Delft University of Technology (TU Delft)

Faculty of Mechanical, Maritime and Materials Engineering (3ME)

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Surface-cell interactions:

Can PEO topography outsmart surface calcium?

by

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Preface

The thesis report includes two parts. In the first part the main research findings are summarised in the form of an article and the second part contains the thesis report with experimental details and findings discussed extensively.

Enjoy reading!

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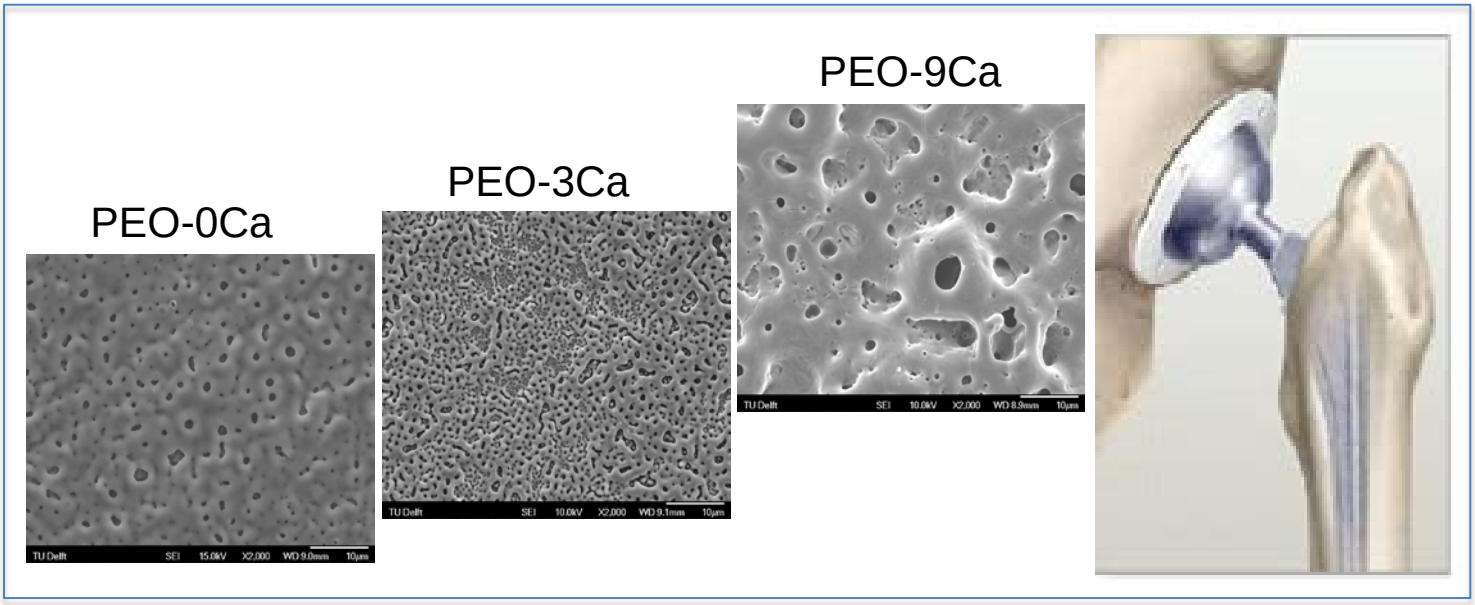

THESIS REPORT

by

Jesu Delihta Liyaa Fernando

Acknowledgements

I owe my heartfelt gratitude to several people who have supported me during this master research and helped me realise my one of my fondest dreams.

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Chapter 1

1 Research background and overview of thesis

1.1 Bone structure and composition

The bone is a vascular, calcified, connective tissue of the vertebrates. It provides mechanical support, protects various organs, regulates mineral homeostasis and is also involved in haematopoiesis (blood cell formation). The bone can be compact or porous. [1]. Compact bone is dense, strong and provides attachment sites for muscles. Cancellous bone is light weight, porous, rich in blood vessels as it contains bone marrow where blood cells are formed (Figure 1).

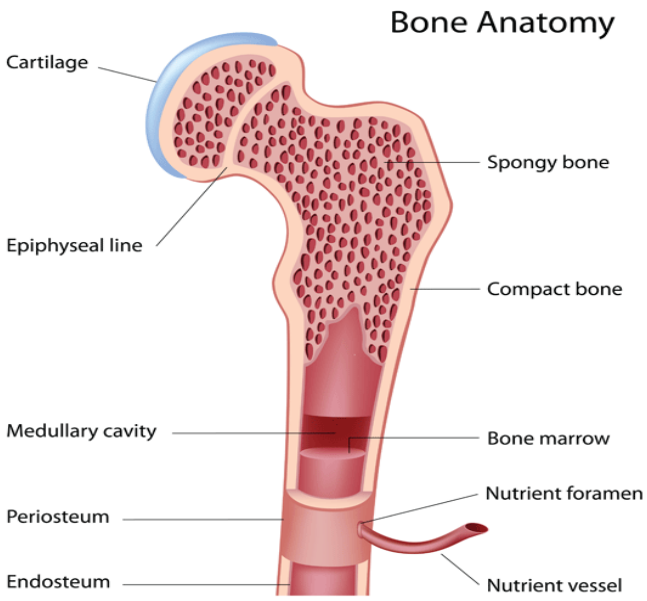

Figure 1: Macrostructure of the bone [2]

The bone is made up of organic collagen matrix within which calcium and other minerals are deposited by bone cells. Calcium, phosphate and magnesium are the most important and abundant mineral in bone with calcium and phosphate combining together in the crystalline complex hydroxyapatite [$Ca_{10}(PO_4)_6$ $(OH)_2$]. This inorganic mineral component (hydroxyapatite) contributes to approximately 65% of the bone [1]. The organic collagen content (mainly collagen type 1) contributes to about 20-25 % of the bone mass. The

remaining 10 % is composed of water that is bound to collagen mineral matrix and the unbound water that freely flows through the vascular channels of the bone [1].

1.1.1 Bone remodeling

The bone is a dynamic tissue that adapts predominantly throughout human life span; this is characterized by bone formation and growth throughout childhood followed bone remodeling which involves resorbing (removing) certain areas of bone and filling the resorbed cavity with new bone in order to preserve skeletal size, shape and regulate mineral homeostasis.

The density of bone is modulated by a group of cells, including osteoclasts which are multinucleated cells that resorb bone and osteoblasts which refill the resorption cavity created by osteoclasts (Figure 2). Osteoclasts anchor themselves to the surface of bone which creates a sealed zone underneath the cells. Within this zone the osteoclasts create an acidic environment that dissolves the mineral matrix of the bone. Once the mineral matrix has been dissolved, the osteoclasts release enzymes that remove the remaining collagen content of the bone matrix that completes resorption of bone in that region [3].

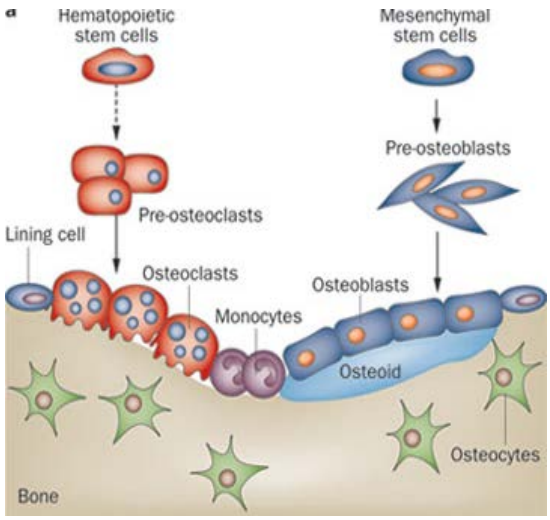

Figure 2 Bone remodeling mechanism [4]

Following resorption, osteoblasts move into the resorption space and deposit an organic matrix termed as osteoid which is predominantly made of collagen. This collagen forms a scaffold like structure in which minerals like calcium and phosphate begin to crystallize. Meanwhile some osteoblasts that get trapped within the mineral matrix become osteocytes that respond to mechanical loading of bone and direct remodeling. Bone remodeling thus

rejuvenates bone by maintaining a fine balanced equilibrium between bone resorption and bone formation.

1.2 The articular cartilage and osteoarthritis

At the ends of bone where joints are formed a smooth, structurally organized, avascular connective tissue known as articular cartilage is present. The articular cartilage glides over each other and provides a friction free environment that is required for the smooth articulation of the joints besides shock absorption and protection of bone ends.

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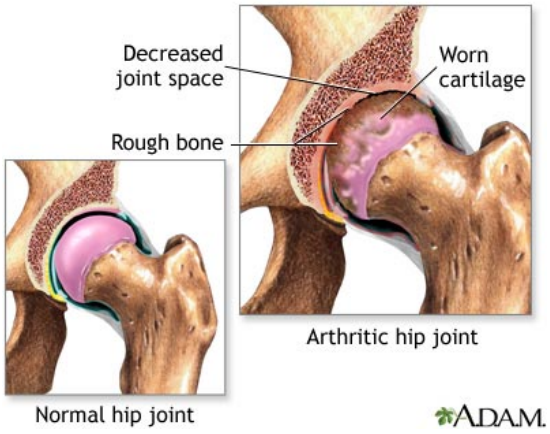

Figure 3 Normal and arthritic hip joint [5]

The wearing and degradation of this articular cartilage can lead to a joint degenerative disease termed as osteoarthritis. Since the articular cartilage has no vascular supply, the self-repair of the articular cartilage is highly limited. Figure 3 shows a normal and an arthritic hip joint. As can be seen from the figure, the degradation of the articular cartilage leads to bone on bone contact causing friction, pain, inflammation compromising the functions of articular cartilage which leads to loss of mobility in advanced stages of osteoarthritis [6].

The drugs used in the treatment of osteoarthritis only help in the alleviation of the symptoms such as pain and inflammation. However, the lost cartilage cannot be recovered which leads research to explore ways to restore normal function of the joints.

1.3 Treatment of osteoarthritis

In the advanced stages of hip osteoarthritis where the patient has limited or loss of mobility, the treatment involves replacement of the diseased hip joint with an orthopedic implant which can restore the function of the joint and eliminate pain (Figure 4). The clinical procedure is known as total hip replacement.

Orthopedic implants are devices made of biomaterials that are able to co-exist with living issues and restore the normal functions in the human body. The basic requirement of a biomaterial is to stay in the body without triggering adverse immune reaction [7]. However a biomaterial has to fulfil various other needs based on the application, to make it a suitable choice.

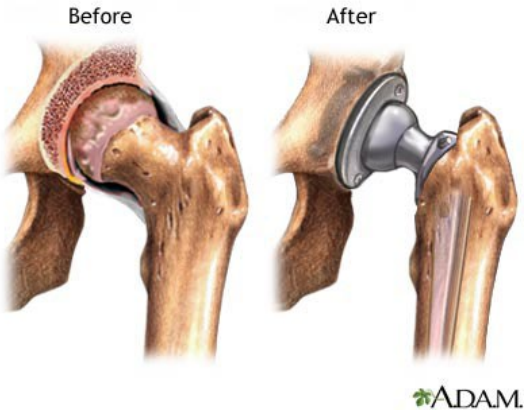

Figure 4 Diseased cartilage and cartilage replaced with implant [8]

The orthopedic implant used in total hip replacement procedure consists of a femoral component (femoral head and stem) and an acetabular component (acetabular cup) (Figure 4). In total hip replacement, the basic procedure is to cut off the femoral head and reshape the femur to accept the femoral stem of the implant and to insert the acetabular cup into the acetabular socket of the hip joint (Figure 5). The implant thus replaces the articular cartilage and helps in the smooth articulation of the hip joint [9].

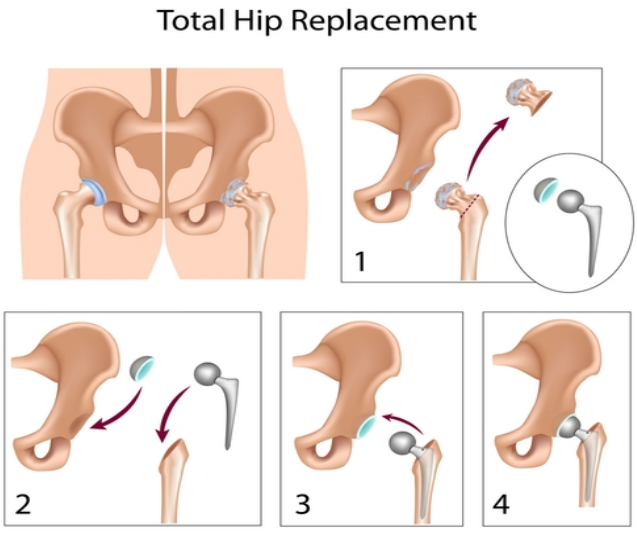

Figure 5 Total hip replacement procedure [10]

1.3.1 Implant fixation

Once the implant is inserted in the bone, the main aspect to be addressed is good fixation of the implant to the bone which is crucial for the success of total hip replacement procedure. Currently, there are two methods used to secure the implant to the bone known as cemented and cementless fixation.

