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Supervisor:

Professor Despoina Deligianni

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University of Patras, Electrical and Computer Engineering Department
[Krousouloudi Maria]
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This thesis is dedicated to my parents, who have encouraged the research in the university despite the difficulties.

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Abstract

Nowadays, bone diseases are constantly on the rise either because of lifestyle change – population aging, increased obesity rates, minimal physical exercise - or because of permanent bone damage. To date, methods for defective bone treatment, such as autografting and allografting present short-term or long-term problems - painful and long-term processes, unpredictable bone resorption, risk of disease and / or infection that may cause a reduction or complete loss of limb - . For this reason, it is necessary to study and apply new biomaterials and techniques for bone regeneration. In this thesis, osteoblasts were differentiated from bone marrow cells isolated from hip and knee bones and cultured under suitable conditions similar to human's body (37 ° C, 5% CO_2, nutrient). Then *in vitro* studies were performed, and the development of osteoblasts on different types of substrates - polystyrene, smooth titanium, titanium dioxide nanotubes, rough titanium - was evaluated. Finally, through an electric stimulation device, manufactured in the laboratory, it was examined whether electrical stimulation of cells on the same substrates, as above, improves their proliferation and differentiation.

The first chapter consists of two parts. The first part analyses the structure and composition of the bones, as well as the types of bone cells. The second part analyses the types of mesenchymal cells, isolated from bone marrow, and the indices of osteogenic differentiation - alkaline phosphatase, total protein. The second chapter refers to biomaterials. Firstly, the desired properties of biomaterials associated with bone engineering are presented. Then the biomaterials used in the present study, are presented, the polystyrene - its evolution over years and its conversion from plain polystyrene to bio- and the titanium - its applications in medicine and its modifications to improve its properties -. The third chapter presents the cell - material interaction. At first, the cellular environment, the extracellular matrix - synthesis and interactions - and the function of adhesion-dependent cells are described. Then the properties of the bio-interface - chemistry, topography, substrate elasticity - and cell growth factor are presented. Finally, a look at the evolution of biomaterials over the years is made.

The fourth chapter is about *in vitro* cell culture. It first describes a cell culture, its usefulness and its conditions - nutrient, CO_2, temperature -. The conditions for cell passaging are also reported, as well as the method for cell counting. Finally, a reference is made to osteoblasts, the type of cells used in our study. The fifth chapter includes electrical stimulation of cells. The human body by itself emits an electrical stimulus that enhances cellular communication.

However, there is the possibility of external electrical stimulation of bone - regenerating tissues - and this chapter refers to electrical stimulation applications in the body. The sixth chapter includes the experimental part. The materials and methods used as well as the preparation of the substrates are presented. The procedure for isolation and differentiation of osteoblasts from their receipt from the hospital to their culture in the laboratory is described below. Moreover, the process of cell degradation and subculturing as well as cell counting is being explained. After that, the experimental part is divided into two sub-sections. The first section describes the cell culture in polystyrene flasks, and then the biocompatibility test on different substrates - polystyrene, titanium, titanium dioxide nanotubes, rough titanium - . The second section describes the osteoblast culture on the same substrates - polystyrene, titanium, titanium dioxide nanotubes, rough titanium - under electrical current stimuli through an electric stimulation device manufactured in the laboratory.

The present investigation aimed to find an appropriate combination of parameters related to substrate characteristics (nature and topography) and an induced electrical field that leads to the proliferation and enhanced activity of osteoblast bone cells. With this purpose, a cell carrying device has been designed and used for the electrical stimulation of the human osteoblast cells. It was found that a combined effect of both applied electrical field (0.3V, 3 hours) and specific substrate nature and topography considerably increase the ration ‘alkaline phosphatase/ total protein’ (ALP/TP), which has been translated as the degree of biocompatibility. When no electric field is applied, the cells tend to increase ALP levels associated to their multiplication and proliferation on soft surfaces, while a light decrease is observed when they are seeded on rough surfaces.

On the opposite, the total protein level is increased cells seeded on rough substrates, since this is related to their adhesion process. The ALP/TP is decisive for the faith osteoblasts population. Generally, a light increase in proliferation on TNTs compared to other substrates is associated with the highly organized nature of the TNTs. Results agree with already reported studies and confirm that substrate nature which includes its chemical composition, its mechanical properties, and surface topography are very important factors, decisive for osteoblasts well-functioning. When an electric field is applied, cells functions are improved. Titanium dioxide nanotubes, due to their nature and highly organized architecture, contribute to the uniform distribution of the electric field in the medium and under the bone cell population. Finally, the effect of an adjusted intensity of the electric field and the appropriate topography of the substrate on cells behaviour demonstrates the importance of these two in

vitro parameters for the activity of bone forming cells and may be considered crucial in the manufacturing of advanced biomaterials for orthopaedic applications.

Extended Abstract (in Greek)

Στις μέρες μας, οι παθήσεις των οστών αυξάνονται συνεχώς είτε λόγω της αλλαγής του τρόπου ζωής του πληθυσμού - αύξηση του προσδόκιμου ζωής, αυξημένα ποσοστά παχυσαρκίας, ελάχιστη φυσική άσκηση - , είτε λόγω ατυχημάτων που προκαλούν μόνιμες παθήσεις στα οστά . Μέχρι σήμερα, η αντιμετώπιση των ελαττωματικών οστών, περιλάμβανε μεθόδους όπως αυτομοσχεύματος (autografting) και αλλομοσχεύματος (allografting), που παρουσιάζουν βραχυπρόθεσμα η μακροπρόθεσμα προβλήματα - επίπονες και μακροχρόνιες διεργασίες, απρόβλεπτη οστική απορρόφηση, κίνδυνος νόσου και / ή μόλυνσης που μπορεί να προκαλέσει μείωση ή πλήρη απώλεια των επαγωγικών παραγόντων του οστού - . Για το λόγο αυτό χρήζεται αναγκαία η μελέτη και εφαρμογή νέων βιοϋλικών και τεχνικών για την ανάπλαση των οστών με πρόβλημα. Στην παρούσα διπλωματική εργασία απομονώθηκαν οστεοβλάστες από μυελό του οστού ισχίου και γονάτου και καλλιεργήθηκαν σε κατάλληλες συνθήκες που προσομοιάζουν του ανθρώπινου οργανισμού (37^{o}C, 5% CO_2, θρεπτικό υλικό). Κατόπιν πραγματοποιηθούν *in vitro* μελέτες και εκτιμήθηκε η ανάπτυξη των οστεοβλαστών σε διαφορετικά είδη υποστρωμάτων - πολυστυρένιο, λείο τιτάνιο, νανοσωλήνες τιτανίου, αδρό τιτάνιο - . Τέλος, μέσω μιας κυψελίδας ηλεκτρικής διέγερσης που κατασκευάστηκε στο εργαστήριο, εξετάστηκε κατά πόσο η ηλεκτρική διέγερση των κυττάρων στα ίδια υποστρώματα βελτιώνει τον πολλαπλασιασμό και τη διαφοροποίησή τους.

Το πρώτο κεφάλαιο αποτελείται από δύο μέρη, τα οστά και τα μεσεγχυματικά κύτταρα, τα οποία μπορούν να απομονωθούν από μυελό οστού. Στο πρώτο μέρος αναλύεται η δομή και η σύνθεσή των οστών, καθώς και τα είδη των οστικών κυττάρων. Στο δεύτερο μέρος αναλύονται οι τύποι των μεσεγχυματικών κυττάρων και οι δείκτες οστεογενικής διαφοροποίησης - αλκαλική φωσφατάση, ολική πρωτεΐνη. Το δεύτερο κεφάλαιο αναφέρεται στα βιοϋλικά. Πρώτα παρουσιάζονται οι επιθυμητές ιδιότητες των βιοϋλικών που σχετίζονται με τη μηχανική οστών. Κατόπιν παρουσιάζονται τα βιοϋλικά που χρησιμοποιήθηκαν στην παρούσα εργασία, πολυστυρένιο - η εξέλιξή του ανά τα χρόνια και η μετατροπή του από απλό πολυστυρένιο σε βιο, κατάλληλο για καλλιέργεια κυττάρων πάνω του - και τιτάνιο - οι εφαρμογές του στην ιατρική και οι τροποποιήσεις του για βελτίωση των ιδιοτήτων του - . Στο τρίτο κεφάλαιο παρουσιάζεται η αλληλεπίδραση κυττάρου και υλικού. Αρχικά περιγράφεται το κυτταρικό περιβάλλον, η εξωκυττάρια μήτρα - σύνθεση και αλληλεπιδράσεις - και η λειτουργία των κυττάρων που αναπτύσσονται προσκολλημένα στην επιφάνεια του υποστρώματος. Έπειτα αναλύονται οι ιδιότητες της βιο-διεπαφής - χημεία,

