cells were seeded in 96-well plates in complete α -MEM. After 24 hours the complete media was replaced for the α -MEM+ β -Gly+Aa media obtained from calvarial osteoblast-like cultures seeded over P or L Ti surfaces, every 48 hours the fresh conditioned media was collected from the calvarial osteoblasts cultured in parallel to the BMCs cultures, filtered and used to stimulate BMCs.

Alamar blue

Resazurin (Alamar Blue, Sigma Aldrich, St. Louis, MO) assay was performed every other day after the first day of culture for 20 days, according to the manufacturer's protocol, to analyse the BMC's viability and proliferation. The 1:10 Alamar Blue solution was added directly into the culture wells and the plates were incubated for 1 hour at 37°C. Cell viability was assessed by the proportional absorbance at 570nm in a microplate reader (Power Wave XS, BioTek Instruments, USA).

Alkaline phosphatase activity

After 7 and 14 days, the osteoblasts were lysed using 500µL of a 50mmol/L Tris-HCl solution (pH 7.4) containing 0.5% of Nonidet P40. Total protein content was quantified using a bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Scientific, Rockford IL, USA). ALPase activity was assessed by measuring the release of thymolphthalein from thymolphthalein monophosphate using a commercial test kit (Labtest Diagnostica SA, Belo Horizonte MG, Brazil). According to the measured protein amount, 20 µg of total protein was added to a solution of thymolphthalein monophosphate (22 mmol/L) and diethanolamine buffer solution (0.3 mmol/ml, pH 10.1) and incubated for 40 min at 37°C. 200µL of a Na₂CO₃ (0.09 mmol/ml) and NaOH (0.25mmol/mL) solution was added and incubated at 37°C for additional 10 minutes, the color gradient was measured by the absorbance at 590nm in a microplate reader (Power Wave XS, BioTek Instruments, USA). ALPase activity was calculated from a standard curve using alkaline phosphatase as standard in a range varying from 22.5 to 900 µU.

Alizarin red staining

The mineral deposition of osteoblast-like cells treated with α -MEM+ β -Gly+aAa was accessed by Alizarin Red staining at 14 and 21 days after the cells were cultivated in 96-well plates. The cells were rinsed twice with PBS at 37°C and then fixed with 150 uL of 4% paraformaldehyde for 10 minutes. After rinsing the cells again with PBS, 50 uL of 10% w/v Alizarin Red (Sigma Aldrich, St. Louis, MO) staining solution was added to each well at room temperature for 10 minutes, the staining solution was then removed and the wells rinsed twice with PBS. The mineralization was quantified by dissolving stained nodules with 200uL of 10% w/v cetylpyridinium chloride (Sigma Aldrich, St. Louis, MO) solution. The dissolved nodules

were transferred to 96-well plates and read at 562 nm in a microplate reader (Power Wave XS, BioTek Instruments, USA).

Real-time PCR

Total RNA was extracted from seeded BMC's treated with α -MEM+ β -Gly+Aa after 14 days using an affinity column method according to the manufacturer's protocol (RNAqueous-micro Kit, Thermo Scientific, Rockford IL, USA). The RNA from each well was quantified by UV absorbance (Eppendorf, Hamburg, Germany) and considered acceptable when the 260/280nm absorbance ratio was higher than 1.8. Three-hundred nanograms of total RNA were converted into cDNA using random hexamer primers and reverse transcriptase in a total reaction volume of 20uL (High capacity cDNA synthesis kit, Applied Biosystems).

Quantitative real-time PCR (qPCR) reactions were performed to assess the expression levels of the genes encoding the transcription factors *Runx2* (forward primer, 5'-CCTGAACTCTGCACCAAGTCCT-3'; reverse primer, 5'-TCATCTGGCTCAGATAGGAGGG-3') and Osterix (*Sp7*) (forward primer, 5'-TGCTTGAGGAGGAAGTTCAC-3'; reverse primer, 5'-AGGTCACTGCCCACAGAGTA-3'), and bone-specific proteins osteopontin/secreted phosphoprotein (*Spp1*) (forward primer, 5'-TCACCATTCGGATGAGTCTG-3'; reverse primer, 5'-ACTTGTGGCTCTGATGTTCC-3'), alkaline phosphatase (*Alpl*), α 1chain of collagen type 1 (*Colla1*) (forward primer, 5'-CCTCAGGGTATTGCTGGACAAC-3'; reverse primer, 5'-CAGAAGGACCTTGTTTGCCAGG-3') and osteocalcin/bone gamma-carboxy glutamic acid protein (*Bglap*) (forward primer, 5'-GCAATAAGGTAGTGAACAGACTCC-3'; reverse primer, 5'-CCATAGATGCGTTTGTAGGCGG-3'). The 20uL total volume reactions included TaqMan qPCR master mix (Applied Biosystems), cDNA, deionized water and species-specific pre-designed and optimized pairs of primers and probe (TaqMan gene expression assays).