1.3.1.1 Cemented Implants

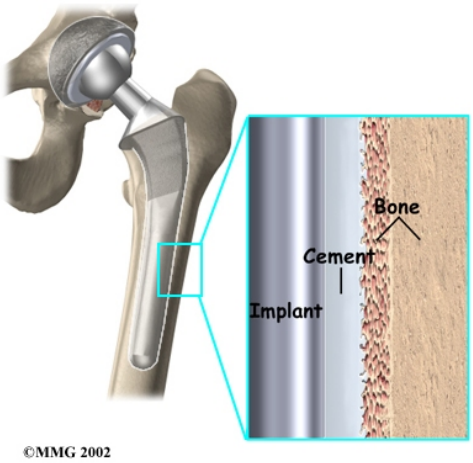

Figure 6 Cemented hip implants [11]

In a cemented procedure, bone cement (polymethylmethacrylate) is used as a fixative which forms a mantle between the acetabular cup and the acetabulum, as well as the femoral stem and the femur, providing stability of the prosthesis (Figure 6). Even though cemented fixation

is a well-established procedure, it is known to result in aseptic loosening and infection associated with the bacteria trapped in the cement [12].

1.3.1.2 Cementless Implants

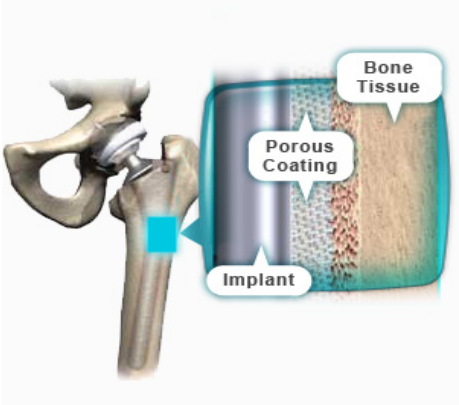

Figure 7 Cementless hip implants [13]

A more recent development introduced in the field of orthopedic implants is cementless or un-cemented fixation between the implant and surrounding bone. This method relies on interactions between the surface of the implant and the surrounding bone leading to osseointegration. The process is often assisted by the use of a coating made of the bone apatite (calcium and phosphorous) in order to make the surface osteoconductive [14].

1.3.2 Implant failure

Orthopedic implants have tremendous impact on the quality of human life over the recent years owing to the ageing population and active lifestyle. In United States alone about 1 million hip and knee replacements procedures are performed yearly and the number of annual total hip and knee replacements in Netherlands is around 50,000 [15].

Poor implant fixation leading to subsequent loosening of the implants is an important cause for the failure of implants besides failure related to infection, design error, inappropriate material choice and fabrication [12,16]. Once an implant fails the patient has to undergo a revision surgery which not only affects the life quality of the patient but also imposes financial concerns.

Hence there is an immense need for research to be focused on enhanced fixation which is required for the long term success of the hip implants.

1.4 **Osseointegration**

Osseointegration is a natural fixation of the implant to bone which forms the basis of cementless fixation. In the total hip replacement procedure, the femoral head is cut off and a hole is drilled in the femur. This is followed by insertion of the femoral component of the implant into the drilled hole in the femur.

Once the implant is inserted in the bone there is mechanical interlocking between the implant surface and bone which provides the primary stability of the implant. Simultaneously, the drilling tears open the blood vessels in the bone releasing blood in the surrounding area. This attracts various inflammatory cells and also, the proteins from the surrounding region are adsorbed on to the implant surface which directs the migration of osteoblasts toward surface. This leads to further strengthening of the primary fixation by a finely tuned communication of osteoblasts with the implant surface resulting in the secretion of new bone mineral matrix which bonds the implant to bone. Hence, cell-material interactions represent an important stage of osseointegration that leads to stable fixation of implants.

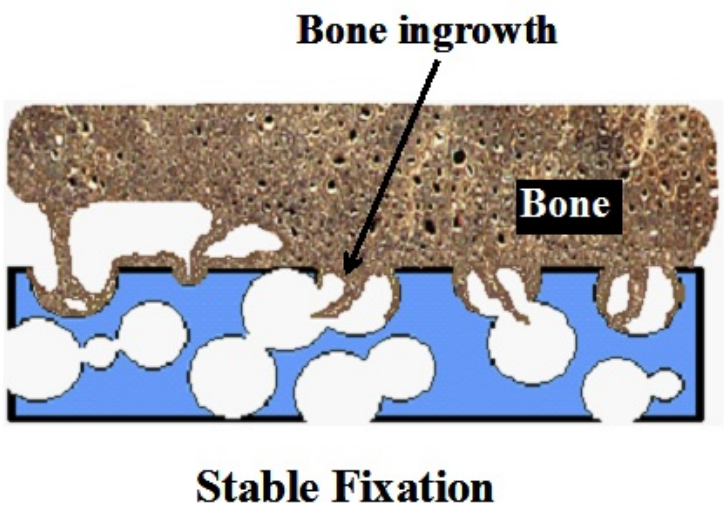

Figure 8 Fixation of orthopedic implants by osseointegration [17]

Once the cells migrate at the implant surface they should perform a series of functions that lead to bone formation. The main cell-surface interactions are presented in Figure 9.

At first, the cells adhere to the implant surface. This process is facilitated by various proteins such as vinculin, fibronectin adsorbed on the surface of implant that act as focal points for

cells adhesion. Once the cells anchor themselves on the surface of the implant they start migrating and proliferating on the surface. During proliferation, several extracellular matrix proteins such as procollagen 1, TGF-β and fibronectin are secreted by the cells [18].

Later, the cells commit themselves to a particular lineage, in this case as osteoblasts. This process is known as osteogenic differentiation where the mature cells get transformed into osteoblasts (bone forming cells). Differentiation of cells into osteoblasts is mainly characterised by the maximal expression of alkaline phosphatase marker by the cells [19].

Finally, the osteoblasts deposit the mineral matrix on the implant surface, a process termed as mineralization which bonds the bone to the implant surface. There are various proteins such as bonesialoprotein, osteopontin, osteocalcin that mediate differentiation and subsequent mineralization [19].

However, the process is not as simple as it seems since the implant is a metal surface unlike the bone. Hence, for the cells to secrete bone matrix on the implant, the surface should favour the above mentioned interactions. Therefore, the implant surface requires modification such that they allow the osteoblast cells to attach and produce bone (osteoconductive) or even better, induce osteogenic differentiation of stem cells leading then to matrix deposition (osteoinductive). Though osteoinductive surface is the ideal surface that we seek for enhanced osseointegration, making the surface osteoconductive has been so far the basic requirement for surfaces used in THRs.

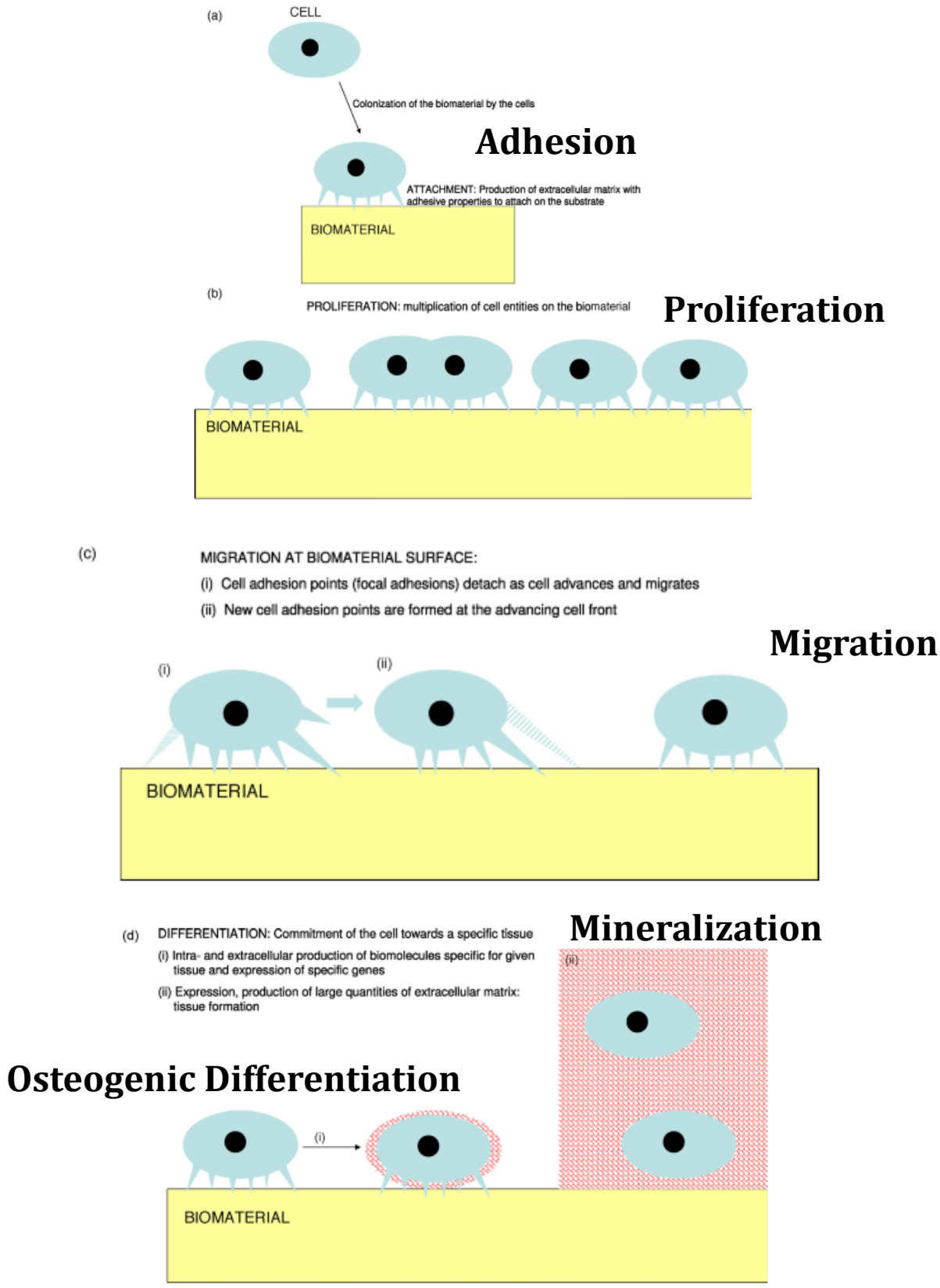

Figure 9 Initial stages of osteoblasts interaction on biomaterial [18]

1.5 Surface modification of titanium for osseointegration

The choice of material is of crucial importance for orthopedic applications. The biomaterial used for hip implants should possess characteristics that make it suitable for load bearing applications, due to which metals are the preferred choice.

Titanium stands out as the most widely used metal for orthopedic applications owing to its excellent corrosion resistance and relatively optimal Young's modulus [20]. However when titanium is exposed to air, a native oxide layer of few nanometer thickness is formed on the surface. This native oxide layer of titanium enhances the corrosion resistance of this metal but also makes the surface bio inert (less reactive to cells). Further, to ensure primary fixation, a rough/ porous surface is needed. Therefore, titanium requires surface modification for osseointegration.

There are various surface modification techniques such as sand-blasting, acid-etching, plasma spraying and plasma electrolytic oxidation that can be used to modify the surface chemistry and topography of the implant material [21].

Plasma spraying of hydroxyapatite is the most widely employed technique in clinical applications for surface modification of cementless implants. This method is based on thermal spraying where hydroxyapatite based particles are sprayed onto the surface of the implant material using a plasma jet at very high temperature. This forms a micro-scale level osteoconductive coating on the implant surface which promotes bone healing and accelerates osseointegration [20].

Despite the impressive clinical success of plasma spraying as a well-established method to promote osseointegration, it is known to be presented with weak bonding of the ceramic coating to the metal substrate causing coating delamination. Moreover, the high process temperature and rapid solidification process lead to uncontrolled changes in the chemical structure of the hydroxyapatite coating [20]. Therefore, there is a clinical concern over the long-term stability of implants coated by this method.

Plasma electrolytic oxidation (PEO) is a relatively new surface modification technique that is used to enhance the surface oxide properties of titanium by creating a porous, bioactive oxide layer that could be enticing to osteoblasts compared to surfaces modified by other techniques. Unlike plasma spraying, PEO converts the surface of titanium into a thick oxide layer with

additional surface features (topography and chemistry) that have the potential to promote osseointegration [22].

1.6 Plasma electrolytic oxidation process

During the PEO process, a high current /voltage is applied to the titanium sample made anode in an electrolytic bath (Figure 10). At a certain voltage (breakdown voltage), the oxide layer breaks open generating microdischarges. The generation of microdischarges is explained by various effects such as electron avalanches, local heating and stress generation [23].

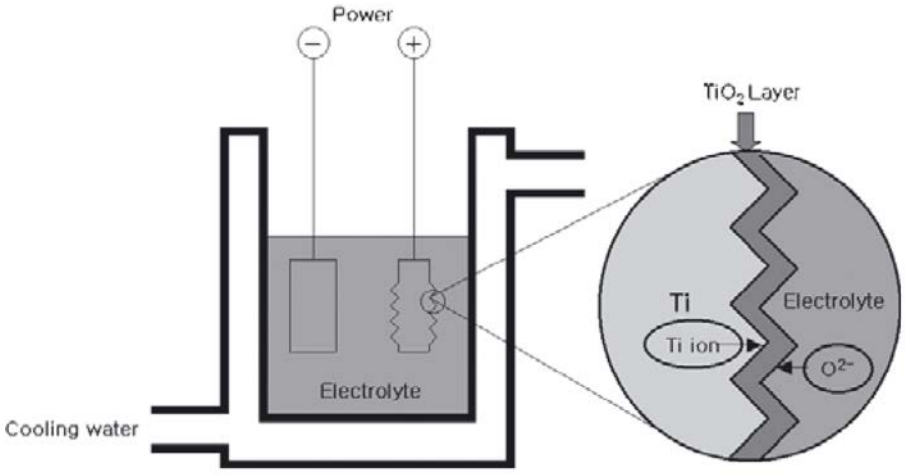

Figure 10 Schematic of PEO process [24]

The growth of the layer in the presence of microdischarges leads to formation of a porous oxide layer with the components of the electrolyte being incorporated in it (Figure 11). If Ca/P based electrolytes are used, there is potential to make the oxide layer osteoconductive. Depending on the applied current/voltage, electrolyte and oxidation time, the surface features (topography and chemistry) of the oxide layer can be changed. A typical PEO surface morphology is presented in Figure 11.