τοπογραφία, ελαστικότητα υποστρώματος - και ο παράγοντας ανάπτυξης των κυττάρων. Τέλος γίνεται μία αναδρομή στην εξέλιξη των βιοϋλικών ανά τα χρόνια. Το τέταρτο κεφάλαιο πραγματεύεται την *in vitro* καλλιέργεια κυττάρων. Αρχικά περιγράφεται μια κυτταροκαλλιέργεια, η χρησιμότητά της και οι συνθήκες που πρέπει να επικρατούν σε αυτήν - θρεπτικό υλικό, CO_2, θερμοκρασία - . Έπειτα αναφέρονται οι συνθήκες για διαχωρισμό των κυττάρων και νέα καλλιέργεια καθώς και η μέθοδος υπολογισμού των κυττάρων της καλλιέργειας. Τέλος γίνεται αναφορά στη συγκεκριμένη κατηγορία κυττάρων, οστεοβλαστών, η οποία χρησιμοποιήθηκε στην παρούσα μελέτη και γιατί επιλέχθηκε. Το πέμπτο κεφάλαιο περιλαμβάνει την ηλεκτρική διέγερση των κυττάρων. Ο ανθρώπινος οργανισμός από μόνος του εκπέμπει ηλεκτρικά ερεθίσματα που συμβάλλουν στην επικοινωνία. Υπάρχει όμως και η δυνατότητα εξωτερικής ηλεκτρικής διέγερσης ιστών - οστικών οι οποίοι έχουν τη δυνατότητα ανάπλασης - και σ αυτό το κεφάλαιο αναφέρονται εφαρμογές ηλεκτρικής διέγερσης στον οργανισμό. Το έκτο κεφάλαιο περιλαμβάνει το πειραματικό μέρος. Αρχικά παρουσιάζονται τα υλικά και οι μέθοδοι που χρησιμοποιήθηκαν καθώς και η προετοιμασία των υποστρωμάτων. Στη συνέχεια περιγράφεται η διαδικασία απομόνωσης και διαφοροποίησης των οστεοβλαστών από την παραλαβή τους από το νοσοκομείο μέχρι την καλλιέργειά τους στο εργαστήριο. Έπειτα εξηγείται η διαδικασία αποκόλλησης των κυττάρων και υποκαλλιέργειας (subculturing) καθώς και ο υπολογισμός κυττάρων της κυτταροκαλλιέργειας. Κατόπιν, το πειραματικό μέρος χωρίζετε σε δύο υπομέρη. Στο πρώτο σκέλος περιγράφεται η καλλιέργεια οστεοβλαστών σε φλάσκες πολυστυρενίου, και μετά ο έλεγχος βιοσυμβατότητας σε διαφορετικά υποστρώματα - πολυστυρένιο, τιτάνιο, νανοσωλήνες διοξειδίου του τιτανίου, αδρό τιτάνιο - . Στο δεύτερο σκέλος περιγράφεται η καλλιέργεια οστεοβλαστών αντίστοιχων υποστρωμάτων - πολυστυρένιο, τιτάνιο, νανοσωλήνες διοξειδίου του τιτανίου, αδρό τιτάνιο - υπό την επήρεια ρεύματος μέσω μιας κυψελίδας ηλεκτρικής διέγερσης που κατασκευάστηκε στο εργαστήριο. Η παρούσα έρευνα στοχεύει στην εξεύρεση ενός κατάλληλου συνδυασμού παραμέτρων που σχετίζονται με τα χαρακτηριστικά του υποστρώματος (φύση και τοπογραφία) και ένα επαγόμενο ηλεκτρικό πεδίο που οδηγεί στον πολλαπλασιασμό και την ενισχυμένη δράση των οστεοβλαστών. Με αυτό το σκοπό, έχει σχεδιαστεί μια συσκευή για την ηλεκτρική διέγερση των ανθρώπινων οστεοβλαστών που απομονώθηκαν από οστικό ιστό που ελήφθη κατά τη διάρκεια ορθοπεδικής χειρουργικής επέμβασης. Ένα πεδίο συνεχούς ρεύματος εφαρμόστηκε στο μέσο καλλιέργειας και μια σειρά πρωτοκόλλων για την ηλεκτρική διέγερση των οστεοβλαστών έχουν δοκιμαστεί για να βρεθεί ο κατάλληλος συνδυασμός παραμέτρων (τάση, χρόνος, υπόστρωμα) που ενισχύουν τη δραστηριότητά τους. Διαπιστώθηκε ότι η

συνδυασμένη επίδραση τόσο της εφαρμοζόμενης ηλεκτρικής περιοχής (0.3V, 3 ώρες) όσο και της ειδικής φύσης υποστρώματος και τοπογραφίας αυξάνει σημαντικά την αναλογία «αλκαλική φωσφατάση / ολική πρωτεΐνη» (ALP / TP), η οποία ερμηνεύεται ως ο βαθμός βιοσυμβατότητας. Όταν δεν εφαρμόζεται ηλεκτρικό πεδίο, τα κύτταρα τείνουν να αυξάνουν τα επίπεδα της ALP που σχετίζονται με την διαφοροποίησή τους σε μαλακές επιφάνειες, ενώ παρατηρείται ελαφρά μείωση σε τραχείες επιφάνειες. Αντίθετα, το επίπεδο ολικής πρωτεΐνης είναι αυξημένο σε κύτταρα σε ακατέργαστα υποστρώματα, καθώς αυτό σχετίζεται με τον πολλαπλασιασμό και τη διαδικασία προσκόλλησης. Το ALP / TP είναι καθοριστικό για τον πληθυσμό των οστεοβλαστών. Γενικά, μια ελαφρά αύξηση στον βαθμό διαφοροποίησης σε TNTs (νανοσωλήνων διοξειδίου τιτανίου) σε σύγκριση με άλλα υποστρώματα συνδέεται με την εξαιρετικά οργανωμένη φύση των TNTs. Τα αποτελέσματα συμφωνούν με ήδη υπάρχουσες μελέτες και επιβεβαιώνουν ότι η φύση του υποστρώματος (χημική σύνθεση, μηχανικές ιδιότητες και επιφανειακή τοπογραφία) είναι καθοριστική για την καλή λειτουργία των οστεοβλαστών. Όταν εφαρμόζεται ένα ηλεκτρικό πεδίο, βελτιώνονται οι λειτουργίες του κυττάρου. Οι νανοσωλήνες διοξειδίου του τιτανίου, λόγω της φύσης τους και της εξαιρετικά οργανωμένης αρχιτεκτονικής, συμβάλλουν στην ομοιόμορφη κατανομή του ηλεκτρικού πεδίου στο μέσο και κάτω από τον πληθυσμό των οστικών κυττάρων. Τέλος, η επίδραση μιας προσαρμοσμένης έντασης του ηλεκτρικού πεδίου και η κατάλληλη τοπογραφία του υποστρώματος στη συμπεριφορά των κυττάρων καταδεικνύουν τη σημασία αυτών των δύο *in vitro* παραμέτρων για την κυτταρική δραστηριότητα οστεοβλαστών και μπορούν να θεωρηθούν κρίσιμα για την κατασκευή προηγμένων βιοϋλικών για ορθοπεδικές εφαρμογές.

Acronyms

ALP – Alkaline Phosphatase

AMSC – Adipose Marrow Stem Cells

BMSC – Bone Marrow Stem Cells

Ca – Calcium

CO_2 – Carbon Dioxide

DLC – Diamond-Like Carbon

ECM – Extracellular Cell Matrix

ESCs – Embryonic Stem Cells

HA – Hydroxyapatite

hMSC – Human Mesenchymal Stem Cells

MAO – Micro-Arc Oxidation

MSC – Mesenchymal Stem Cells

NSC –Neural Stem Cells

O_2 - Oxygen

P – Phosphorus

PEO – Plasma Electrolytic Oxidation

PS – Polystyrene

TCPS – Tissue Culture Polystyrene

Ti – Titanium

TiO_2 – Titanium Dioxide

TP – Total Protein

Chapter 1: Bones and Mesenchymal Cells

1.1. Bones

A bone is a rigid organ that constitutes part of the vertebrate skeleton. Bones provide structure of the body, support and protect internal organs, such as the heart, lungs, brain, kidneys, and uterus, produce red and white blood cells, store minerals and enable mobility. Bones come in a variety of shapes and sizes and have a complex internal and external structure. They are lightweight to allow efficient movement, yet strong and stiff to provide support. This unique mechanical behaviour is because of the primary structural element of the skeletal system, the bone tissue, which is impacted by a composite material of organic proteins and mineral crystals. These elements are intricately organized on many scales to create the material properties that allow bone to serve these functions under the variety of loading conditions experienced during everyday activities **[1][2][3]**. A healthy bone is a living, growing, dynamic material that is endlessly remodelled by a group of specialized bone cells from bone surfaces and marrow, in order to adapt its architecture and serve its functions throughout life. These bone cells digest aged or damaged bone, reform new bone tissue in its place and repair fractures that occur **[2][3]**.

1.1.1. Bone Structure

A typical long bone shows the gross anatomical characteristics of a bone (Figure 1-1). A long bone has two parts: the diaphysis and the epiphysis. The diaphysis is the tubular shaft that runs between the proximal and distal ends of the bone. The walls of the diaphysis are composed of dense and hard compact bone, and the hollow region inside diaphysis, called the medullary cavity, is filled with yellow marrow **[4]**.

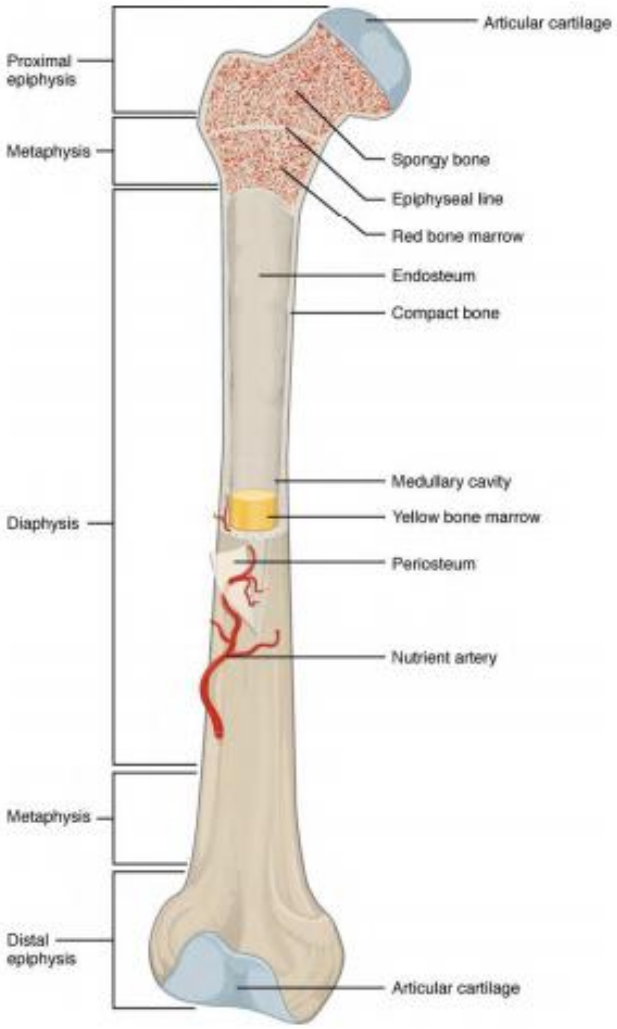


Figure 1-1 *Anatomy of a long bone. The structure of a long bone allows for the best visualization of all of the parts of a bone* ***[4]****.*

The epiphysis which is the wider section at each end of the bone is filled with spongy bone. Spongy bone spaces are filled with red marrow. Metaphysis is the narrow area, where each epiphysis meets the diaphysis. Metaphysis contains the epiphyseal plate (growth plate), a layer of hyaline (transparent) cartilage in a growing bone, that is replaced by osseous tissue when the bone stops growing in early adulthood (approximately 18–21 years) **[4]**.