The determination of the relative levels of gene expression was performed using the quantification cycle by the standard curve method using the gene encoding to β -actin (*Actb*) as reference gene. Results were expressed as fold change over the levels of expression of the normalized target gene determined in cDNA prepared from P samples group.

ELISA

The ELISA tests for Dkk-1 (# MKK100), Sclerostin (# MSST00), BMP-2 (# DBP200), TGF-beta (# MB100B) and Osteopontin (# MOST00) were performed following the

manufacturer's recommended protocol (R&D Systems, Minneapolis, MN, USA). For these assays, aliquots of the supernatants (α -MEM+ β -Gly+Aa) collected at 0, 2, 4, 6, 8, 10 and 12 days were mixed to obtain pooled samples. Six different pools were evaluated per group (n = 6).

Statistics

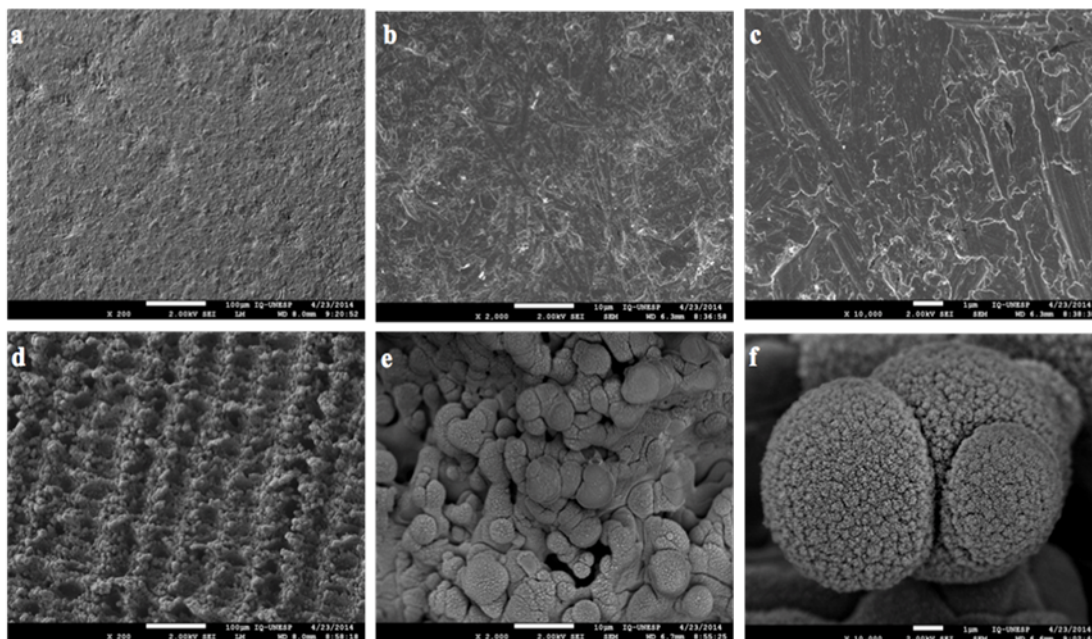
Data from Alamar Blue, qPCR, Alkaline phosphatase activity and Alizarin red were submitted to t-tests since they adhered to the normal distribution (Shapiro-Wilk, $p > 0.05$). The level of significance was 95% ($p < 0.05$). Results were represented as mean+SD.

RESULTS

Ti surface characterization

SEM analysis revealed topographic differences between the polished (P) and laser-irradiated Ti surfaces (L). The P surface was homogeneous with traces of the manufacture process and minor surface roughness (Fig. 1a, b, c). In contrast, L surfaces generally presented a morphologically irregular surface, characterized by the presence of spheres and protrusions of different diameters due to the irradiation pattern (Figure 1d, e, f). Longitudinal channel-shaped depressions with different depths and widths were observed. At high magnification it was possible to detect in the L surface the presence of secondary nano-roughness inside the spheres caused by Ti ablation. Therefore, laser irradiation created a nano-to-micro hybrid surface (Figure 1f).