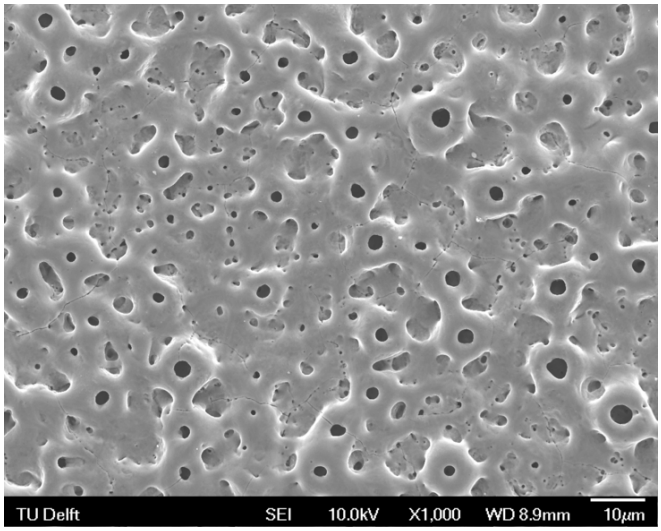

Figure 11 Typical morphology of a porous titanium oxide layer produced by plasma electrolytic oxidation [25]

PEO has certain advantages over plasma sprayed hydroxyapatite such as: increased adhesion strength of the oxide layer, (eliminating the need for pre-treatments such as grit blasting to increase the surface roughness [20]), possibility to change chemistry of the oxide layer by using various electrolytes, generation of unique microporous topographies with potential for novel biofunctionalities. These surfaces are therefore under intense research focus for biomedical applications [26].

1.7 Relevant surface features for cell response

The prime question that drives most research in this field is, how changes in biomaterial surface features can influence cell response and what is the ideal surface for certain application / cell type?

Biomaterial surface features can be broadly classified into surface topography (e.g., surface roughness, porosity, pore size) and surface chemistry (e.g., elemental chemical composition especially Ca/P ratio, phase composition and crystal structure). There are other surface features including wettability and surface energy that are influenced by both topography and chemistry.

Over the past decade, various studies have attempted to address the above questions by understanding behaviour of cells to surfaces with varying features. The results of these studies have highlighted two possible theories related to topography and chemistry mediated

cell response. In the next paragraphs, these are briefly presented in the case of bone applications.

1.7.1 Surface topography mediated cell response

During bone remodelling, the osteoclasts resorb certain areas of bone by acidification and proteinase activity. The irregular acid etching of these osteoclasts creates micro pits with sub-micro scale roughness. The osteoblasts deposit osteoid on these pits created by the osteoclasts [27]. Furthermore, the cell membrane receptors, protein size and features of collagen fibrils are of the nano scale [27]. Surface modification of metallic implants aims to achieve enhanced cell material interaction to promote osseointegration by mimicking this complex hierarchical structural features of the bone. Figure 12 shows the interactions between bone and implant surface at different topographical scales.

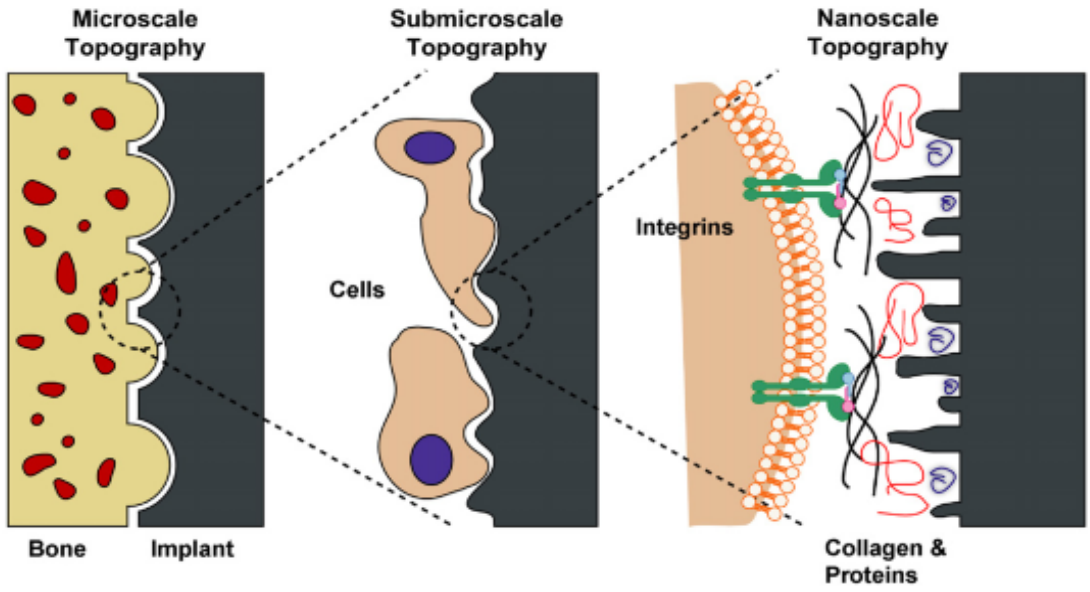

Figure 12 Schematic of the interaction between bones and implant surface at different topographical scales [27]

Cells are highly sensitive to the surface they come in contact with. Therefore, implant surface with sub-micron scale roughness are easily sensed by osteoblasts [28]. This may lead to enhanced attachment of osteoblasts to these surfaces. Furthermore, the pores on the surface may allow for the cells to anchor themselves [25] and also the pores adsorb protein from surrounding regions that can act as ligands for focal adhesion of cells [29].

1.7.2 Surface calcium mediated cell response

Since calcium phosphate is the bone material involved in mineralization, coating an implant with a thin layer of hydroxyapatite or calcium based coating is considered beneficial being used as a most effective way to render the surface of the implant bioactive (i.e., osteoconductive).

The presence of calcium and phosphate in the coating may trigger chemotaxis and drive osteoprogenitor cells to the surface [Figure 13]. Hence, these bioactive calcium phosphate coatings offer an initial support for the natural healing process until the natural osseointegration process fully occurs [30].

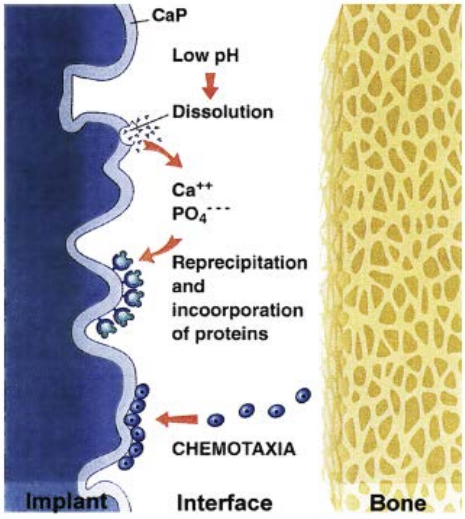

Figure 13 Bioactive coating mediated osseointegration [30]

Though several studies are being performed in this area to understand surface feature mediated cell response, variations in results between studies limits the possibility to attribute the cell response to a particular surface feature. For instance, in the case of PEO surfaces Mohedano *et al*, [31] showed that the surface with high roughness (Ra 1.63 µm) in their study had less proliferation of MC3T3 cells; on the other hand, in another study [32] it was observed that SaOS2 cells proliferated better on surface that had increased roughness (0.8 µm). These variations are not only due to different experimental conditions and type of surface/cells used but mainly due to insufficient assessment of surface characteristics and cell response. It has been observed from the literature that very few studies have looked into mineralization of cells on PEO surfaces which is a key cellular function confirming

osteogenic differentiation and proving the ability of adhered cells to produce bone tissue. Hence, in order to gain an understanding of surface feature mediated cell response, detailed investigation of the most important surface features and cell functions is crucial.

Such a study has been recently performed at TU Delft in cooperation with Erasmus MC [25]. Two different PEO surfaces were produced by varying oxidation time from 1 to 5 minutes. The response of pre-osteoblast cells was assessed over 21 days. The surface oxidised for 1 minute showed smaller pore size, lower roughness and increased porosity whereas the surface oxidised for 5 minutes had larger pore size with less porosity and enhanced roughness. The 5 minutes surface also had higher Ca/P ratio compared to 1 minute surface.

Both PEO surfaces assisted cells adhesion, matrix synthesis and mineralization, as revealed by imaging and staining assays. However, the surface oxidised for 1 minute enhanced cells attachment and spreading as well as matrix mineralization.

Since both, topography and chemistry changed by extending the oxidation time, it was not possible to delineate the effects of these surface features on cells response. In addition, although qualitative analysis gives a broad understanding of cell response in different conditions, quantification of cell response is needed as it would give clear data to compare cells response to various surfaces and also reveal the extent to which variations in surface features could impact cell response.

1.7.3 Goal of the research

The goals of this MSc study have been formulated as follows:

1. Quantification of pre-osteoblasts response to PEO treated titanium surfaces
2. Delineation of the effects of PEO topography and surface calcium on the *in vitro* pre-osteoblasts response.

According to the goals set, the research has been performed in 2 stages (Figure 14).

Stage 1 focused on quantifying cells response to PEO treated surfaces. Therefore, the previous PEO surfaces produced at 1 and 5 minutes in a Ca/P based electrolyte have been considered (PEO1 and PEO5). Pre-osteoblasts and cell attachment, proliferation, growth, differentiation and matrix mineralization were quantified using suitable assays.

Stage 2 involved delineation of the effects of PEO topography and surface calcium on cells response. Therefore PEO1 and PEO5 surfaces have been considered together with a new PEO surface with controlled topography but without calcium.

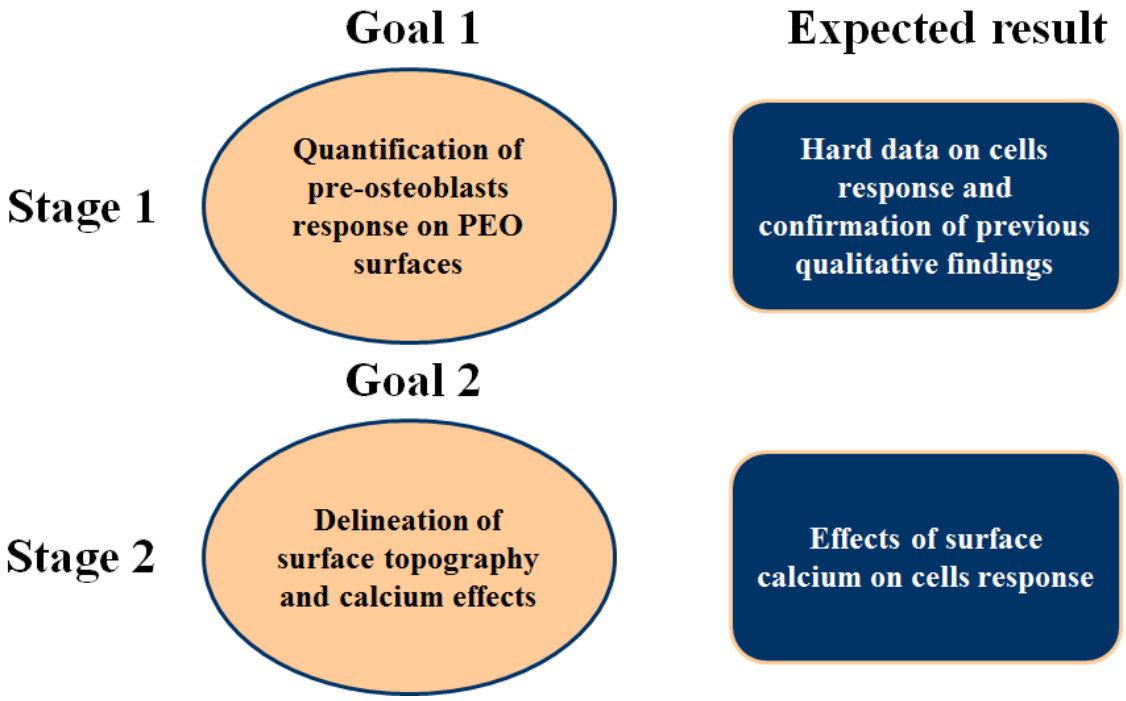

Figure 14 Research scheme

Chapter 2

2 Materials and Methods

All chemicals were purchased from Sigma Aldrich, unless specifically mentioned.

2.1 Sample preparation and plasma electrolytic oxidation (PEO) process

2.1.1 Sample preparation for oxidation

Disks made of Ti6Al7Nb alloy with a diameter of 21mm and a thickness of 7.5mm was used as substrates. The disks were ground with 320, 800 and 1200 grit paper on front, back as well as on the sides. Before oxidation, all samples were cleaned with 99% acetone (5 min), 96% ethanol (5 min) and deionized water (5 min) in an ultrasonic bath. In this research, Ti6Al7Nb samples have been used reused after removal of the oxide layers. In order to remove the PEO layers and recover the substrates, the oxidized samples were immersed in sulfuric acid (99 % H_2SO_4) at 90 °C for about 1-1.5 hours in an ultrasonic bath. After the layer has been removed, the samples were gently transferred to another beaker and rinsed under running tap water and placed in ultrasonic bath for 10 minutes. Thereafter, they were ground and cleaned as described above.