The medullary cavity has a delicate membranous lining called the endosteum, where bone growth, repair, and remodeling occur **[4]**.

The outer surface of the bone is covered with a fibrous membrane, the periosteum, which contains blood vessels, nerves, and lymphatic vessels that nourish compact bone. Tendons and ligaments also attach to bones at the periosteum. The periosteum covers the entire outer

surface except where the epiphyses meet other bones to form joints (Figure 1-2). In this region, the epiphyses are covered with articular cartilage, a thin layer of cartilage that reduces friction and acts as a shock absorber **[4]**.

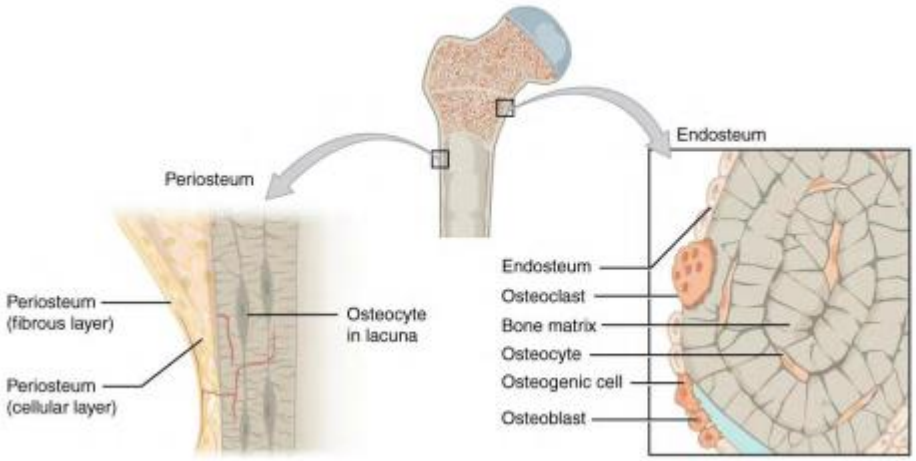


Figure 1-2 *Periosteum and Endosteum. The periosteum forms the outer surface of bone, and the endosteum lines the medullary cavity* ***[4]****.*

1.1.2. Bone Composition

Bone is not uniformly solid, but consists of two phases. The first phase is an organic flexible matrix (about 30%), of which 90 to 95% is composed of elastic collagen fibers and the remainder is ground substance, such as water lipids, non-collagenous proteins and cells. The second phase is bound minerals (about 70%), such as calcium and phosphorus in the form of hydroxyapatite crystals that account for approximately 25% of the total bone volume and 50% of the bone mass. The structure and mechanical behaviour of bone is determined by the quantity and mechanical integrity of each of these phases, the structural organization of the different phases, and the physical interaction between them **[1][2][3]**.

1.1.2.1. Bone Cells and Tissue

Bone cells compose a small amount of the bone volume. They are entrenched in a matrix of collagen fibers that provide a surface for inorganic salt crystals to adhere. These salt crystals form when calcium phosphate and calcium carbonate combine to create hydroxyapatite,

which incorporates other inorganic salts like magnesium hydroxide, fluoride, and sulfate as it crystallizes, or calcifies, on the collagen fibers. The hydroxyapatite crystals give bones their hardness and strength, while the collagen fibers give them flexibility so that they are not brittle. Despite the small number of cells, they are crucial to the function of bones. Four types of cells are found within bone tissue: osteoblasts, osteocytes, osteogenic cells, and osteoclasts (Figure 1-3) **[4]**.

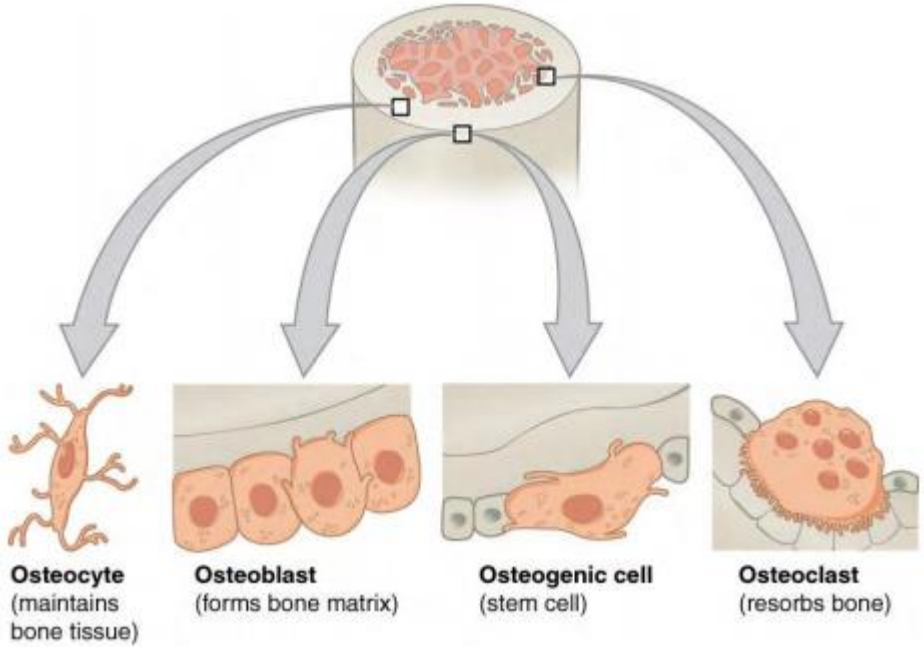


Figure 1-3 *Bone Cells. Four types of cells are found within bone tissue. Osteogenic cells are undifferentiated and develop into osteoblasts. When osteoblasts get trapped within the calcified matrix, their structure and function changes, and they become osteocytes. Osteoclasts develop from monocytes and macrophages and differ in appearance from other bone cells* ***[4]***.

Osteoblast cells are found in the growing portions of bone (e.g. periosteum, endosteum) and are responsible for forming new bone. Osteoblasts, which do not divide, synthesize and secrete the collagen matrix and calcium salts. As the secreted matrix surrounding the osteoblast calcifies, the osteoblast become trapped within it, changing in structure and becomes an osteocyte, the primary cell of mature bone and the most common type of bone cell. Osteocytes are located in a space called a lacuna and are surrounded by bone tissue. Like osteoblasts, osteocytes lack mitotic activity. They can communicate with each other and

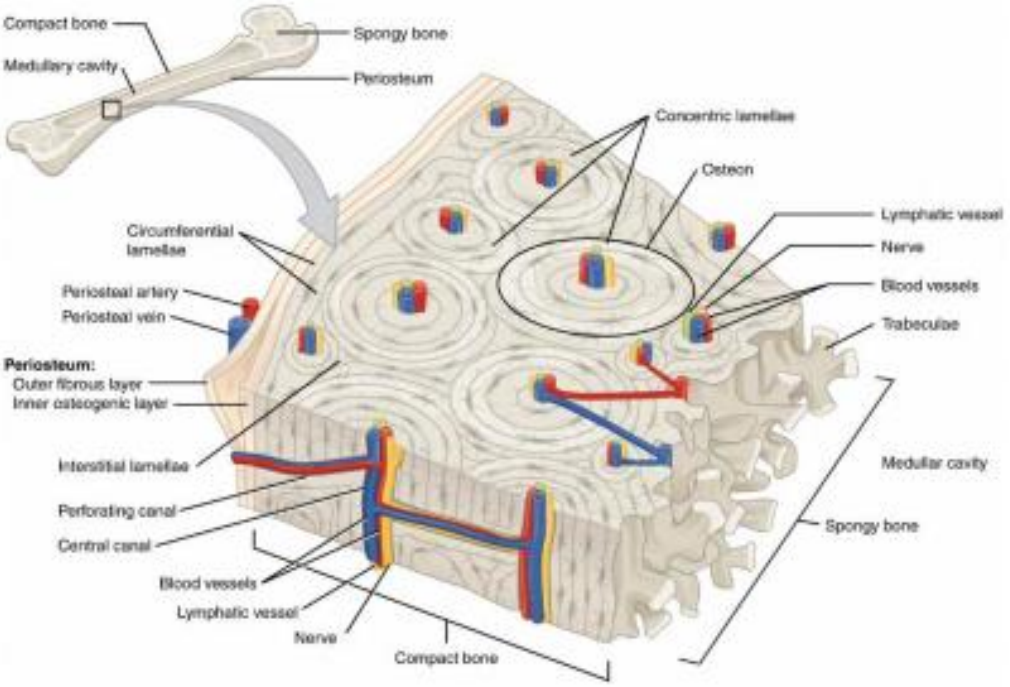
receive nutrients via long cytoplasmic processes that extend through canaliculi, channels within the bone matrix **[4]**.

On the other hand, the third bone cell categories, osteogenic cells are undifferentiated with high mitotic activity and they are the only bone cells that divide. Immature osteogenic cells, found in the deep layers of the periosteum and the marrow, differentiate and develop into osteoblasts. So, the new tissue is constantly formed, and old, injured, or unnecessary bone is dissolved for repair or for calcium release and this shows the dynamic nature of bone. The cell responsible for bone resorption, or breakdown, is the osteoclast. They are found on bone surfaces, are multinucleated, and originate from monocytes and macrophages, two types of white blood cells, not from osteogenic cells. The ongoing balance between osteoblasts, that continually form new bone, and osteoclasts, that continually break down old bone, is responsible for the constant but subtle reshaping of bone **[4]**.