Figure 1 – Representative SEM images of polished P surface in x200 (a), x2000 (b) and x10000 (c); and laser-irradiated L surface in x200 (d), x2000 (e) and x10000 (f).

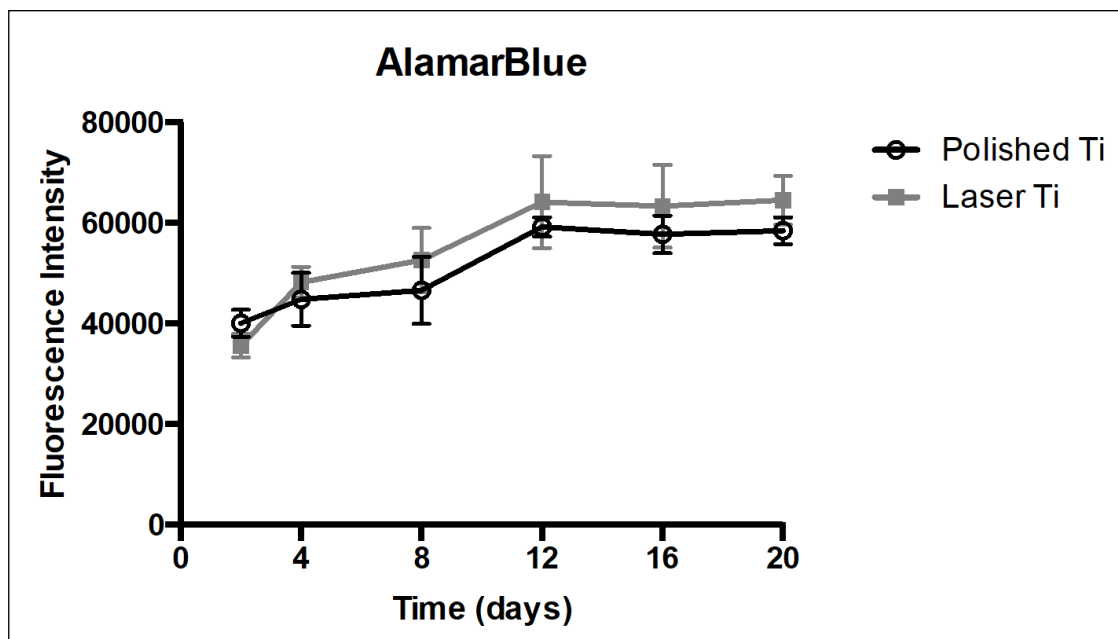


Source: Innovation in Biomaterials unpublished data, 2017.

Cell viability and proliferation

The effect of surface topography was first investigated on BMCs viability and proliferation. The cells were treated with the α -MEM+ β -Gly+Aa medium obtained from osteoblasts cultured in P and L surfaces. For both periods of evaluation, no significant difference between the groups was observed (Figure 2) ($P>0.05$).

Figure 2 – BMCs proliferation and viability assessed by Alamar Blue assay. Mean ($\pm$ SD) of fluorescence intensity at 2, 4, 8, 12, 16 and 20 days ($n = 8$) ($P > 0,05$).

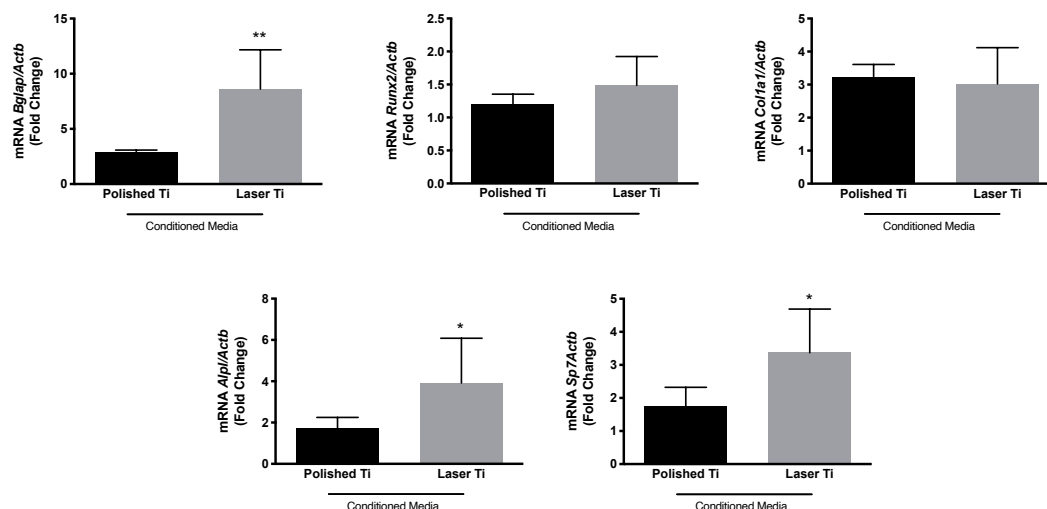


Source: Innovation in Biomaterials unpublished data, 2017.