2.1.2 Synthesis of PEO1 and PEO5 surfaces

The PEO treatment was carried out in a double wall glass electrolytic cell (Figure 15) in an electrolyte containing 0.02M calcium acetate and 0.15M calcium glycerophosphate. The samples were screwed to an insulated metallic rod and suspended in the centre of the electrolytic cell as anode, surrounded by a cylindrical stainless steel cathode. A temperature of 10°C ± 2°C was maintained by an external bath and the electrolyte was continuously stirred by a magnetic stirrer at 500 r.p.m throughout the oxidation process. The PEO process was performed under galvanostatic conditions using a current density of 20 Adm^{-2} for 1 and 5 min. The current was applied by using an AC power supply type ACS 1500 (ET power systems Ltd., UK). The 1 min sample will be referred to as **PEO1** and the 5 min sample will be referred as **PEO5**.

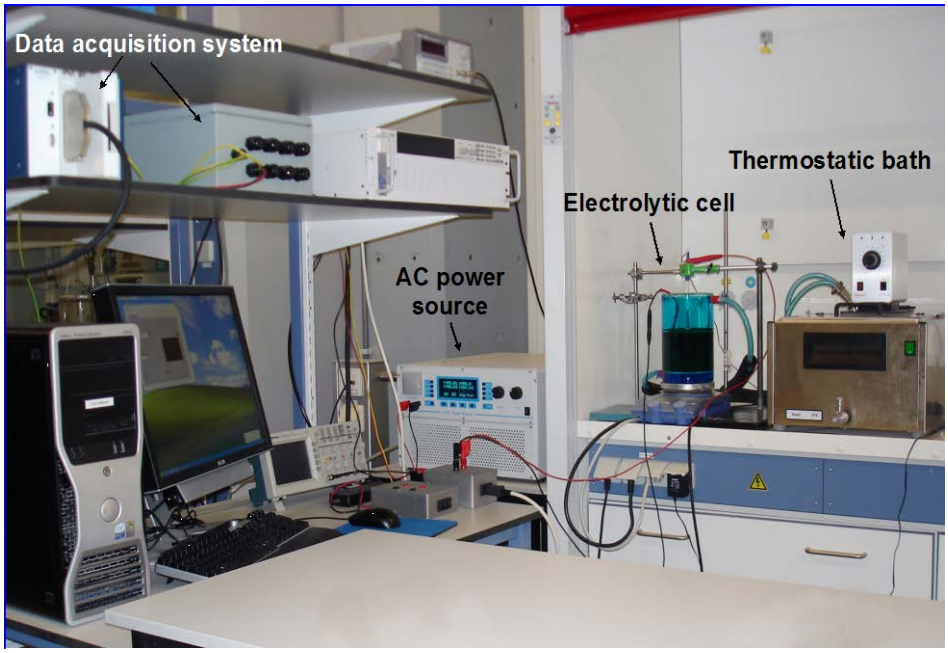

Figure 15 PEO set up used for preparing PEO1 and PEO5 surface

2.1.3 Synthesis of PEO surface with no calcium

The PEO surface without calcium was produced in an electrolyte containing 1M phosphoric acid. The oxidation process was carried out in a glass beaker (Figure 16) containing the electrolyte and the sample screwed to the insulated rod as anode. A platinum counter electrode was placed facing the sample at the same height. A magnetic stirrer with 500 r.p.m. was used to stir the electrolyte throughout the process. The PEO process was performed under galvanostatic conditions using a current density of 1.1 Adm^{-2} for 7 min at room temperature. The current was applied by using AC power supply type ACS 1500 (ET power systems Ltd., UK). The samples will be referred to as **PEO-Ca** surface.

2.1.4 Cleaning and sterilization

After oxidation, the samples were rinsed in running tap water for 5 min, followed by ultrasonic cleaning in 70% ethanol (30 sec), rinsing in deionized water (5min) and ultrasonic cleaning in deionized water (30 sec). Thereafter, the samples were sterilized for 1 hour at 110ºC using a Nabertherm oven.

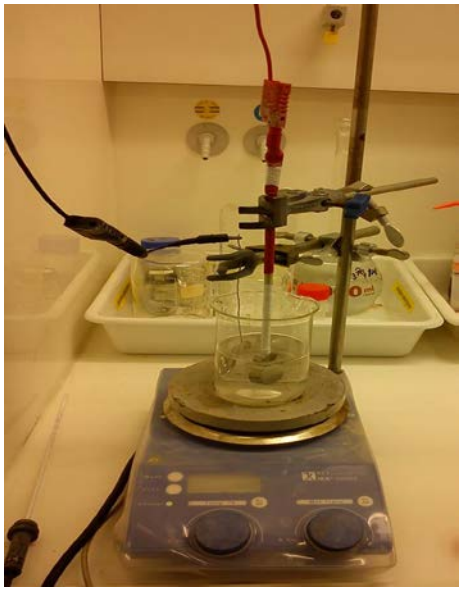

Figure 16 PEO set up used for preparing PEO-Ca surface

2.2 Surface characterization

The surface characterization was performed to analyze the surface morphology and chemical composition of PEO-Ca surface in this research. PEO1 and PEO5 surface characteristics were used from the previous study [25].

2.2.1 Topography

The surface topography was examined by Scanning Electron Microscopy (SEM) on a JEOL JSM-6500F microscope interfaced to a computer. The electrical conductivity on the surface was realized by coating the surfaces with a uniform carbon layer before imaging, using an Auto Carbon Coater type JEC-530. Images were taken at different magnifications for each sample using an accelerated voltage of 10kV and a working distance between 8-10 mm.

2.2.2 Surface roughness

The roughness of the surface was measured by Micro Surface Profilometrey (MSP), using a SURTRONIC3+ Surface Texture meter (Taylor/Hobson, UK). Ten measurements were taken for each sample using a cut-off length (Lc) of 0.6mm and an evaluation length (Ln) of 4mm. The average roughness (Ra) and the maximum peak-to-valley height (Ry) were measured.

2.2.3 Surface porosity

The surface porosity was determined by analyzing the SEM images of the surface at 2000x magnification in Adobe Photoshop CS 5. The open pores were selected using the software.

Thereafter, the pore size and area of the selected pores were measured. The pore size was based on the largest length of the pores. Based on these measurements the porosity was calculated using the formula below.

$$\% \text{ Porosity} = \frac{\text{Total area occupied by pores}}{\text{Total area of image}} \times 100$$

2.2.4 Elemental composition

The chemical characterization of the surface was done by measuring the elemental composition by using Energy Dispersive Spectroscopy (EDS) in combination with SEM. Therefore, a Noran System Six (NSS) software from SEM images taken at 3,000X magnification were used. The composition was measured at 3 different locations on the sample.

2.3 *In vitro* response of pre-osteoblasts to PEO surfaces

Simian Virus 40-Human Fetal Osteoblasts (SV-HFO) cells were used in this research. SV-HFO cells are human pre-osteoblasts that have been well-characterized and are known to differentiate and mineralize in 3 weeks span [19].

2.3.1 Cells pre-culturing

The SV-HFO cells were pre-cultured for a week in α-Minimum Essential Medium (α-MEM), without phenol red, supplemented with 20mM HEPES, 2% streptomycin/penicillin, 1.8 mM $CaCl_2$ and 2% Charcoal Treated Fetal Calve Serum (CT-FCS) with a pH of 7.5. The cells were incubated at 37°C in 5% CO_2. After pre-culturing, the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS), detached with trypsin and counted with a haemocytometer.

2.3.2 Cells seeding

The oxidized samples were placed in 12-well plates (Costar, Sigma Aldrich) and 900µl α-Minimum Essential Medium (α-MEM), without phenol red, supplemented with 20mM HEPES, 2% streptomycin/penicillin, 1.8 mM $CaCl_2$ and 2% Charcoal Treated Fetal Calve Serum (CT-FCS) were added. The cells were seeded on the oxidized samples and also on the polystyrene as positive control (PC) at a concentration of 35,000 cells/well.

The cell culture medium was refreshed on alternate days. 1µM dexamethasone and 10mM β-glycerophosphate were added to the medium starting from day 2 to induce differentiation of SV-HFO cells. Triplicates were used for each experiment.

2.3.3 Cells harvesting

Cells were harvested to perform analysis on the cell lysates. Every step was done on ice. At first the medium was collected. Thereafter, every well was rinsed with 900µl of 1x PBS (Phosphate Buffer Saline). To every well 250 µl of PBS-Triton X-100 was added. Because of this Triton, which is a soapy fluid, cells start to lyse. Cells were then scraped with a spatula and the cell lysate was collected in tubes. Hereafter, wells were rinsed again with 250µl of PBS-Triton and this was also collected in the same tube. Cell lysates were stored in -80° C and medium in -20° C. Before every biochemical assay cells were sonicated. Depending on extracellular matrix size, samples were sonicated for 10-15 seconds at 10-15 micro amps.

2.4 Characterization of SV-HFO cells response

2.4.1 Cells attachment

The attachment of cells to the PEO surfaces and positive control was assessed by counting the cell nuclei at preselected time points following staining with DAPI.

At 5 hours and 24 hours of incubation the medium was removed from the samples and they were washed with PBS. The cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature followed by PBS wash thrice. They were then permeabilized by incubation with PBS containing 0.15% Triton X-100 for 10 minutes at room temperature. Three consecutive washes with PBS were performed followed by dehydration with ethanol: 70% ethanol (1 min, RT) and 100 % ethanol (1 min, RT). The cell nuclei were stained with 1:50000 DAPI solutions in PBS (2 min, RT) after which the cells were left to air dry in the dark. The fluorescence microscopy was performed on a microscope at 10 x magnification by taking 8 images of the stained nuclei at different areas of each sample. Triplicates were used for each surface. The number of nuclei was counted manually and expressed in number of nuclei/cm^2.

2.4.2 Cells proliferation

The proliferation of cells was assessed by analysis at day 2, 5, 7 and 9. The medium in each well was discarded and each well was washed with 700µl PBS. Next, 100µl of trypsin was

added to each well and the plate was incubated at 37°C in 5% CO_2 for about 5 minutes. Then the plate was removed from the incubator and 300µl of α-Minimum Essential Medium (α-MEM) with 10% Fetal Calf Serum (FCS) was added in each well to stop the lysation. The cells were then collected in 1.5 ml eppendorfs. The cells lysates were centrifuged at 300 RPS for 5 minutes at 4°C and supernatant was removed. The cells were fixed by adding 500µl of 70% EtOH drop-wise to the pellets and were gently mixed. The samples were then placed in the -20ºC freezer until the analysis was done.

During analysis the cells were centrifuged at 300 RPS for 5 minutes at 4°C and the supernatant was removed. Each pellet was dissolved in 115µl of PBS+1% BSA + 5µl of Ki-67 antibody. The samples were incubated at room temperature in the dark for 30 minutes. After incubation 500µl of PBS+1% BSA were added and the samples were centrifuged at 300 RPS for 5 minutes at 4°C. The supernatant was gently removed with a pipette and each pellet was dissolved in 100µl of PBS+1% BSA. The samples were then vortexed and analysed by fluorescence activated cell sorting (FACS). The results are expressed in % proliferative cells.

2.4.3 DNA quantification

The DNA measurement was done to get an indication of cells growth. Therefore, 50µl of SV-HFO cell lysates were treated with 100µl of heparin solution (5000IU/ml in PBS) and 50µl of ribonuclease A (5mg/ml in PBS) for 30 min at 37°C. Heparin was added to remove proteins and RNase was added to remove RNA, so only DNA was left in every sample. This was followed by adding 50µl of ethidium bromide solution (5mg/ml in PBS) to stain DNA. Ethidium bromide intercalates with base pairs in the double helix. Absorbance was measured with a plate reader at an excitation of 340 nm and an emission of 590 nm. The standards were prepared by using DNA stock (0.025mg/ml in PBS triton). The DNA is expressed as $\mu g/cm^2$.

2.4.4 Differentiation of pre-osteoblasts

Alkaline phosphatase (ALP) is an enzyme on the membrane of a cell and is a marker for the differentiation of osteoblasts. A high ALP activity means that there is high osteogenic differentiation. In this assay para-nitrophenylphosphate (PNPP) was converted to para-nitrophenol and phosphate under the influence of ALP. Para-nitrophenol has a yellow colour.

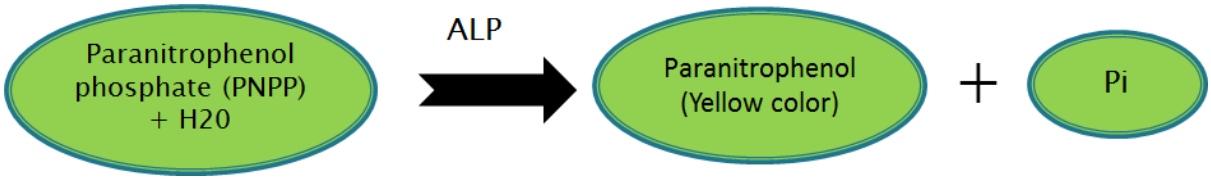

Figure 17 ALP assay

ALP activity was determined by measuring the release of paranitrophenol from paranitrophenylphosphate (20mM in 1M diethanolamine buffer supplemented with 1mM $MgCl_2$ at pH 9.8) in SV-HFO cell lysates for 10 min at 378° C. 0.06M NaOH was added to stop the reaction and the absorbance was measured at 405nm on a plate reader. The ALP is expressed as mU/cm^2.