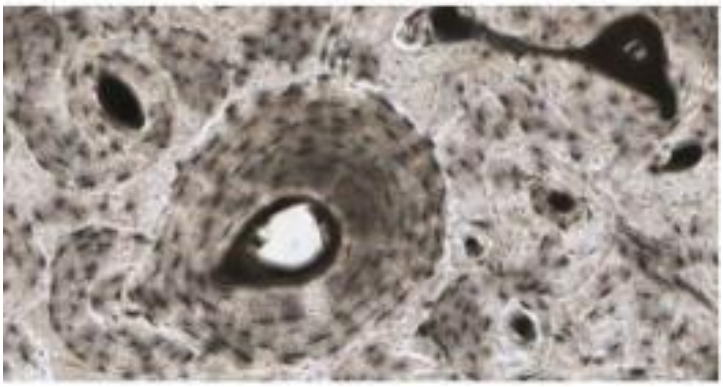
1.1.2.2. Compact and Spongy Bone

Another difference in bone composition at a more macroscopic level has to do with their histology. The two different bone types are: compact bone that is dense so that it can withstand compressive forces, while spongy (cancellous) bone that has open spaces and supports shifts in weight distribution **[4][5]**.

The hard outer layer of bones, that is the denser and stronger of the two types of bone tissue, is composed of cortical bone also called compact bone (Figure 1-4). The cortical bone gives bone its smooth, white, and solid appearance, and accounts for 80% of the total bone mass of an adult human skeleton. Cortical bone is covered by a periosteum on its outer surface, and an endosteum on its inner surface, which is the boundary between the cortical bone and the cancellous bone. The microscopic structural unit of compact bone is called an osteon, or Haversian system. Each osteon is composed of lamellae which is concentric rings of calcified matrix. Running down the center of each osteon is the central canal, or Haversian canal, which contains blood vessels, nerves, and lymphatic vessels. The osteocytes are located inside spaces called lacunae, found at the borders of adjacent lamellae. Canaliculi connect with the canaliculi of other lacunae and eventually with the central canal, so that nutrients are transported to the osteocytes and wastes are removed from them **[1][4][5]**.



(a)



(b)

Figure 1-4 *Diagram of Compact Bone. (a) This cross-sectional view of compact bone shows the basic structural unit, the osteon. (b) In this micrograph of the osteon, you can clearly see the concentric lamellae and central canals. LM × 40. (Micrograph provided by the Regents of University of Michigan Medical School © 2012)****[4].***

The internal tissue of the skeletal bone that is an open cell porous network is called cancellous or trabecular or spongy bone (Figure 1-5). Cancellous bone has a higher surface-area-to-volume ratio than cortical bone; it is less dense, and so it is weaker and more flexible. The

greater surface area also makes it suitable for metabolic activities such as the exchange of calcium ions. Cancellous bone, found at the ends of long bones, near joints and in the interior of vertebrae, is highly vascular and often contains red bone marrow where hematopoiesis, the production of blood cells, occurs. The primary anatomical and functional unit of cancellous bone is the trabecular, aligned towards the mechanical load distribution that a bone experiences within long bones such as the femur. Trabecular bone accounts for the remaining 20% of total bone mass but has nearly ten times the surface area of compact bone **[1][5][6][7]**.

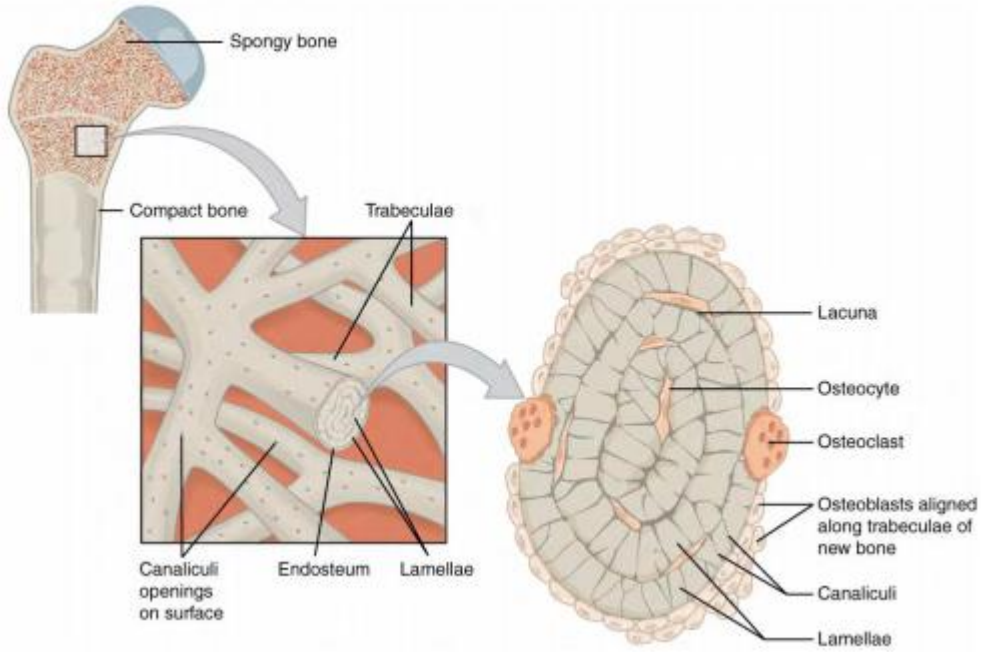


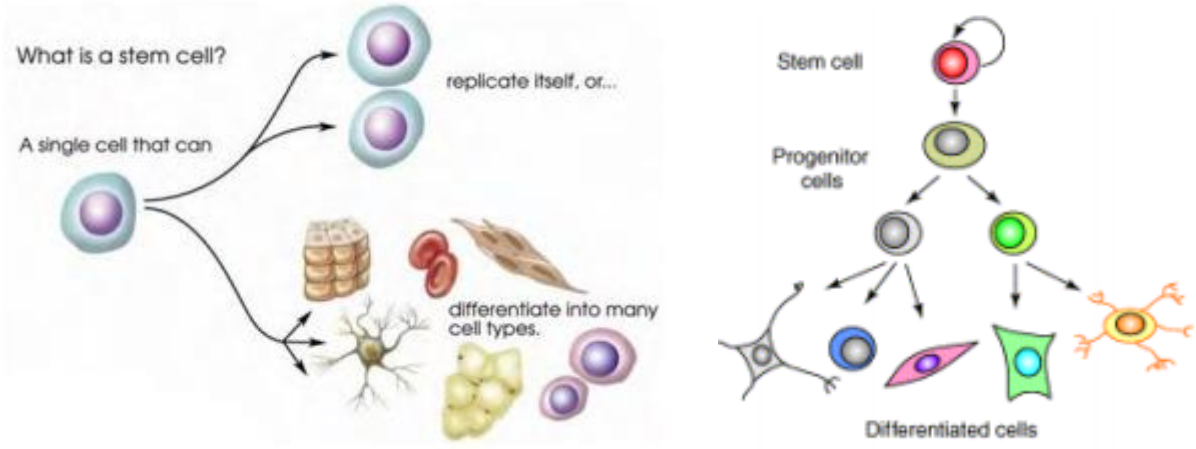
Figure 1-5 *Diagram of Spongy Bone. Spongy bone is composed of trabeculae that contain the osteocytes. Red marrow fills the spaces in some bones* ***[4]***.

1.2. Mesenchymal Stem Cells

1.2.1. Types

Stem cells are cells that can differentiate into other types of cells, and can also divide in self-renewal to produce more of the same type of stem cells, when stimulated to do so (Figure 1-6 (a)) **[8][9]**. Stem cells can be divided into mesenchymal stem cells (MSCs) and into embryonic stem cells (ESCs). Human mesenchymal stem cells (hMSC) may be harvested from bone marrow (BMSC) and adipose (AMSC) tissue **[10]**.

Stem cells are similar to progenitor cells, which have the ability to differentiate into a specific type of cell. However, progenitor cells are already more specific than stem cells and can only be pushed to differentiate into its "target" cell (Figure 1-6 (b)). On the other hand, stem cells have the ability to differentiate into many different types of cells **[11]**.



(a) (b)

Figure 1-6 *Stem Cells (a) Definition of a stem cell* ***[12.]*** *(b) Progenitor cells and differentiated cells* ***[11].***

1.2.2. Osteogenic Differentiation

One of the problems of using mesenchymal progenitor cells for bone tissue engineering is the heterogenicity of the cultures, but there are some widely recognized biochemical marker for osteoblast activity. Alkaline phosphatase is related to osteoblasts early differentiation, while total protein is related to the proliferation and the degree of adhesion of the cells on the substrate.

1.2.2.1. Alkaline Phosphatases (ALP)

Alkaline Phosphatases (ALP) are a group of membrane-bounded enzymes found primarily in the liver and bone. It is an important component in hard tissue formation, highly expressed in mineralized tissue cells. The primary importance of measuring alkaline phosphatase is to check the possibility of bone disease or liver disease, but there are also other associated

organs/glands such as adrenals, uterus, prostate, and intestine. The screening can be done either by using staining methodologies or by means of quantifying the level of ALP present in the supernatant or after lysing the cells. Both of the methodologies are straight forward, and can be easily implemented by using commercially available standard kits **[3]**.

1.2.2.2. Total Protein (TP)

Protein makes up roughly 50% of the volume of a bone and about one-third its mass. Total protein is a known marker of the osteoblast phenotype. Protein has been identified as being both detrimental and beneficial to bone health. Depending on a variety of factors, including the level of protein in the diet, the protein source, calcium intake, weight loss, and the acid/base balance of the diet. In the adhesion of the osteoblast on the biomaterial there are several proteins involved. During osteoblasts - implant interactions, their structure is modified according to surface characteristics of the materials involved. More precisely, the proteins play a major role in osteoblastic response to mechanical stimulation and they are of significance for the development of future biomaterials **[3]**.