Laser-modified surface affects osteoblast genes expression in BMCs

The effects of laser-modified Ti surface on osteoblast differentiation and function were then investigated on expression of mRNAs, encoding for transcription factors (*Runx2* and *Sp7*) and osteoblasts phenotypic markers (*Alpl*, *Colla1*, and *Bglap*), in BMCs treated with the α -MEM+ β -Gly+Aa medium obtained from osteoblasts cultured on P and L surfaces. After 14 days, the L surface α -MEM+ β -gly+aa medium up regulated the mRNA expression of the transcription factor *Sp7* and the phenotypic markers *Bglap* and *Alpl* (Figure 3).

Figure 3 – mRNA relative expression of osteoblast phenotypical marks after 14 days of culture of BMCs treated with P and L surface conditioned media. Mean ($\pm$ SD) of mRNA expression of *Bglap*, *Runx2*, *Col1a1*, *Alpl* and *Sp7* (n = 8). Real-time PCR (* p <0.05; ** p <0.01).

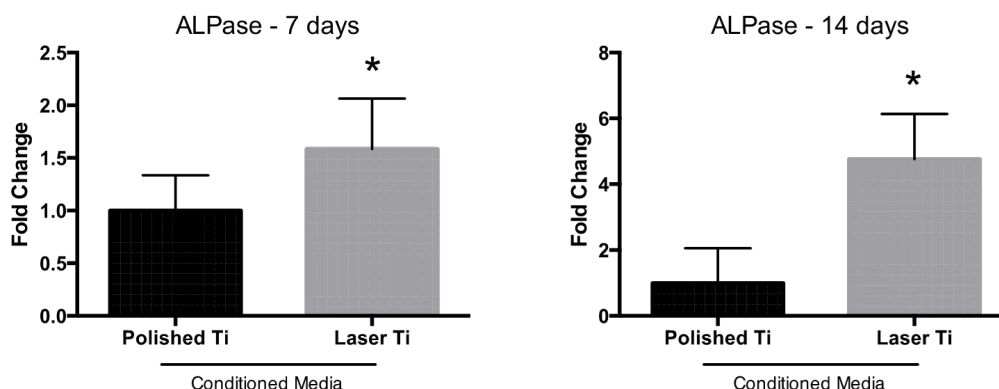


Source: Innovation in Biomaterials unpublished data, 2017.

Laser-modified surface stimulates ALPase activity in BMC

After 7 and 14 days the activity of ALPase was increased in BMCs treated with the conditioned media obtained from the culture of osteoblasts grown on L Ti-irradiated surfaces, compared to BMCs treated with the media obtained from osteoblasts grown on the P surfaces (Figure 4).

Figure 4 – ALPase activity of BMCs treated with P and L surfaces conditioned media at the 7th and 14th day of culture. Mean ($\pm$ SD) of spectrophotometric quantification of ALPase activity in BMCs cultures at 7 and 14 days ($n = 8$) (* $P < 0.05$).