2.4.5 Extracellular matrix (ECM) mineralization by osteoblasts

The extracellular mineral matrix deposited by the osteoblasts was characterized by measuring the calcium content in cell lysates, calcium in medium and also by Alizarin Red staining of cultures at predetermined time points.

2.4.5.1 Calcium in cell lysates

This assay is based on the cresolphthalein complexone method. Cresolphthalein complexone reacts with calcium and magnesium in alkaline environment to form an intense violet colored complex which maximally absorbs at 577 nm. 8-hydroxyquinoline is incorporated into this reagent to preferentially bind with magnesium and prevent interference from this cation. The resulting increase in the absorption of the reaction mixture is directly proportional to calcium concentration in the sample.

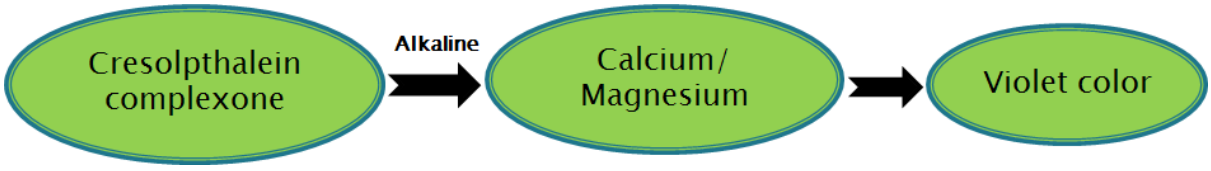

Figure 18 Calcium from cell lysates: principle of the assay

Therefore, the cell lysates were placed overnight in 0.24M HCl at 48°C. Calcium content was colorimetrically determined after addition of 1M ethanolamine buffer (pH 10.6), 0.35 mM – cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline and 0.6 M hydrochloric acid at 595

nm. Calcium in the cell lysates was measured at day 16, 21 and 27. The calcium content is expressed in nM/cm^2.

2.4.5.2 Calcium in medium

The calcium in medium give secondary information about the matrix mineralization by osteoblasts as cells take up calcium from the medium and deposit it as mineral matrix. Therefore the lesser the calcium in the medium removed from cells at each time point relative to the medium used to refresh cells (control), the more calcium has been used by cells to deposit mineral matrix.

The medium removed from cells was treated with 1M ethanolamine buffer (pH 10.6), 0.35 mM –cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline and 0.6 M hydrochloric acid and the calcium was measured colorimetrically at 595 nm on a plate reader. Calcium in the medium was measured at day 16, 21 and 27. Calcium concentration in medium is expressed in mM.

2.4.5.3 Alizarin Red staining

Alizarin Red staining is done to determine the presence of calcium deposition by cells of an osteogenic lineage. As such it is an early stage marker (day 10–16 of *in vitro* culture) of matrix mineralization, a crucial step towards the formation of calcified extracellular matrix associated with true bone. Alizarin Red is an anthraquinone dye which readily forms a complex with calcium during a process of chelation resulting in a birefringent end product.

Alizarin Red staining was performed on PC and PEO1 surface at day 16 and 21 and on PC, PEO1 and PEO5 at day 27. The Alizarin Red staining was done by washing the cells with PBS followed by fixing with 70 % ethanol for at least 60 minutes in 4°C. The cells were then stained with Alizarin Red solution for 5 to 10 minutes and carefully washed three times with distilled water. Photos of the stained samples were taken.

2.4.6 Statistics

All values are presented as average ± the standard deviation. Statistical analysis was performed using Student's t-test ($P<0.05$).

Chapter 3

3 Results and Discussion

3.1 *In vitro* SV-HFO response to PEO1 and PEO5 surfaces: A quantitative study

From the previous study [25] it has been qualitatively observed that variations in surface features of PEO treated titanium surfaces (PEO1 and PEO5) have an influence on SV-HFO response.

- Therefore, quantification of SV-HFO response was the next needed step in order to better compare and further understand the effects of these PEO surfaces on these cells.

The main cellular functions assessed included: attachment, proliferation, growth, differentiation and matrix mineralization. The quantification has been performed using relevant biological assays and culture time, as described in the Materials and Methods section.

3.1.1 Attachment of SV-HFO cells to PEO surfaces

The attachment of pre-osteoblasts quantified by counting the nuclei at 5 and 24 hours after seeding of SV-HFO cells on PEO1, PEO5 surface and on PC is presented in Figure 19.

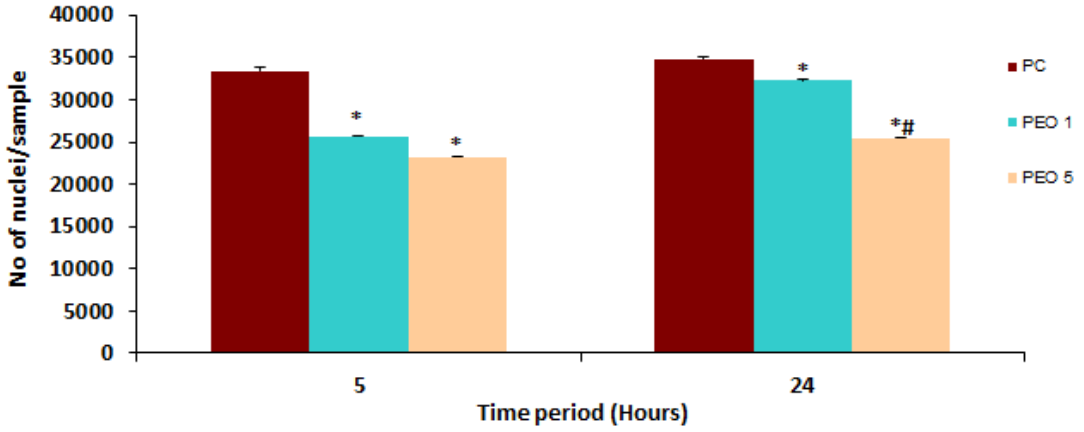

Figure 19 Number of nuclei/ sample at 5 hours and 24 hours on PEO1, PEO5 and PC; Statistical difference ($p<0.05$) from control samples are indicated by * and between samples is indicated by #

The number of nuclei of pre-osteoblasts on both PEO1 and PEO5 surfaces was significantly less than on the positive control at both time points. This indicates that attachment of cells on PEO surfaces is decreased relative to the PC. In addition, a significant difference was evidenced between the PEO surfaces after 24 hours suggesting that PEO1 surface favors SV-HFO adhesion relative to PEO5 surface.

3.1.2 Proliferation and growth of SV-HFO cells on PEO surfaces

The percentage of proliferative cells was quantified by Ki-67 marker expression of proliferating cells on PEO surfaces and PC up at days 2, 5, 7 and 9. It can be observed from the results (Figure 20) that the proliferation of cells on PEO1 surface was less than on the positive control after 2 days and 5 days but there was no significant difference at later time period (day 7 and 9). By comparison, the proliferation of cells on PEO5 surface was significantly less than on positive control over the entire period. However, there was no significant difference in proliferation between PEO1 and PEO5 surfaces.

The findings indicate a slower proliferation of pre-osteoblasts on PEO surfaces relative to PC, especially in the first 5 days. In addition, the trend suggests that cells on the PEO1 surface keep proliferating at a higher rate than on the PEO5 surface between 5 and 9 days.

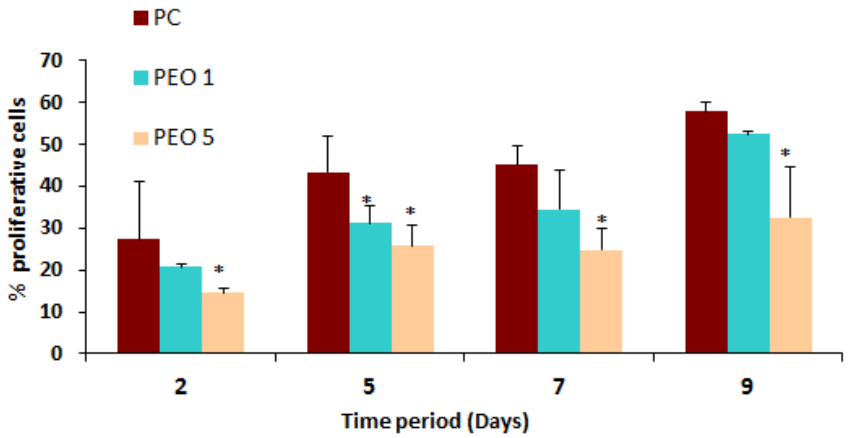

Figure 20 Proliferation of pre-osteoblasts on PEO1, PEO5 and PC at day 2, 5, 7 and 9; Statistical difference ($p<0.05$) from control samples are indicated by * and between samples is indicated by #

The DNA content was measured up to day 27 to assess the growth of pre-osteoblasts on PEO surfaces. As can be seen from the result (Figure 21) DNA on the PC is significantly higher than on PEO surfaces. This could be the consequence of enhanced attachment and proliferation of cells on PC.

However, there was a significant difference in cells growth between the two PEO surfaces up to day 9, with less DNA measured on PEO5 surface. At later time period, no significant difference was observed on both surfaces albeit the average DNA values were always higher for PEO1 surface.

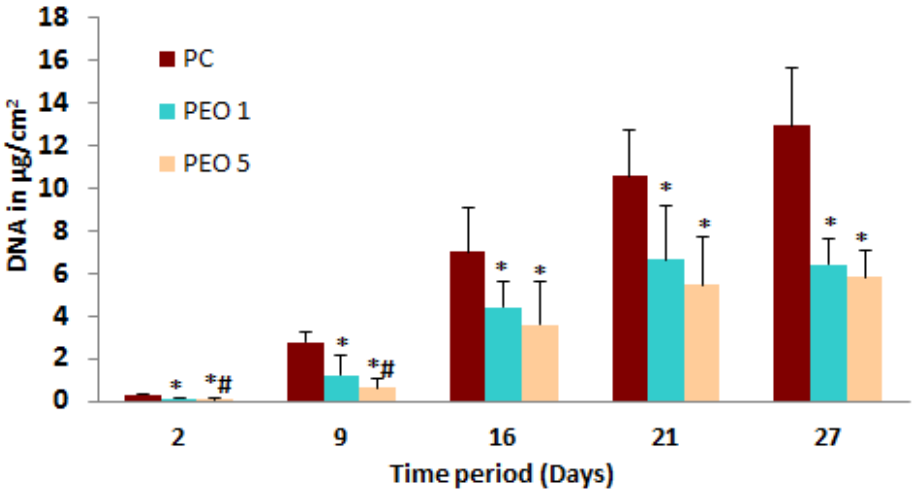

Figure 21 DNA content of pre-osteoblasts on PEO1, PEO5 and PC at day 2, 5, 7 and 9; Statistical difference ($p<0.05$) from control samples are indicated by * and between samples is indicated by #

3.1.3 Differentiation of SV-HFO cells on PEO surfaces

Differentiation of cells into osteoblasts is one of the most important cell functions that we need the cells to perform on the surface. Surfaces that have the capacity to promote osteogenic differentiation of cells are a prime requirement for orthopedic applications. The ALP activity of SV-HFO cells was quantified over 27 days to assess the differentiation of these cells on PEO surfaces relative to the PC. It can be seen from Figure 22 that ALP activity of cells on PC was higher than on PEO surfaces only at day 9, with no significant difference between the groups at later time period (i.e., day 16 and 21).

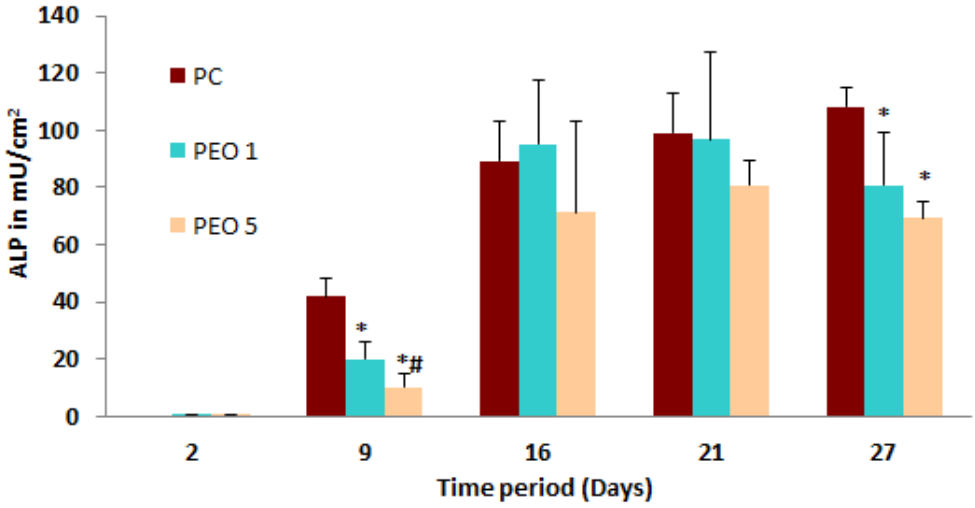

Figure 22 Differentiation of pre-osteoblasts on PEO1, PEO5 and PC at day 2, 5, 7 and 9; Statistical difference ($p<0.05$) from control samples are indicated by * and between samples is indicated by #

This indicates that even though there are fewer cells on PEO surfaces compared to PC, the cells express high differentiation ability on PEO surfaces. Thus, PEO surfaces have potential to enhance the osteogenic differentiation of cells. But it is interesting to note that the difference in surface features between PEO1 and PEO5 surface affects differentiation only at early time period (day 9) and similar to cell growth, the differentiation of cells is comparable on both surfaces at later time period. At day 27 it seems that differentiation slows down.