Chapter 2: Biomaterials

2.1. Introduction

Aging, increased obesity, poor physical activity and accidents are bone disorders and conditions of the population being increased day by day. The traditional biological methods of bone-defect management; autografting and allografting cancellous bone, applying vascularized grafts of the fibula or iliac crest and other bone transport techniques, present short or long-term problems (e.g. laborious and lengthy processes, unpredictable bone resorption, risk of disease and/or infection which may cause a lessening or complete loss of the bone inductive factors). So, there are multiple clinical reasons to develop bone tissue-engineering alternatives, including the need for better filler materials, via the synergistic combination of biomaterials, cells, and factor therapy, which can be used in the reconstruction of large orthopedic defects and the need for orthopedic implants that are mechanically more suitable to their biological environment **[13]**.

2.2. Desired Features of a Bone Tissue-Engineering Material

Generally, a biomaterial is any substance that has been engineered to interact with biological systems for a medical purpose - either a therapeutic (treat, augment, repair or replace a tissue function of the body) or a diagnostic one. In order to concern a material as biocompatible it has to facilitate cell adhesion, spreading and potentially induce differential cell function, based on the application. Biomaterials can be derived either from nature or synthesized in the laboratory using a variety of chemical approaches utilizing metallic components, polymers, ceramics or composite materials. A biomaterial may be used *in vitro* for laboratory testing or *in vivo* for an autograft, allograft or xenograft used as a transplant material. Bone implant materials are often designed to promote bone growth while dissolving into surrounding body fluid **[14]**. Thus for many biomaterials good biocompatibility along with good strength and dissolution rates are desirable. Biomaterials must be compatible with the body, and there are often issues of biocompatibility which must be resolved before a product can be placed on the market and used in a clinical setting. Biocompatibility is related to the behavior of biomaterials in various environments under various chemical and physical conditions. The term may refer to specific properties of a

material without specifying where or how the material is to be used. For example, a material may elicit little or no immune response in a given organism, and may or may not able to integrate with a particular cell type or tissue. Immuno-informed biomaterials that direct the immune response rather than attempting to circumvent the process is one approach that shows promise **[15]**. The ambiguity of the term reflects the ongoing development of insights into how biomaterials interact with the human body and eventually how those interactions determine the clinical success of a medical device (such as pacemaker or hip replacement). Modern medical devices and prostheses are often made of more than one material—so it might not always be sufficient to talk about the biocompatibility of a specific material **[16][17]**.

2.3. Polystyrene

2.3.1. Evolution of Polystyrene

Polystyrene (PS) is considered to be the fundamental substrate for adherent animal and human cell culture for over 50 years **[18]**. Although the glass was the choice for imaging due to its lower refractive index **[19]**, the two-dimensional tissue culture polystyrene (2D TCPS) has become the basic platform for adherent cell culture due to the mass production of PS through injection molding. It is a transparent, low-cost, high-culture volume, alternative to glass, material which is compatible with many cell strains and contrast agents **[20][21]**. In the 1830s, styrene was discovered and the first observations of polymerization were documented (Figure 2-1) **[22][23]**. Major development of the plastic occurred during and after World War II with the need for a consistent synthetic rubber supply **[23][24]**. Modern applications for PS harness the inherent material properties, such as highly recyclable injection molded or thermoformed plastic, to achieve research and consumer goals **[22][25]**. The formability of the material eases manufacturing constraints, both as a compounded and pure material, due to its second-order glass transition temperature near the boiling point of water (95–105°C, with some molecular weight dependence) **[26-29]**. Nowadays, the business drove to mass-produce culture plastics, such as PS, in bio-manufacturing and cell-based research activities as an injection-molded, cast, embossed, electrospun and, more recently, three-dimensional (3D) printed polymer and move away from glass. However, the simple homopolymer lacks appropriate surface chemistry for cellular recognition: phenyl groups do not readily provide

anchoring points for cells as they are not normally expressed in the human body **[30]**. This has dictated the need to modify and develop PS-based surfaces to facilitate cell anchorage *in vitro* by incorporating surface functionality, which cells will bind to and grow on, a major theme of this review. Five decades of 2D PS spearheading *in vitro* cell culture approaches has built a foundation of knowledge, understanding mechanisms to expand cells generally **[31]**.

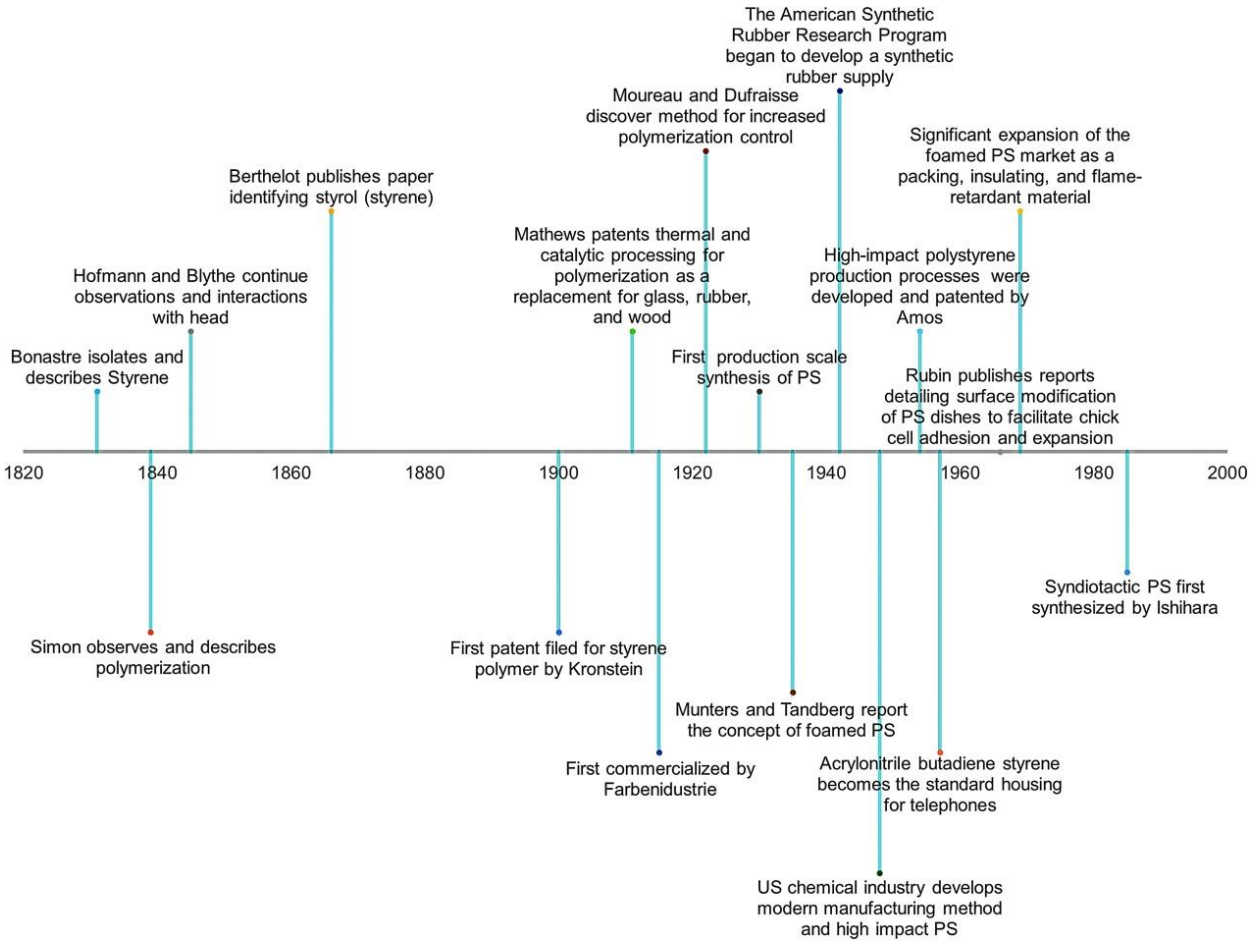


Figure 2-1 *Timeline for major developments of PS, from the initial discovery to custom-compounded polymers. PS has played a pivotal role in many industries, with nearly 200 years of research attributed to this single material* ***[31]****.*

2.3.2. 2D PS: The Basis of *In Vitro* Cell Culture

Fabricating mass-produced flat and finely topographically detailed 2D tissue culture surfaces both employ the same basic technologies: casting, embossing, or molding. With the fewest

technical and equipment challenges, industrial scale injection molding has served to fabricate parts with features traditionally at the hundreds of micron scale and above, where improvements continue to push these boundaries **[32][33]**. Since the 1970s, injection-molded PS, followed by an oxygen plasma treatment, has remained the most prolific method for manufacturing TCPS culture surfaces **[34]**. However, custom in-house injection molding equipment is generally unavailable to research groups and mass-produced TCPS may not possess surface characteristics of interest. This is not to say laboratory bench embossing is out of reach. Imprinting fine channels, through holes, and other microfluidic features have been demonstrated for cell-based assays, although it may require a 15-ton hydraulic press with heating plates capable of reaching 125°C **[35]**. Such equipment may aide in embossing PS surfaces, but is not necessary to achieve anisotropic surface behavior. Using a silicon template, nanoimprinting has been used to align osteoblasts, finding that deeper grooves improved cell alignment along the channels, and increased migration rate along 150 nm grooves by a factor of 1.46 **[36-39]**. Hot embossing has been used to create many fine features, including pillars, grooves, and microwells, which successfully spatially segregated cells by patterning the surface chemistry and topography **[40]**. As well, micro-pattern width has been seen to influence differentiation of human mesenchymal stem cells to vascular smooth muscle cells, where finer widths aid in cell alignment **[40]**. As the cells adhere to the micro-pattern, internal mechanical stresses likely act to differentiate the cell, working to match features to function **[41][42]**. However, while sequestering the cells with micro-channels may be beneficial to guide cells, the reduction in cell spreading and change in morphology may also generate genetic abnormalities (such as forming micronuclei) **[43][44]**, making cell culture platforms with relevant length scales larger than the cells still more desirable. With this quality in mind, casting and embossing remain two of the more reproducible means to produce PS parts, especially on the industrial scale with injection molding equipment, and remain the method of choice for consumer and industrial parts. The major drawback to casting is the prohibitive cost of the tooling and equipment, limiting the most practical uses to large volume part production. On the laboratory bench, the lack of specialized tooling or standardized technique can result in poor part resolution and high cycle times. Other fabrication methods, such as electro-spinning and 3D printing, provide alternate ways to achieve resolution that cannot be accomplished by casting and embossing with the advantage of developing complex 3D structures. Following fabrication, PS must be surface treated to facilitate cell adhesion. Over time, these approaches have evolved **[31]**.