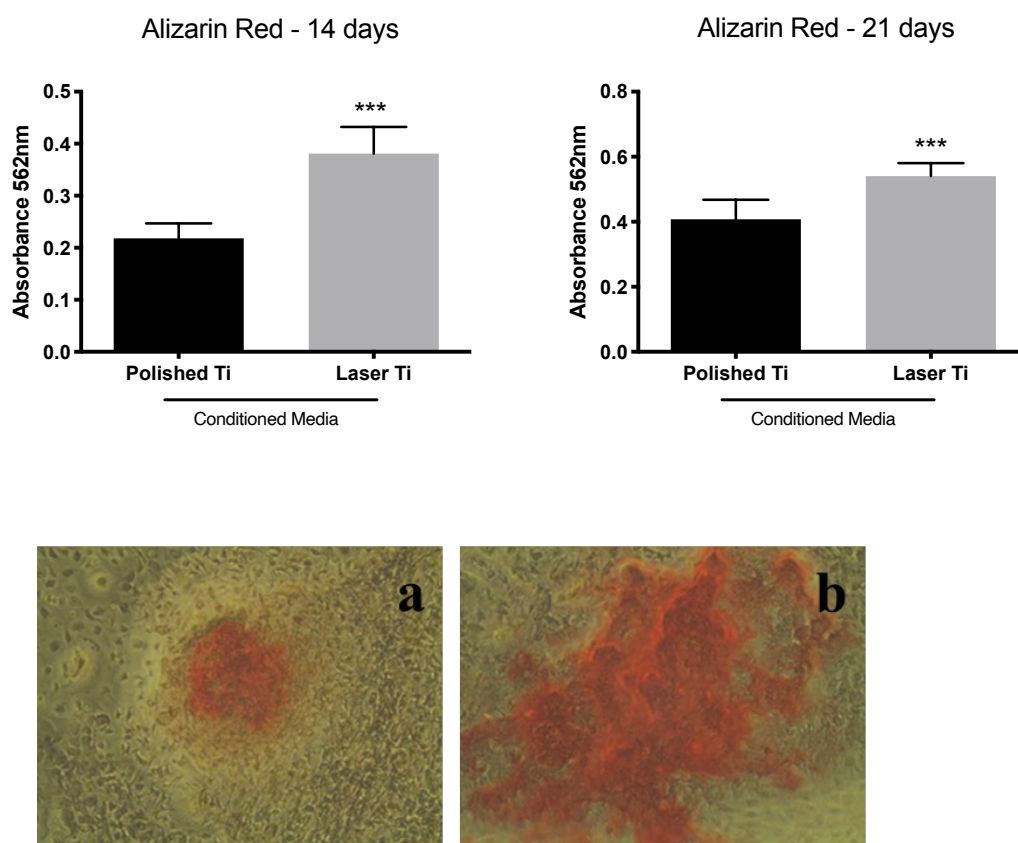


Source: Innovation in Biomaterials unpublished data, 2017.

L surface stimulates mineralized nodules formation

The formation of a mineralized extracellular matrix is what defines fully differentiated and active osteoblasts. To investigate how the laser-modified Ti surface affected osteoblasts maturation and function, we quantified calcified nodules formed in osteoblasts cultures treated with P or L Ti surfaces conditioned α -MEM+ β -Gly+Aa medium after 14 and 21 days of culture. Active formation of mineralized nodules was observed in both groups and experimental periods. After 14 and 21-day culture, osteoblasts treated with L surface conditioned α -MEM+ β -Gly+Aa medium showed higher calcified matrix formation compared to cells treated with P surface conditioned medium (Figure 5) ($P < 0.05$).

Figure 5 – Mineralized matrix deposition of osteoblasts maintained in P or L conditioned media after 14 and 21 days of culture. Mean ($\pm$ SD) of the absorbance after the mineralized nodules dissolution ($n = 8$) (***) $p < 0.001$). Photomicrographs of nodules found in P (a) and L (b) groups.

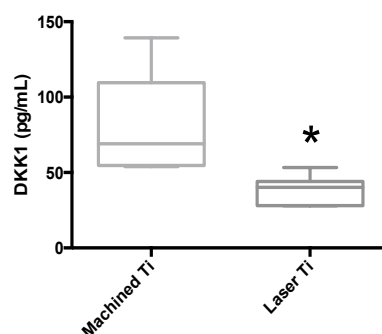


Source: Innovation in Biomaterials unpublished data, 2017.

L surface downregulates Wnt signaling inhibitor in osteoblasts

To investigate the impact of the laser-modified surface on expression of Wnt signaling inhibitors, we performed an ELISA assay for Dkk-1 and Sclerostin using the supernatant of osteoblasts cultured on P and L surfaces. The expression of Dkk-1 was decreased in the L surface group compared to the polished P surface group. The protein Sclerostin was not detected in all groups.

Figure 7 – Dkk-1 protein measured by ELISA is downregulated in L surface samples. Levels of Dkk-1 were measured by ELISA in osteoblasts cultured on P and L Ti surfaces. Mean ($\pm$ SD) expression of Dkk-1, OPN and TGF-beta proteins ($n = 6$) ($*P < 0,05$).



Source: Innovation in Biomaterials unpublished data, 2017.