3.1.4 Matrix mineralization

The synthesis of mineral matrix by osteoblasts is the most crucial stage of cell-material interaction that assists in osseointegration. Yet, very limited studies in this field have presented data on mineralization. Assessment of matrix mineralization on these surfaces is therefore an important step taken in this study. Mineralization by osteoblasts was evidenced

qualitatively by Alizarin Red staining and quantitatively by measuring calcium content in the cell lysates and culture medium at various time points.

The results of Alizarin Red (Figure 23) show that even though mineralization on PEO1 surface was visible on day 16 and 21 as it did on PC, substantial amount of mineralization occurred only at later stages (between day 21 and 27).

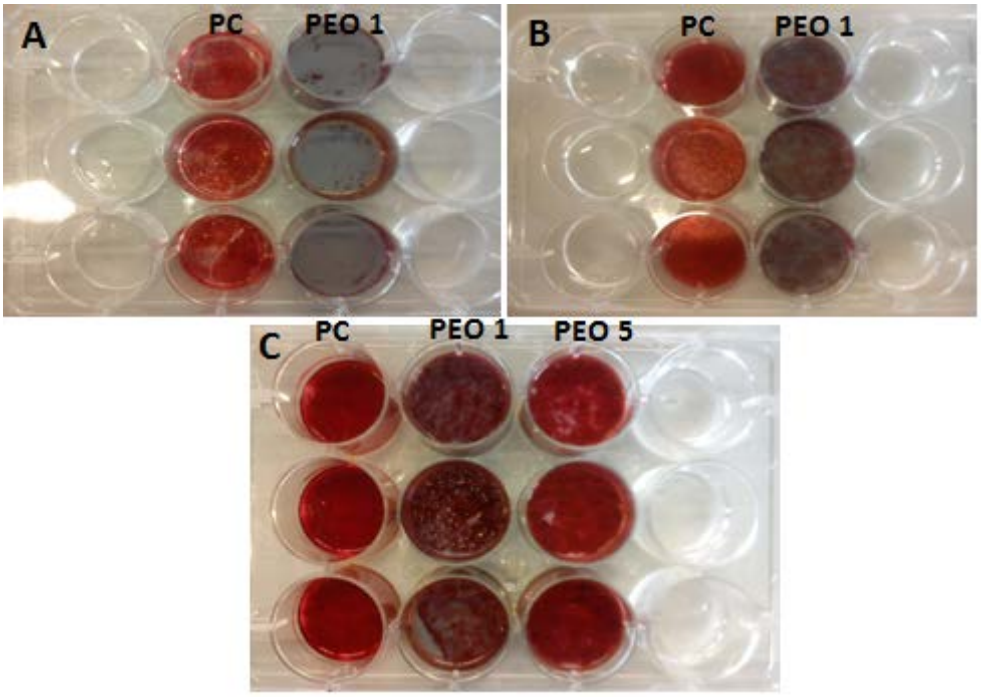

Figure 23 Alizarin Red staining of mineral matrix on PC and PEO1 at day 16 (A), day 21 (B) and on PC, PEO1 and PEO5 surfaces at day 27

Due to lack of data on PEO5 surface at early time points, no qualitative comparison was possible between the PEO surfaces.

The findings were confirmed by quantification of calcium content in cell lysates (Figure 24) which showed highest calcium on PEO surfaces at day 27. However, the mineralization on PEO surfaces was significantly less than on PC at all time points.

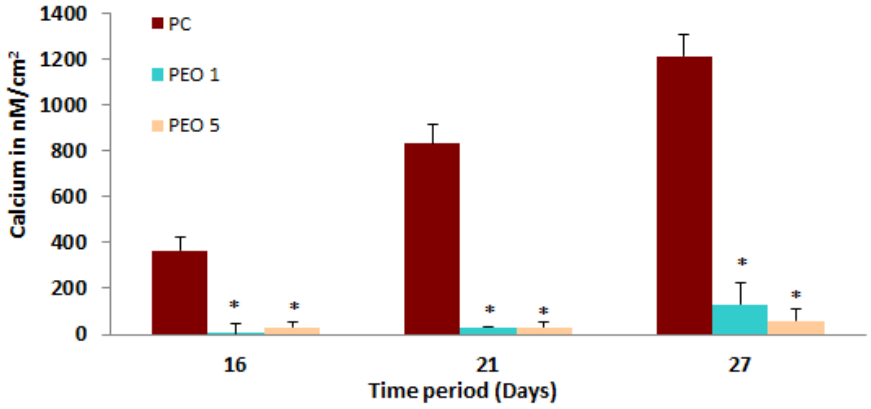

Figure 24 Mineralization of osteoblasts on PEO1, PEO5 and PC at day 16, 21 and 27 quantified by calcium in cell lysate ; Statistical difference ($p<0.05$) from control samples are indicated by * and between samples is indicated by #

This observation was further confirmed by quantifying the calcium drop in the culture medium which is an indirect measure of mineralization. As can be seen in Figure 25, strong mineralization occurred on PC already at day 16 but on PEO surfaces meaningful mineralization was evidenced by this assay only at day 27.

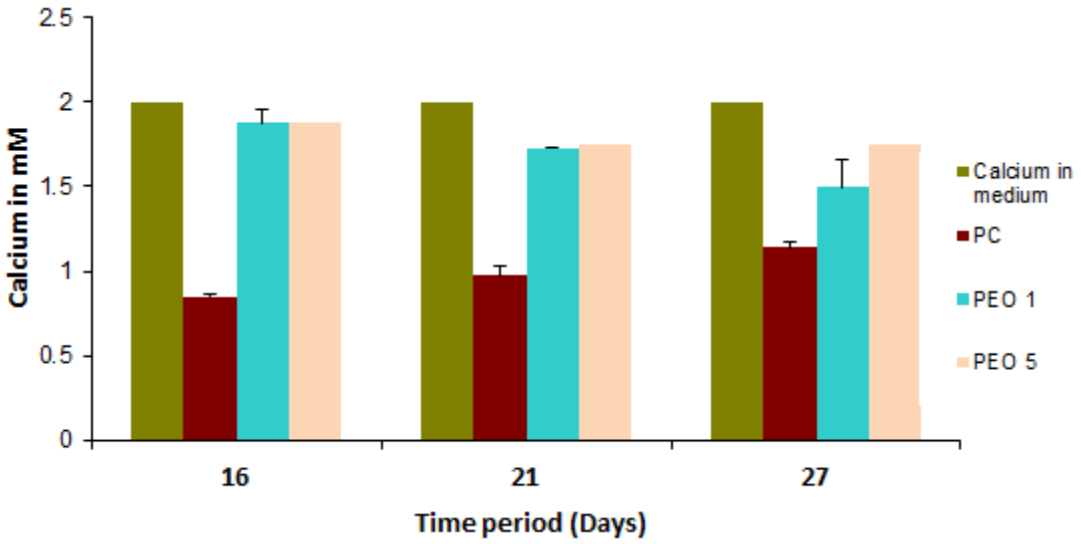

Figure 25 Mineralization of osteoblasts on PEO1, PEO5 and PC at day 16, 21 and 27 quantified by calcium drop in the culture medium; Control represents the calcium content in the medium used throughout the experiment to refresh cells.

Furthermore, there was no significant difference observed in mineralization between the PEO surfaces.

The results on mineralization obtained by Alizarin Red, calcium in cell lysate and culture medium also highlight the importance of combining qualitative findings with quantitative findings in order to capture early mineralization which may not be clearly evident in the quantitative analysis.

3.1.5 Discussion

This part of the research was focused towards quantification of SV-HFO cells response to PEO1 and PEO5 surfaces in order to better compare the two surfaces and further understand the effects of surface features on cells response. It is clearly demonstrated that both PEO surfaces assist the most important functions of SV-HFO cells, namely attachment, proliferation, differentiation and matrix mineralization over an incubation period of 27 days. Nevertheless, when compared to the PC surfaces (polystyrene surfaces), the cells seeded on the PEO surfaces need an adaptation time for interacting with a completely different surface (a porous TiO_2 surface). This has been evidenced by the delay in cells attachment observed on PEO surfaces after 24 hours relative to the PC (Figure 19).

When comparing the two PEO surfaces, the largest differences in cells response have been observed at early time points (mostly up to 9 days) i.e., during attachment, proliferation and early differentiation. The results indicate that PEO1 surface is having more favorable effects on initial cells response. At later time points, i.e., during late differentiation and mineralization stages, the differences between the two surfaces are not significant albeit the trend is similar.

One of the reasons for the enhanced cells attachment on the PEO1 surface after 24 hours relative to the PEO5 surface may be related to the effects of surface topography on cells. It has been evidenced in the previous study [25] by SEM imaging that SV-HFO cells can attach and spread easily on the PEO1 surfaces using the pores as anchorage sites for their extensions (Figure 26). By comparison, the larger pores protruding from the PEO5 surface acted as obstacles for cells attachment and spreading.

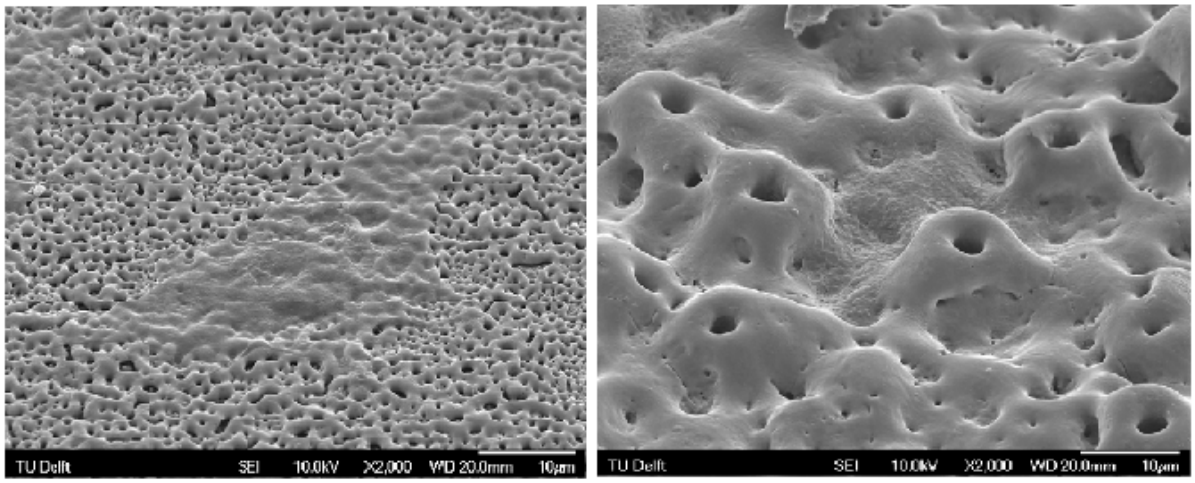

Figure 26 SEM imaging of SV-HFO cells on PEO1 and PEO5 surfaces at 2000x magnification after 24 hours of incubation [25].

It seems like the surface with fine pores and lower roughness is more favorable for cells attachment. Nevertheless, the PEO1and PEO5 surfaces differ not only in surface topography but also in surface chemistry, especially the Ca/P ratio (Table 1). Therefore, in this part of the study it was not possible to delineate between the topography and possible chemistry effects on cells response.

The higher DNA levels on PEO1 surface relative to PEO5 surface up to day 9 (Figure 21) may be the result of enhanced initial cells attachment on PEO1 surface and higher proliferation rate observed on this surface in this time interval. This may further explain the larger ALP activity measured on this surface at day 9. Nevertheless, it seems that after a slower start on PEO5 surface, cells are able to continue their growth and stabilize at later time points with beneficial effect on subsequent differentiation (Figure 22) and mineralization (Figure 23).

Table 1 Surface characteristics of PEO1 and PEO5 surfaces [25]

Surface characteristics	PEO1	PEO5
Average roughness Ra (µm)	0.19±0.03	1.43±0.08
Maximum peak- to valley height Ry (µm)	1.64±0.27	10.32±0.80
Surface porosity (%)	14.5	4.0
Ca/P atomic ratio	0.99	2.02

Notably, at extended culturing time (> 9 days), cells on both PEO surfaces showed ALP activity comparable with that on the positive control despite the slower cells growth indicating that these surfaces strongly favor osteogenic differentiation of SV-HFO cells.

Mineralization data showed that the differentiated cells were able to produce bone matrix on PEO surfaces. Experiments with longer culturing times are needed to follow the mineralization process and assess quality of the mineral matrix on the two PEO surfaces. The lack of sufficient data on mineralization form previous studies in this field and the different cell lines and experimental conditions used in few studies that have shown mineralization [33,34] limit the possibility to relate and analyze mineralization on PEO surfaces used in this study to other surfaces. Further, the findings should be compared with clinically relevant titanium surfaces. Nevertheless, from the few previous studies, PEO surfaces produced in Ca/P electrolytes could enhance mineralization by HFO cells relative to plasma sprayed titanium surfaces [34]. Further, PEO surfaces produced in acid electrolytes promoted proliferation, differentiation and mineralization by MC3T3-E1 cells when compared to non-oxidized titanium surfaces [33].