2.3.3. The Transformation of PS to TCPS

Custom growth surfaces used for *in vitro* cell deposition continue to evolve, using material properties to modulate adhesion and testing specific cell types **[31]**. Transforming native PS surfaces to include chemistry other than phenyl groups can increase the hydrophilicity and surface charge, modulating the deposition of extracellular matrix, cells, and proteins in order to incite cell adhesion **[45-47]**. In general, functionalization methods can be broadly divided into two groups: liquid phase and plasma-based treatments. Liquid phase treatments have the advantage of quick large surface treatments elaboration and the disadvantage of limited functionality due to surface oxidation and often require highly corrosive substances. On the other hand plasma-based treatments most commonly used to manufacture TCPS in bulk, broaden the surface chemistries achievable, but require ionizing energy to modify the surface. New surface modification techniques provide targeted cell interaction mechanisms, by grafting specific binding regimes, such as DNA and proteins, but may have long-term stability issues and higher costs limiting their broad appeal. A discussion of these techniques along with the treatment fluid choices will examine mechanisms for depositing specific chemistry, modulating contact angle, and roughening surfaces to facilitate cell adhesion, extracellular matrix deposition, and cell expansion **[31]**.

2.4. Titanium

2.4.1. Titanium in Medical Applications

Increased use of titanium and its alloys as biomaterials comes from their properties; good biocompatibility, high strength to weight ratio, low density, and improved corrosion resistance with good plasticity and mechanical behaviour close to those of bones. Light, strong and totally biocompatible, titanium is one of the few materials that naturally match the requirements for implantation in the human body. The mainly used materials as hard tissue replacements in artificial bones, joints and dental implants are the commercially pure titanium (cp Ti, grade 2) and Ti-6Al-4V (grade 5) alloy **[48][49]**. As a hard tissue replacement, the low elastic modulus of titanium and its alloys is generally viewed as a biomechanical advantage because the smaller elastic modulus can result in smaller stress shielding. Another criterion for the excellent biocompatibility and corrosion resistance of titanium and its alloys is the

spontaneously formation of an extremely thin, adherent, protective titanium oxide film in the passivation or repassivation process. Concerning the medical applications of these materials, the use of pure titanium is more limited to the dental implants because of its limited mechanical properties whereas Ti-6Al-4V alloy is being used for better mechanical characteristics needed such as in knee implants, hip implants, plates and bone screws. One of the most common applications of titanium alloys is artificial hip joints that consist of an articulating bearing (femoral head and cup) and stem **[50]**, where metallic cup and hip stem components are made of titanium. As well, they are also often used in knee joint replacements, which consist of a femoral and tibial component made of titanium and a polyethylene articulating surface **[51]**.



(a) (b)

Figure 2-2 *Schematic diagram of (a) artificial hip joint and (b) knee implant* ***[51]****.*

2.4.2. Surface Treatments of Titanium (Ti) and Titanium Alloys

Surface engineering can play a significant role in improvement of titanium's natural capabilities, such as the tribological behaviour, corrosion resistance and osseointegration of the implant. Surface modifications can be applied by different techniques; surface oxidation, thermo-chemical surface treatments like boriding, carburizing and nitriding, as well as physical deposition methods such as plasma spray coatings and ion implantation are some of the techniques **[52][53]**. Surface topography plays a fundamental role in regulating cell behaviour (adhesion, orientation, shape of cells), so great efforts have been devoted to thickening and stabilizing surface oxides on titanium to achieve desired biological responses.

Oxidation is the most popular technique for the surface modification of Ti alloys, in which oxide layers on titanium are commonly produced by either electrolytic anodizing **[54]** or heat treatment **[55-57]**. Thermal oxidation results in the formation of a 15-30 μm thick titanium dioxide layer of the rutile phase. However, due to their long-term high temperature action, thermal diffusion processes can also lead to the formation of a diffusion sub-layer consisting of an oxygen solid solution in α-Ti, and development of phase segregation and coalescence which may cause substrate embrittlement and worsened mechanical and/or corrosion performance **[51]**.

Conventional anodic oxidation generates thin films of amorphous hydrated oxide or crystalline TiO_2 in the anatase form that exhibit poor corrosion resistance in some reducing acids and halide solutions, while rutile generally possesses much better protective properties **[58]**. However, recent developments in high voltage anodizing allow the production of crystalline rutile/anatase films at near to ambient temperature **[59]**. By anodic oxidation the micro-topography can be varied through regulating electrolyte and electrochemical conditions because elements such as P and Ca can be imported into the surface oxide on titanium. *In vivo* data show implant surfaces containing both Ca and P enhance bone apposition on the implant surface **[51]**.

Furthermore, there are alternative methods to improve the biocompatibility such as Diamond-Like Carbon (DLC) coatings, biocompatible chemicals **[60]** and materials such as ceramics for coating. DLC coatings basically consist of a mixture of diamond (sp3) and graphite (sp2). DLC films are attractive biomedical materials due to their relatively high hardness, low friction coefficient, owing to the solid lubricant because of its graphite and amorphous carbon contents **[61]**, good chemical stability and excellent bio and hemocompatibility **[62-65]**. Phosphoric acid was used as modification of titanium and results indicated that pretreatment of the implant with phosphoric acid caused no citotoxicity to the osteoblasts **[66]**.

Hydroxyapatite (HA) coating is another proven method for high osseointegration and implant mechanical bonding improvement **[67-68]**. The process of osseointegration depends upon the surface properties such as surface chemistry, surface topography, surface roughness and mainly the surface energy. In general, the higher the degree of osseointegration, the higher is the mechanical stability and the probability of implant loosening becomes smaller. TiO_2, calcium phosphate, titania/hydroxiapatite composite and silica coating by the sol-gel method can be applied on the surface of the titanium and titanium alloys. Micro-Arc Oxidation (MAO) or Plasma Electrolytic Oxidation (PEO) technique is used to synthesize TiO_2 layer. This technique is based on the modification of the growing anodic film by arc micro-

discharges, which are initiated at potentials above the breakdown voltage of the growing oxide film and move rapidly across the anode surface. This technology provides a solution by transforming the surface into a dense layer of ceramic which not only prevents galling but also provides excellent dielectric insulation for contact metals, helping to protect them against aggressive galvanic corrosion. PEO process transforms the surface of titanium alloys into a complex ceramic matrix by passing a pulsed, bi-polar electrical current in a specific wave formation through a bath of low concentration aqueous solution. A plasma discharge is formed on the surface of the substrate, transforming it into a thin, protective layer of titanium oxide, without subjecting the substrate itself to damaging thermal exposure **[51]**.

Among all the above mentioned surface treatments, Diamond-Like Carbon coating and Plasma Electrolytic Oxidation are the most promising ones applied on titanium surfaces **[51]**.

Chapter 3: Cell-Material Interaction

3.1. Introduction

Like all multicellular organisms the human body is structured following a hierarchical scale of levels of complexity. Our body consists of 10^{13} cells, classified into more than 200 different cell types; similar cell types together with extracellular matrix (ECM) form tissues and tissues form organs. Within a tissue, cells adopt well-defined positions, orientations, morphologies, and gene expression patterns. This hierarchy of cells and ECM leads to physiological processes, such as development, angiogenesis, wound healing and tissue maintenance and not malfunction and disease of cells. Researches have shown that cells communicate with each other and their environment to build-up organized structures through many different channels. Cell responses like adhesion, proliferation, differentiation, spreading and migration have been related to both specific (e.g., specific molecules or molecular structures) and nonspecific (e.g., hydrophilicity/hydrophobicity, wettability and charge) surface chemical properties **[69]**, physical surface properties (e.g., topography, roughness, elasticity, pattern dimensions, order) and specific immobilized cell proteins (e.g., laminin) that promote cell adhesion to artificial surfaces **[70] [3]**.

3.2. The Cellular Environment

In order to create a man-made material that can imitate precisely the properties of the natural cellular environment, we have to understand cellular interactions with their environment. This includes both the natural cellular environment and the synthetic bio-interface that is created when bringing an artificial material in contact with living cells. The interaction of cells with their natural environment is highly complex and dynamic, involving a host of interconnected signalling pathways **[69][71][72]**To mimic even part of these interactions, the composition and properties of the natural cellular environment and the communication pathways between the cell and its surroundings have to be understood **[70]**.

3.2.1. Extracellular Cell Matrix (ECM) Composition and Interactions

Cell's environment is heterogeneous and dynamic, consisting of a large number of proteins (forming the ECM) and freely diffusing molecules that mediate interactions between neighbouring cells. The interaction of cells with their local environment and other cells is essential for the structure and cohesion of tissue and affects cellular processes like proliferation, differentiation, spreading and migration. This interaction is mediated by proteins and carbohydrates present in the cell membrane and by the secretion of soluble factors into the extracellular environment, for example, via exocytosis. There are different types of proteins participating in cell-cell and cell-ECM communication. 1) There are membrane-bound molecules, restricted to the immediate surroundings of the cell, but their secreted, soluble factors may interact with both the local and the wider cellular environment. 2) Neighbouring cell to cell interactions are mediated by cell junction proteins like ephrins and cadherins that are involved in cell–cell adhesion, signal transduction and mechanical coupling between cells. 3) There are membrane proteins such as fibronectin, vitronectin and laminins who display binding sites for other proteins, including growth factors and adhesion molecules from the cell membrane. The interaction of cells with the ECM is mediated by membrane receptors such as integrins (Figure 3-1), heterodimeric membrane proteins composed of α- and β- subunits whose specific pairing determines the affinity of the integrin to ligands presented by ECM proteins. 4) In addition to membrane proteins, membrane bound carbohydrate conjugates also contribute to the interaction of cells with their surroundings. The carbohydrate conjugates (glycoproteins, glycolipids and proteoglycans) in the cell membrane form a layer of varying composition and thickness around the cell's exterior, the glycocalyx, that is indicated by the proteoglycans. Through these membrane- bound carbohydrates, cells are able to establish connections with other cells and the ECM. Cells do not only respond to cues presented by their environment; they also actively remodel it. Through exocytosis and other pathways, cells secrete molecules that reshape the ECM and interact with other cells (e.g., collagen and fibronectin are cell-secreted proteins that contribute to the construction of the ECM) **[70]**.