DISCUSSION

The implant osseointegration is environment-dependent and largely influenced by Ti surface topographical properties^{217, 282}. Several techniques aiming micro- and nanostructured surface modifications have been proposed to modulate this biological process⁵⁴. Laser surface treatments have been often described as a highly controllable approach to improve the cellular response to the implant surface^{264, 266}. Recently, our group has characterized a nano-to-micro roughened surface produced by Yb:YAG laser ablation. It increased the outer layer of TiO₂ adding no residue contaminants to the surface, and induced osteoblast differentiation and activity¹¹¹. Besides the well documented direct effect on bone cells in contact to the implant surface, the laser ablation might influence the expression and release pattern of cytokines and growth factors that dictates a paracrine osteogenesis in the bone/implant microenvironment²⁸³⁻²⁸⁵. The distance osteogenesis takes place on the host bone at the peri-implant area, and it is a crucial event for fully osseointegration and endosseous healing^{255, 271}. Here, we report that the laser ablated surface decreased the expression of Wnt modulator Dkk-1 in osteoblast cultures. As well as the conditioned media obtained from these cultures was able to induce osteogenic differentiation and activity of BMCs cells in paracrine way.

Although the L-surface conditioned media had no effect on cell proliferation and viability, it enhanced BMCs ALPase activity and mineralized nodules formation compared to the P group. Suggesting that ALPase activity of bone marrow stromal cells present in host bone plays an important role in bone matrix calcification of peri-implant area. It has been demonstrated that Ti-conditioned medium enhance pre-osteoblast adhesion and activate pivotal signalling proteins related to bone metabolism ²⁸³. The L-surface conditioned medium also increased mRNA expression of Sp7, Bglap and Alpl. It was recently reported that when stimulated with conditioned medium obtained from RAW cells cultured on microstructured Ti topographies, bone marrow stem cells exhibited nearly 1.5-fold higher expression of osteogenic genes and production of mineralized nodules compared to direct stimulation of the Ti surfaces ²⁸⁵. The increased release of bone-related proteins induced by laser-irradiated Ti surface may explain the modulation of BMCs differentiation ¹¹¹.

The Wnt/ β -catenin signaling pathway is an important mechanism that manipulate cell proliferation, cell polarity and cell fate determination during embryonic and postnatal development ²⁷². Bone diseases, such as osteoporosis, pseudoglioma syndrome, sclerosteosis and van Buchem's disease have been associated with atypical Wnt signaling ²⁸⁶. Williams & Insogna (2009) reported that this cell signaling pathway is one of the most critical regulators of bone tissue formation and homeostasis; they showed that Wnt/ β -catenin inhibitors, SOST and Dkk-1, negatively regulate bone mass and the reduction of these molecules results in increased bone mass in mice ²⁸⁷. Herein, the expression of Dkk-1 decreased on osteoblasts cultured onto L surface group, suggesting that besides the direct mechanical stimulation on cellular cytoskeleton and its integrin receptors ²⁸⁸; the laser treatment of the surface might modulate osteogenesis occurring within implant/bone microenvironment by downregulating Wnt canonical signaling inhibitor. However, SOST was not detected in any of the experimental groups (data not shown). Since osteocytes are the main producers of SOST and several growth factors and bone-related proteins have been showed to be deposited in ECM during mineralization process ^{289, 290}. We believe that the levels of SOST present in the osteoblasts culture supernatant were not enough to be reactive in ELISA measurement.

As demonstrated, the laser-modified Ti surface is capable of affecting bone cells at distance, enhancing paracrine mineralized tissue formation by downregulating Dkk-1 and modulating osteoblastic lineage transcription factors and key proteins expression. Additionally, the conditioned media seems to be a worthy tool for stimulating bone regeneration. Cultured MSCs secrete a number of growth factors and cytokines into the medium with defined potential to stimulate tissue regeneration ²⁹¹. Pioneering studies have reported that conditioned media

improved the bone regeneration in critical-sized calvaria defects in rats ²⁹², enhanced the osteoblastic differentiation on collagen membranes ²⁹³ as well as on the inorganic bovine bone graft ²⁹⁴. However, further molecular investigations, gene editing tools and *in vivo* studies are still needed to extend the understanding of the underlying mechanisms governing surface-induced paracrine osteogenesis.

CONCLUSION

Considering the methodology used in the present study one may conclude that the modification of Ti surface with Yb:YAG laser has a positive impact on osteoblast behavior, enhancing the expression of key osteoblastic markers and downregulating the Wnt signaling inhibitor Dkk-1. The pre-conditioned medium stimulated the bone marrow cells into an osteogenic phenotype showing that laser ablated surfaces might affect the bone tissue regeneration process occurring at distance in the bone/implant microenvironment.

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Araraquara, 19 of March of 2021

Jorge Felipe Lima Teixeira