This part of the research generated new and quantified data on SV-HFO cells response to the PEO1 and PEO5 surfaces. The findings supported the previous qualitative results [25] and showed that both PEO surfaces assist cells functions from attachment to matrix mineralization. Nevertheless, the PEO1 surface had a relatively more beneficial impact on the cells initially favoring their attachment, growth and early differentiation. This could be a potential advantage in the “race for the surface” following implantation.

To further understand the role of PEO1 and PEO5 surface features on cells response it is necessary to delineate between the topography and chemistry effects. The presence of Ca on these surfaces may affect cells response. Mohedano *et al,* [31] varied the Ca/P ratio from (1.71 to 4) and showed that MC3T3 cells proliferation was enhanced on surface with high calcium. Recently Hu *et al,* [35] explained that calcium present on the surface promotes protein adsorption due to charge variations and thereby enhances the proliferation of cells. It was also highlighted in another study [36] that high Ca/P ratio (1.66-2.16) promotes differentiation of MC3T3 cells on PEO surfaces. This brings us to the question whether the higher calcium content of the PEO5 surface contributes as a supportive factor to promote cell response at later stages by counteracting the less positive initial influence.

Therefore, in the second part of the research, a new surface has been considered which had an intermediate topography to the PEO1 and PEO5 surfaces but no calcium.

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3.2 Effects of PEO surface calcium on SV-HFO response

This part of research was focused towards understanding the effects of surface calcium on cell response. Therefore, besides the PEO1 and PEO5 surfaces that were used so far, another PEO treated surface (PEO-Ca) that had no surface calcium but topographical features intermediate to PEO1 and PEO5 surface was introduced. The main functions of SV-HFO cells (growth, differentiation and mineralization) were analyzed to identify the effect of surface calcium on the response of these cells.

3.2.1 PEO surface characteristics

The surface morphology of PEO 1, PEO 5 and PEO-Ca surface taken at 2000x magnification by SEM is shown in Figure 27. It is seen from the morphology that while PEO1 and PEO5 surfaces appear extremely different from each other, the PEO-Ca surface shares the topographical features of both PEO1 and PEO5 surfaces.

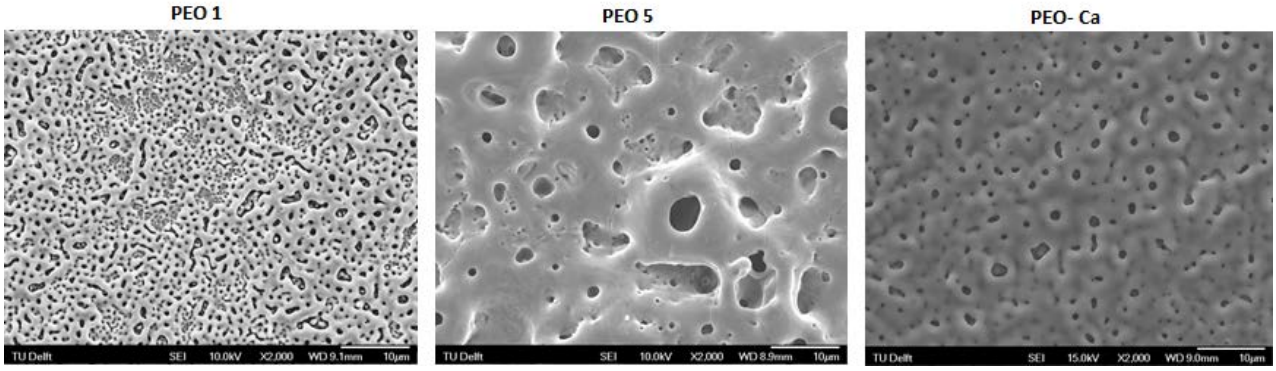

Figure 27 SEM images of PEO1, PEO5 and PEO-Ca surface at 2000x magnification

Table 2 summarizes some of the main surface characteristics of the three PEO surfaces. It is confirmed that the surface without calcium (PEO-Ca) has intermediate porosity compared to PEO1 and PEO5 surface. In addition, Ra and Ry of this surface are closer to the PEO1 surface.

Table 2 Surface characteristics of PEO1 [25], PEO5 [25] and PEO-Ca surface

Surface characteristics	PEO1	PEO-Ca	PEO5
Average roughness Ra (µm)	0.19±0.03	0.26±0.09	1.43±0.08
Maximum peak- to valley height Ry (µm)	1.64±0.27	0.65±0.30	10.32±0.80
Surface porosity (%)	14.5	9.1	4.0
Ca/P atomic ratio	0.99	0	2.02

Figure 28 shows the pore size distribution of PEO1, PEO5 and PEO-Ca surfaces. Most of the pores (48-77%) on the three different surfaces are in the submicron range. While PEO1 surface has the least amount of bigger size pores (> 2 microns) and PEO5 surface has the maximum amount of bigger size pores, PEO-Ca surface has an intermediate pore size distribution.

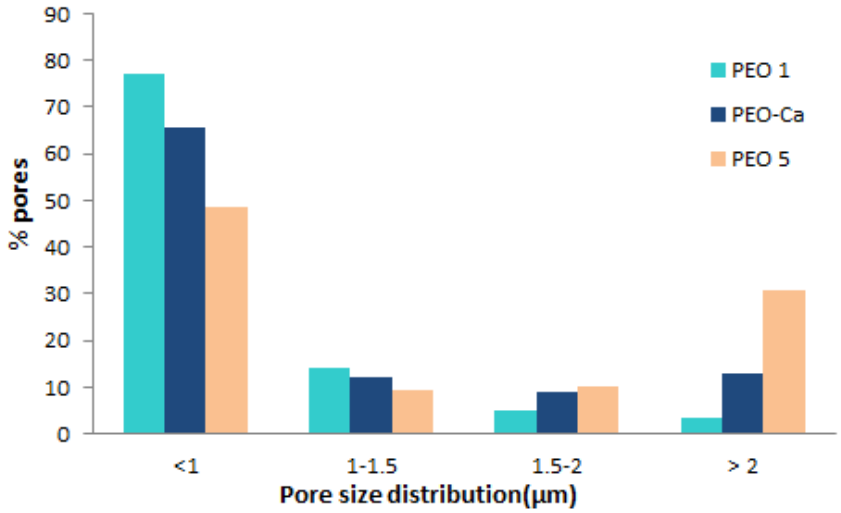

Figure 28 Pore size distribution of PEO1, PEO5 and PEO-Ca surface

Figure 29 shows the EDS spectra for the three surfaces and it can be seen that PEO-Ca surface has no calcium peaks but a relatively high peak of phosphorous because of the oxidation in a phosphorous based electrolyte. The highest calcium peak was found on PEO5 surface followed by PEO1 surface.

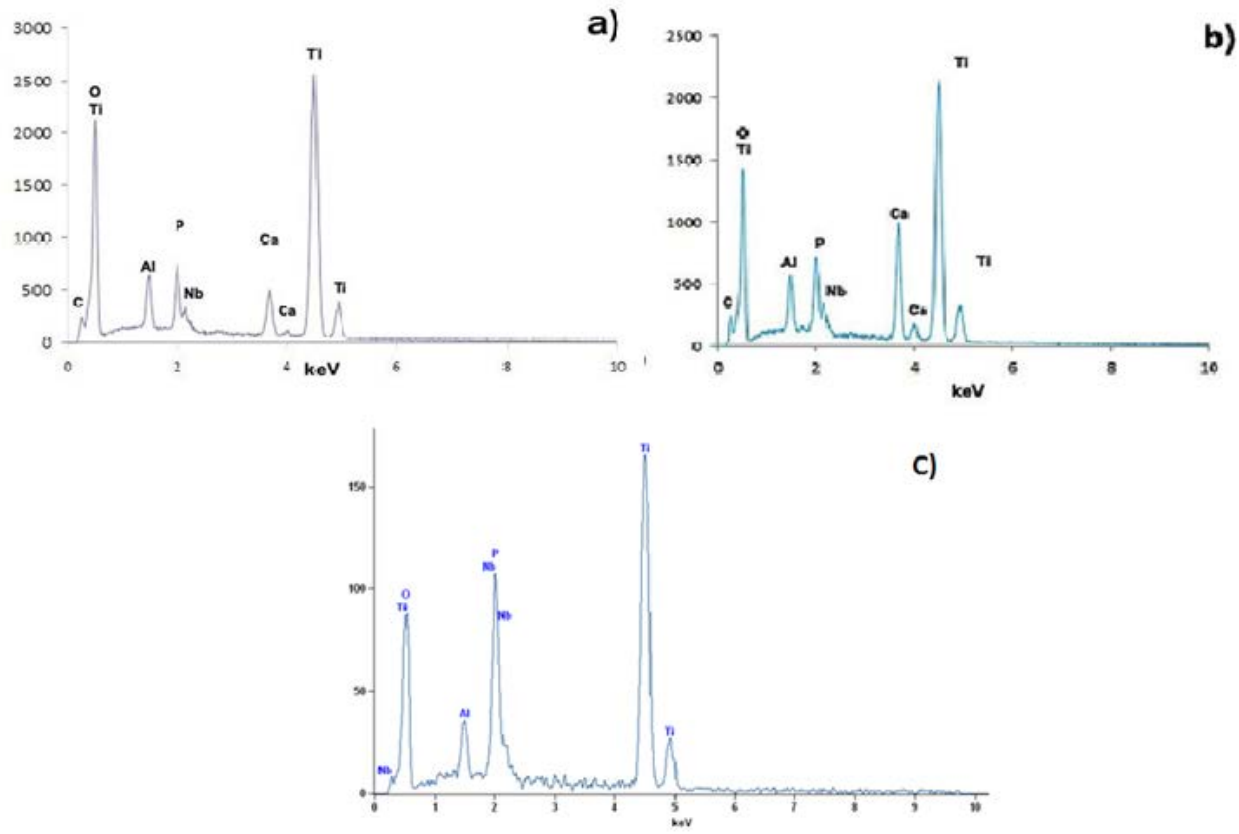

Figure 29 EDS spectra for (a) PEO1, (b) PEO5 and (c) PEO-Ca surfaces

The average calcium concentration ranged from 0 on PEO-Ca to 3.1at% on PEO1 and 8.7 at % on PEO5 surface.

The surface characterization thus shows that the PEO-Ca surface introduced in this study to analyze the effects of surface calcium on cell response has no surface calcium yet the surface morphology is in between PEO1 and PEO5 surfaces. Thus, the effects of surface calcium on cells response can be studied in a systemic manner.

3.2.2 Growth of SV-HFO cells

Cells growth in terms of DNA content from day 2 to day 30 on PEO-Ca, PEO1 and PEO5 surfaces is shown in Figure 30. Once again it was observed that pre-osteoblasts growth on

PEO5 surface was less than on PEO1 but also less than on PEO-Ca surface at initial time period (up to day 9). There was no significant difference between the three surfaces at later stages. Interestingly, PEO-Ca surface that had no calcium did not have a negative influence on cell growth over the entire time period.

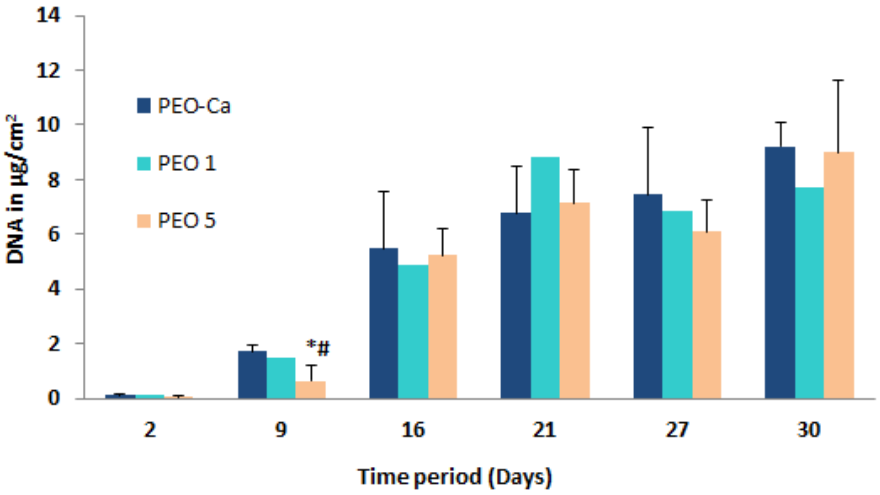

Figure 30 Growth of pre-osteoblasts on PEO1, PEO5 and PEO-Ca surfaces by DNA; Statistical difference ($p<0.05$) from PEO-Ca surface is indicated by * and between PEO1 and PEO5 surface is indicated by #

3.2.3 Differentiation of SV-HFO cells

ALP activity of SV-HFO cells on PEO-Ca, PEO1 and PEO5 surface (Figure 31) clearly showed that surface calcium has no profound influence on differentiation, as the ALP activity of cells on PEO-Ca surface was not affected due to the absence of calcium on the surface. Furthermore, it was also seen that the differentiation at day 9 on PEO5 surface that has relatively high level of calcium, was significantly less than on PEO-Ca surface and PEO1 surfaces. However, at later time period there was no significant difference in differentiation of cells on all 3 surfaces. As previously observed (Figure 22), differentiation seems to slow down from day 27 when mineralization takes over.