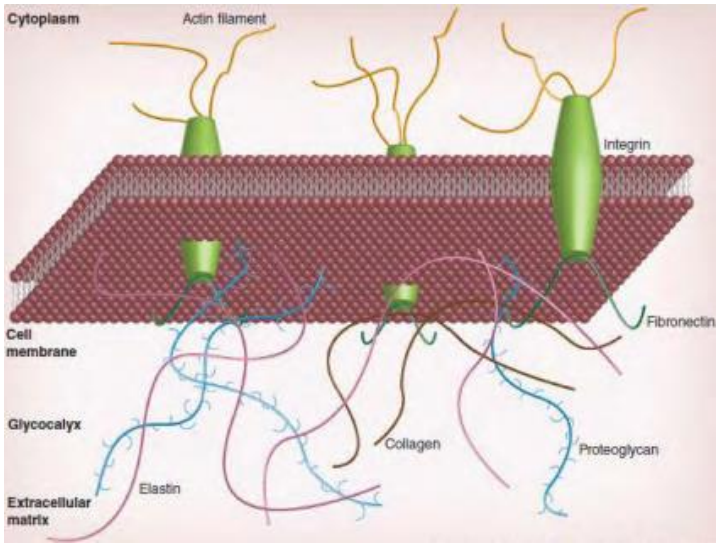


Figure 3-1 *Schematic of a cell membrane showing the connection between actin filaments of the cytoskeleton and the extracellular matrix through integrins* ***[73]****.*

The chemical and physical properties of the ECM, both spatially and temporally are affected by dynamic changes in the composition and structure of the ECM. Changes in the type of functional groups presented by the ECM, the mechanical properties and the topographical structure of the ECM can result in alterations in cell adhesion and other cellular processes. This dynamic behaviour is an integral part of a functioning biological system and underlies essential biological processes including stem cell differentiation and cell migration, as well as the development of diseases **[70]**.

3.2.2. Cells Adhesions

Adhesion-dependent cells rely on the formation of cell adhesions to survive and function. Understanding the mechanisms by which the cell anchors to its surroundings is essential to designing and engineering functional bio-interfaces. The role of cell adhesion is mostly studied in 2D surfaces and is suggested to be different in a 3D culture environment. The formation of cellular adhesions has been described in four distinct stages: surface recognition stage; early attachment stage; intermediate attachment stage and late adhesion or cell spreading stage. These ultimately result in the formation of mature adhesions (molecular complexes referred to as focal adhesions) through which integrins establish connections between the cell and their surroundings. These processes are dynamic and reversible. Cell

migration, for example, requires the controlled assembly and disassembly of focal adhesion complexes to enable the cell to make and break adhesions with its surrounding. The cytoplasmic components of integrin molecules are connected to the actin cytoskeleton via intermediate proteins such as talin. Focal adhesion complexes thus contribute to both the formation and regulation of cell–material interactions and translate information about mechanical, chemical and topographical cues from the extracellular to the intracellular environment. The properties of the cytoskeleton affect essential cellular processes such as elasticity, migration, division and differentiation. The focal adhesion-mediated connection between the cytoskeleton and the local cellular environment provides a direct link between the cell and the ECM **[70]**.

3.3. Bio-interface Properties

The introduction of artificial materials into a living, biological environment either *in vivo* as biomedical implants and devices or *in vitro* as cell culture materials affects the cellular environment and consequently the cellular function and processes. These materials may be metals, polymers, ceramics and glasses. The selection of a suitable material has to include both bulk and surface material properties. In the present context, we deal only with material's surface because it's the first point of contact with the cell and has an important role in determining the biological response. When brought into contact with live cells, the material surface is rapidly coated by proteins, so the cell–material interaction is typically mediated by a surface adsorbed protein layer and cells rarely interact with the material surface directly.
A multitude of factors, like protein size, specific protein–surface interaction, solution protein composition and concentration, determine the composition and properties of the protein layers absorbed onto a surface. On cell adhesive surfaces, the resulting protein layer typically includes cell adhesion promoting proteins such as vitronectin, fibronectin, laminin and fibrinogen **[70]**.

3.3.1. Chemistry, Topography and Elasticity of the Substrate

The properties (e.g., type, concentration, distribution and motility) of the cell surface absorbed proteins depend on the substrate surface properties layer and thus provides control over the response of cells to the biomaterial. Three surface properties have been identified as main

determinants of cell behaviour and cell fate: surface chemistry, surface topography and surface elasticity or stiffness **[69]**.

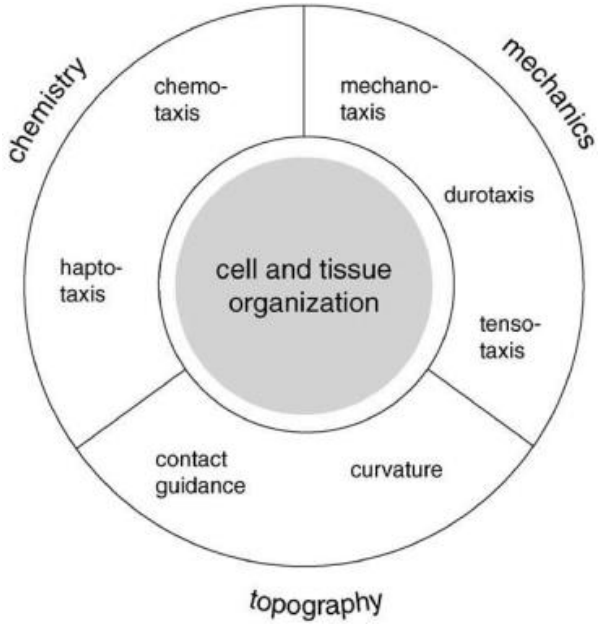


Figure 3-2 *An overview of known factors influencing cell and tissue organization. The main mean of communication is release and capture of biochemical molecules. In cell migration, this leads to chemotaxis, that is directed movement along a chemical gradient* ***[74]****.*

Many different cell types employ chemotaxis and direct their movements according to certain chemicals in environment. In the context of tissue organization, chemotaxis is involved for example in morphogenesis, angiogenesis, and wound healing. By using the lock-and-key principle of ligand-receptor binding, cells can exchange very specific information. When cells are suspended in solution, they can capture signaling molecules which are distributed according to the physical laws of diffusion and reaction. In contrast, when cells adhere to an extracellular structure, in addition they can bind biochemical ligands which are attached to external surfaces, for example to the plasma membrane of other cells or the proteins and sugars of the ECM. Therefore, in cell adhesion, the biochemical information is supplemented by several additional degrees of information, including spatial distributions of ligands which are not determined by diffusion and the topographical and mechanical properties of the structure the cell attaches to **[69]**.

Weiss was the first to observe that cells preferentially orient along ECM-fibres, an organization principle he termed contact guidance. Moreover, he observed that tissue explants

condense the collagen gel between them into aligned parallel fibre bundles, which then guide cell migration out of the explants. Contact guidance therefore could serve as a large-scale organization principle in tissue development by guiding motile cells along aligned ECM-bundles. Although Weiss associated contact guidance with differential adhesiveness and interfacial tensions **[69][75]**, the term has now gained a strong topographical connotation. In 1976, Dunn demonstrated that cells react to surface curvature and prefer to align along the axis of minimal curvature, where minimal distortion of the cytoskeleton occurs. This mechanism also favors orientation of cells along fiber bundles. Contact guidance also manifests itself as cell alignment along micro-fabricated grooves. During recent years, it has also been shown that even 1topographical features in the nanometer range influence cell behavior. It is important to note, though, that contact guidance through elongated topographic features provides only a bidirectional cue for cell migration **[69]**.

In contrast, a unidirectional cue can originate from spatial variations in adhesiveness, for example by immobilized ligand gradients (haptotaxis) **[69]**. Contact guidance and haptotaxis were among the first cell organizing principles discovered for adherent cells that attributed a role to physical cues in the environment. In addition, it has long been known, especially in the medical and bioengineering communities, that certain cell functions are strongly determined by mechanical stimuli. For example, endothelial cells in blood capillaries, osteocytes in bone and alveolar cells of type II in lung only function properly when subject to a certain level of strain indicating proper body functioning (namely the one resulting from blood pulsation, body movements and breathing, respectively). A widely used method to study these effects is the use of cyclically stretched substrates (typically with a physiological frequency of 37 1 Hz). These studies showed that cyclic mechanical forces induce progression through the cell cycle. In regard to changes to the cytoskeleton, it was found that cells tend to orient away from the direction of stretch, presumably because they try to avoid the perturbations arising from the passive deformations. In order to study the underlying mechanotransduction processes in more detail, different experimental techniques have been used to apply external forces to single cells in a localized and well controlled way, including magnetic twisting cytometry (see article by Dobson in this issue) and use of micropipettes. In these studies, forces were applied to integrin-based cell-matrix contacts, and the main effect found was contact upregulation, that is structural reinforcement and increased signaling. In general, a large body of evidence now demonstrates that external forces trigger different signaling cascades determining cell behavior **[69]**.