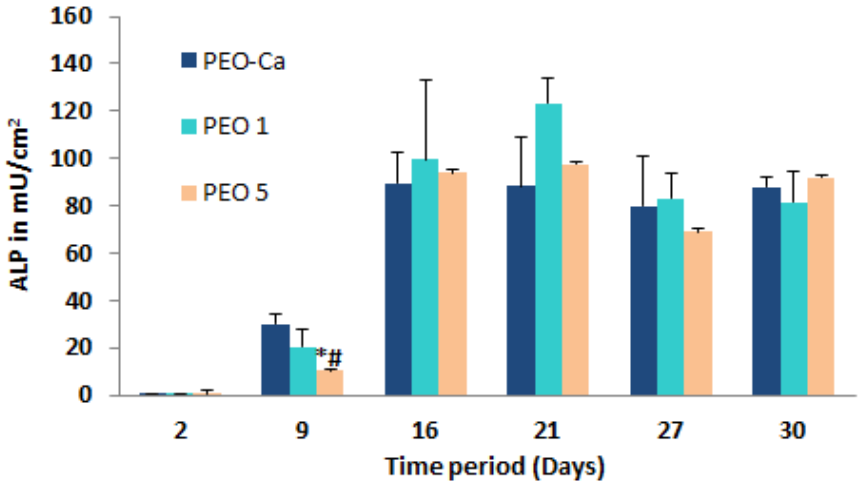

Figure 31 Differentiation of pre-osteoblasts on PEO1, PEO5 and PEO-Ca surfaces by ALP activity; Statistical difference ($p<0.05$) from PEO-Ca surface is indicated by * and between PEO1 and PEO5 surface is indicated by #

3.2.4 Matrix mineralization

Quantification of calcium content in the cell lysates up to day 30 on PEO-Ca, PEO1 and PEO5 surfaces (Figure 32) also evidenced that mineralization was not affected by surface calcium as there was no significant difference in calcium concentrations of cell lysates on all three surfaces (except at day 27).

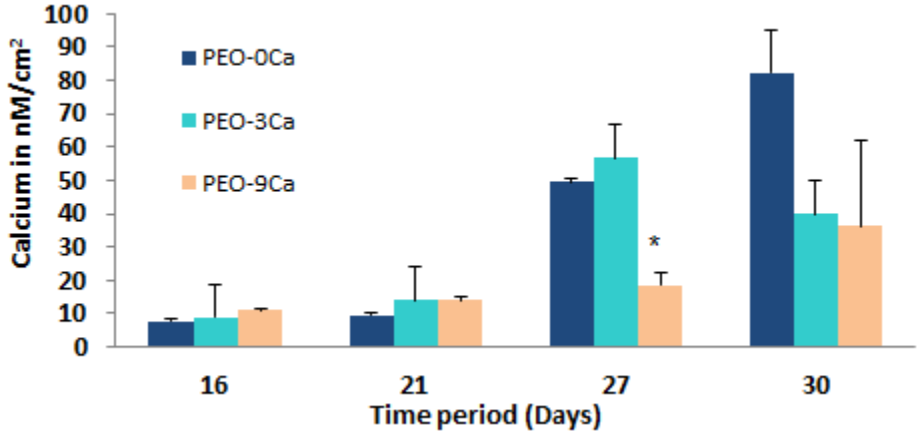

Figure 32 Mineralization by pre-osteoblasts on PEO1, PEO5 and PEO-Ca surfaces by calcium content in cell lysate; Statistical difference ($p<0.05$) from PEO-Ca surface is indicated by * and between PEO1 and PEO5 surface is indicated by #

It was also very interesting to note that mineralization on surface without calcium (PEO-Ca) was significantly higher than on the PEO5 surface that had highest calcium of all three

surfaces at day 27. In general, it could be noticed that the response of SV-HFO cells to PEO-Ca surface followed closely the response to PEO1 surface.

3.2.5 Discussion

In the first part of this research study it was observed that variations in surface features between PEO1 and PEO5 surfaces influenced cell response with enhanced cell attachment on PEO1 surface and lower growth and differentiation on PEO5 surface at early stages. However, at later stages both surfaces appeared stable for cells response. Since PEO5 surface had relatively high Ca/P ratio, it was important to clarify the contribution of surface calcium on cells response.

Therefore, the main idea behind the second part of the research study was to analyse the effects of surface calcium on cells response in a very systematic manner in order to understand if surface calcium could favor cells response at later stages. This was done by introducing a surface that had no calcium (PEO-Ca) but topographical features (i.e., pore size, porosity) intermediate to PEO1 and PEO5 surfaces. In addition, the surface roughness (Ra, Ry) was closer to PEO1 surface.

The pre-osteoblast response from growth to mineralization analysed on these three surfaces clearly indicates that the surface calcium does not play the main role on the cells response as the growth, differentiation and most importantly mineralization of osteoblasts were not hindered on PEO-Ca surface due to absence of calcium.

In addition, it was observed that the growth and differentiation of pre-osteoblasts on PEO5 surface was significantly less than on PEO1 and PEO-Ca up to day 9. Furthermore, it was also interesting to observe that mineralization on PEO5 surface was significantly less than on PEO–Ca surface at day 27 despite its high Ca/P ratio.

PEO-Ca surface shared intermediate topographical features to PEO1 and PEO5 surfaces and the cell response was closer to PEO1 surface at all time period.

Considering further the surface chemistry, PEO-Ca surface had a higher peak of phosphorous in the EDS spectra which means that the surface is relatively rich in phosphorous. Feng *et al*, [37] studied the rabbit osteoblasts response to titanium plates immersed in calcium phosphate or phosphorous solutions and reported that the proliferation and differentiation of cells were

reduced on surfaces that were coated with phosphorous relative to calcium phosphate coated surfaces [37].

Furthermore, in another study, mesenchymal cells differentiation and mineralization on plasma sprayed titanium surfaces in medium containing additional dissolved calcium and inorganic phosphorous ions was assessed and the results signified that additional phosphorous had a negative influence on differentiation and mineralization of cells [38].

Though above findings were on other modified titanium surfaces, it gives an understanding that phosphorus may not favor cells response.

Despite the limiting influence of phosphorus on cells response, the PEO-Ca surface that had high level of phosphorous relative to PEO1 and PEO5 and no calcium resulted as a surface supportive for cells response in this study.

These results clearly imply that surface calcium in the range used in this study is not the main player in cell response. However, it is important to note that the Ca/P ratio used in this study falls under the range of hydroxyapatite which is clinically relevant. Having ruled out the effects of surface calcium, (and from the knowledge of less favourable effects of surface phosphorous on cells response) the findings of this study indicate that topographical features of the PEO treated surfaces under investigation had the largest effect on the measured cell response.

However, it is now important to reason which topographical features provided positive guidance for cells. By taking into account the topographical characteristics of these three surfaces it suggests that the PEO1 and PEO-Ca surface have relatively high porosity, less roughness (Table 2) and less percentage of large sized pores compared to PEO5 surface. Furthermore, the pore size distribution of all three surfaces (Figure 28) shows that even though PEO5 surface has relatively high percentage of pores (approx. 30%) with bigger size (> 2µm), all the three surfaces have most of their pores (48-77%) in the sub-micron range (<1µm).

Nevertheless, the large pores present on PEO5 surface protruded from the surface leading to a significantly higher roughness (Table 1). As evidenced by SEM imaging (Figure 26) and described under section 3.1, the SV-HFO cells could readily attach and spread on the PEO1 surface having finer pores and lower roughness whereas the protruding pores on PEO5

surface acted as obstacle against cells attachment and spreading. However, the cells that were attached to the smaller pores (almost 50 %) on PEO5 surface may have benefited from the pore size and proliferated quite well. Therefore, it seems that even though there was an initial delay in cell response on this surface the cells stabilize at later stages. In other words, an extended adaptation period was needed for cells on PEO5 surface.

Another interesting aspect to address is the strong osteogenic differentiation of cells (comparable to the PC) observed on all three surfaces after 9 days despite the variations in roughness and calcium level. Karzakala *et al*, 2013 [39] reported that variations in average roughness of PEO surface from < 1 µm to 4 µm along with Ca/P ratio affected the viability of adult mesenchymal cells but there was no significant difference in differentiation of these cells on surfaces with varying roughness and calcium level. Likewise, another study with SaOS2 cells [32] showed that the spreading and proliferation of cells were influenced by variations in roughness, pore size, porosity and Ca/P ratio but the differentiation of cells remained the same on the surfaces that had varying characteristics.

These findings are in line with the results of our study but also highlight the importance for detailed investigation of surface features to understand the factor that contribute to enhanced differentiation. Most studies only consider the average roughness, pore size and Ca/P ratio of the surfaces but neglect other aspects such as the pore size distribution, pore height, amplitude roughness parameter and organization of roughness which may have answers to the findings that remain intriguing. The dominant sub-micron pore size distribution in our study may have been a contributing factor in influencing the differentiation of cells on these surfaces. However, this reasoning needs further investigation and experimental proof.

Nevertheless, the results of this study clearly indicate that surface calcium in the range of the concentration investigated is not the main factor and therefore strongly points to PEO topography as key player in influencing cells response. It is the only study known to our knowledge so far that delineates the effects of PEO topography and calcium for a certain type of cells assessed under similar conditions up to and including mineralization. Based on these findings, intense research focus should be directed towards understanding the effects of PEO topographical features on each cell function down to molecular level, in order further optimize these surfaces that could assist in osseointegration leading to enhanced implant fixation.

Chapter 4

4 Conclusions

PEO surfaces have attractive physico-chemical characteristics that may favor osseointegration of bone implants. This is highly needed for extending the life time of these implants. The present research was focused on studying cells response to PEO surfaces with the final goal to properly delineate the PEO topography and calcium effects on cells behavior.

The research was performed in two stages. The focus of first stage was to study cells response to PEO surfaces in a quantitative manner. This was further extended into next stage to delve deeper into the surface features and investigate the effects of surface calcium on cells response.

In the first stage of research two PEO surfaces (PEO1 and PEO5) with different surface characteristics were included. PEO1 surface had higher porosity, lower roughness, lower percent of bigger pores and lower Ca/P ratio. As opposed to PEO1 surface, PEO5 surface had lower porosity but higher roughness, higher percent of bigger size pores and higher Ca/P ratio. Pre-osteoblasts response was assessed over 27 days and included attachment, proliferation, growth, differentiation and mineralization. In the second stage of the research, an additional surface with no calcium but intermediate topography has been included.

The main findings of this study are as follows:

- All functions of the SV-HFO cells have been assisted by PEO surfaces under investigation.
- Up to day 9, cells response is different between the PEO1 and PEO5 surfaces. Cells on PEO1 surface showed enhanced attachment, growth and differentiation. After day 9, no significant difference was observed in cells response between the two PEO surfaces.

- The absence of calcium from the surface did not did not detrimentally affect cells response. On the contrary, early growth and differentiation were enhanced on the surface without calcium relative to the surface with highest calcium level.
- The beneficial effects of PEO-Ca and PEO1 surfaces on cells response could be explained by the difference in topographical features of these surfaces relative to PEO5 surface, mostly by the finer pores and lower surface roughness (Table 3).
- The results of this study show for the first time that PEO topography can be used as a strong surface cue for modulating cells response towards enhanced osseointegration.

Table 3 SV-HFO cells response to the PEO surfaces investigated in this study

SV-HFO's response	PEO1	PEO-Ca	PEO5
Growth	+	+	-
Differentiation	+	+	-
Mineralization	+	+	±
Surface features	High porosity (9-15 %), fine pores (< 1 µm) and low average roughness (approx. 0.2 µm)		Less porosity (4%), protruding pores and high average roughness (1.43 µm)

Chapter 5

5 Limitations and Future suggestions

In the present research, the response of SV-HFO cells to PEO surfaces was investigated. The focus was placed on:

- Quantification of main cellular functions from attachment to matrix mineralization.
- Delineation between the effects of topography and surface calcium.

The findings brought new fundamental insights into the role of PEO surface characteristics on cells response paving the way towards generation of novel surfaces for enhanced osseointegration.

The limitations of the present study include:

1. The lack of titanium surface as control, preferably clinically relevant surface modified titanium (plasma sprayed hydroxyapatite) which would have given insights about the performance of the surfaces used in this study relative to clinical surfaces. As there are limited data's from studies in this field on mineralization, and this study had focused on quantification of mineralization, a comparison with clinically relevant surface under same conditions would have given valuable information for interpreting the results in clinical terms. This would be strongly recommended for follow up research.
2. Even though a negative control check was performed on PEO1 surface for biochemistry assays during this research (not included in report), it is recommended to use negative controls for all surfaces, preferably on polystyrene (PC) surfaces as well, as a check for background influence from the assays in future studies.

In this research the effects of surface calcium were investigated in a simple yet clear manner. Based on the findings, this research can be further expanded in several ways to address several aspects that still remain unclear.

- Firstly, as previously mentioned in the report (Section 3.1.5), an experimental investigation of the effects of pore size distribution on preosteoblasts response would give further in depth information on various features of topography. This could be performed by using another surface that has high percent of pores (more than 70 percent) with greater than 2 micron pore size and comparing the effects of cell response on this surface relative the PEO1, PEO-Ca, and PEO5 surface that has increasing percent of pores with bigger size.
- Next, the preosteoblasts response in this study was studied in an osteogenic condition that induces differentiation of preosteoblasts into osteoblasts. The same can be done in a standard medium to check for the *in vitro* osteoinductive effect of surface features on cell response and mostly importantly PEO-Ca surface should be included in this study for the benefit of doubts with regard to surface calcium effect in a non-osteogenic condition.
- Furthermore, this research should be carried out using mesenchymal stem cells as to check for the potential of the surfaces to promote osteogenic differentiation and matrix mineralization of the clinically relevant cells

The above mentioned future aspects would answer several important research questions in this field in a refined way which would also pave way for more suggestions and recommendations.

Good luck!

Chapter 6

6 References

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