Cells not only react in a specific way to external forces; they also use actively generated internal forces in order to explore the properties of their environment (active mechanosensing). Using fibronectin-coated beads held in an optical trap, it was shown that cells strengthen integrin mediated contacts to the beads in proportion to the force restraining it. The authors concluded that this mechanism might allow cells to navigate in the extracellular matrix in response to its mechanical resistance, a phenomenon they termed mechanotaxis. During recent years, this notion has been confirmed by several other experiments. In particular, the sophisticated use of elastic substrates has shown that cells sense even purely elastic features in their environment. Elastic substrates for the study of cell traction have been introduced in the early 1980s by Harris and coworkers, who found that anchorage-dependent cells like fibroblasts show a remarkable degree of mechanical activity and that they react to mechanical changes in their environment caused by traction of other cells. In another early study with elastic substrates, it was shown that fibroblasts actively sense and align parallel to the axis of stretch of prestrained elastic substrates. More recently, it was found that cells more strongly upregulate cytoskeleton and cell-matrix adhesion on stiffer substrates and locomote in favor of stiffer or strained substrates, a phenomenon which has been termed durotaxis. The underlying process is active mechanosensing through cell-matrix contacts, which have been shown to transduce force biochemically. Conceptually, durotaxis should be distinguished from cell migration in favor of strained substrates, because in general, rigidity and prestrain are not equivalent. Therefore, the later effect might be termed tensotaxis, a term which has been proposed to describe the migration of ectodermal cells in favour of strained regions in Xenopus embryos. .A similar phenomenon has been observed for neutrophil migration in a physiological hydrogel **[69]**.

While traditionally much attention has been focused on the role of biochemical determinants of cell organization, the results described above clearly show that physical cues like topography, forces or the mechanical properties of the environment might be equally important for cellular decision making. Recent advances in materials science (including microcontact printing, soft lithography, microfluidics, and nanotechnology) now provide tools to study the physical determinants of cell organization in much more detail than formerly possible. Moreover, they allow to design new artificial and biomimetic environments for cells, which open up new perspectives for biomedical applications, in particular in tissue engineering. It is important to note that these tools allow not only for better control, but also for quantification of cell adhesion, for example of spreading area as a function of ligand density and substrate rigidity. There are two main reasons why quantification is essential in

this field. First, it allows to single out the relevant mechanisms from a large list of possible explanations which emerge from the intrinsic complexity of biological systems. Second, quantification might be essential for guiding the design process for artificial cellular environment, because there are too many degrees of freedom as to succeed simply by trial-and-error **[69]**.

3.3.2. Growth Factor

Stem cells are influenced by interior and exterior forces, either being cultured *in vitro* or *in vivo*. This type of cells has more force-coupled signaling pathways to adjust itself to environments that range physically, from solid tissues of varied elasticity to flowing fluids and strained tissues. This suggests that carefully made materials can prime the expansion of specific progenitors. For example, myogen markers of MSC are upgrated when grown on firm gels that mimic the elasticity of muscle and being coated with collagen-I, whereas MSC present osteogenic behaviour, when grown on rigid gels that mimic pre-calcified bone. As for NSC, differentiation into glial is promoted on harder matrices that typify glial scars, whereas neuron differentiation is favored on soft matrices that mimic normal brain. (Figure 3-3) In general, in a gradient of elasticity, cells are found to adhere, spread, assemble their cytoskeleton (Figure 3-4), and anchor more strongly to stiff substrates compared to soft substrates in a process called ‘durotaxis’, which might constitute a biophysical basis for why MSC home to sites of injury and fibrosis **[76]**.

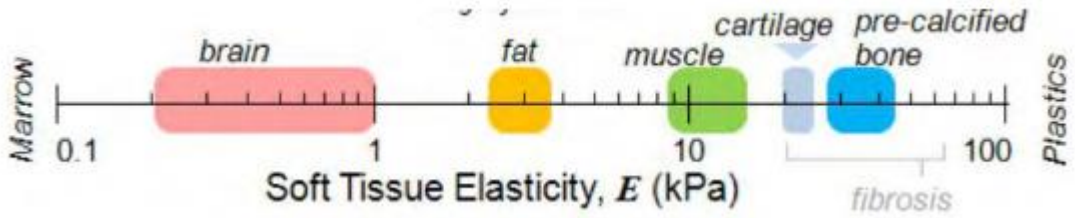


Figure 3-3 *Soft tissue elasticity scale ranging from soft brain, fat, and striated muscle, to stiff cartilage (E ~ 20–30 kPa at the scale of adhesions) and precalcified bone* ***[76]****.*

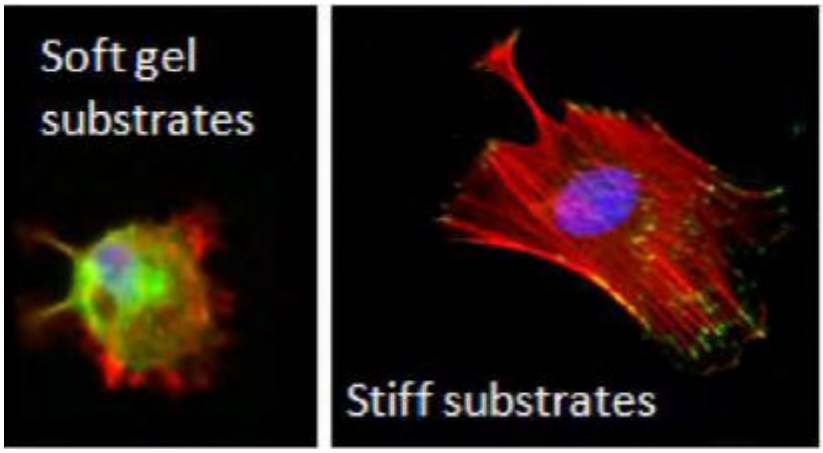


Figure 3-4 *In vitro substrates that mimic soft and stiff tissue microenvironments show that cells anchor more strongly to stiff substrates, building focal adhesions and actin-myosin stress fibers* ***[76]****.*

Conventional cell injections or infusions present poor delivery and retention of cells at the intended site or cell death due to loss of anchorage (anoikis), so the development and use of material systems that create specialized niches for the cells is required. Vehicles for stem cell transplantation, including synthetic polymers and naturally-derived materials, were developed, providing control over the presentation of adhesion sites (e.g., density of peptides to bind integrins) for interacting cells and participating in tissue regeneration. There has not yet been a demonstration that substrate mechanical properties regulate stem cell fate *in vivo*, although the demonstration that gel mechanical properties regulate capillary formation *in vivo* supports this concept. One complication is that the mechanical properties and degradation rates of synthetic niches are typically coupled, and the degradation rate has been demonstrated to enhance bone regeneration by transplanted stem cells **[76]**.

Growth factors (or cytokines) can be provided in a localized manner either from the material scaffolds used as synthetic stem cell niches or with controlled release particle systems. Materials presenting angiogenic factors can enhance local vascularization, and increase the survival of transplanted stem cells and subsequent regeneration. Potentially useful cell populations already exist in the body and attracting these cells to a desired anatomic site has the potential to provide new therapeutic options. Cost and complexity should be reduced compared to approaches with ex vivo bioreactors. With bone repair for example, implantation of simple biomaterials without growth factors has shown how potent the *in vivo* milieu can be in generating native-like bone, in spite of risks of implantation injury. Mobilization of

endothelial progenitor cells from bone marrow with either G-CSF or with drugs such as Statins is also being tested in ischemic conditions **[76]**.

3.4. Biomaterial Evolution

Biomaterial research started 50 years ago and as time went on, the definitions and requirements for a biomaterial have changed considerably. Initially, the focus in biomaterial selection was on biocompatibility, with the goal to completely suppress or reduce cell–material interactions to minimize undesired responses (e.g., toxicity, inflammation) of cells or tissue to the material. An ongoing need to improve the performance of biomaterials and mimic the dynamic processes within the ECM more closely led to the emergence of responsive biomaterials (or smart biomaterials) that are able to change their properties dynamically and, in some cases, reversibly in the presence of an external stimulus. In this case, information flow proceeds from the environment through the material to cell. The inverse process, where information about the cell is collected by the material and transduced to an external processing unit represents processes in sensing devices **[70]**.

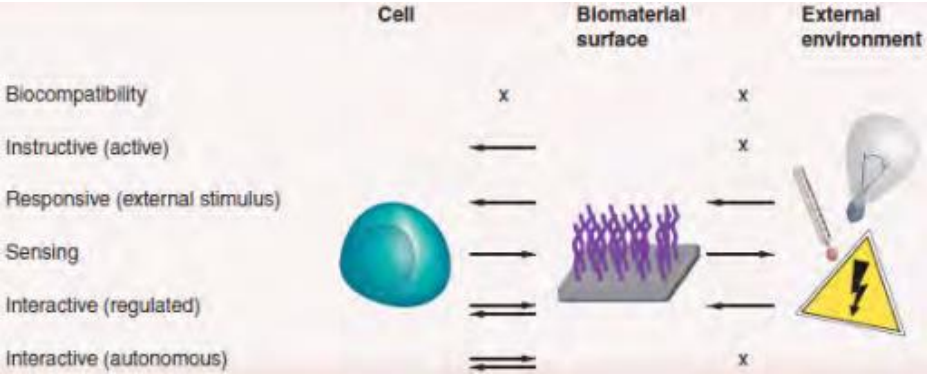


Figure 3-5 *Interaction pathways for biomaterial surfaces with cells and the external environment* ***[70]****.*

Modern biomaterials show high functionality and complexity, but they are still unable to mimic the complex and intricate interactions of a natural cellular environment. The role of the ECM is to provide signals to the cell and also respond to the cell, for example, via enzyme catalyzed degradation of the ECM. By contrast, most biomaterials to date are cell-instructive, in other words, they are designed to elicit a specific desired response from the cells with the

aim to control cellular processes. In a landmark study, a synthetic polymer hydrogel, cross-linked via enzyme cleavable peptide sequences, mimicked the enzyme responsiveness of the ECM. The polymer hydrogel was sensitive to cell-secreted enzymes, establishing the concept of cell responsive biomaterials. Subsequently, enzyme responsiveness was also accomplished on 2D surfaces, leading to the development of peptide surfaces that respond to cell-secreted phosphatase. This now paves the way for interactive biomaterials wherein both the material and the cells are able to dynamically respond to stimuli presented by the respective other system. For the design of interactive interfaces, we distinguish two types of interfaces which we label externally regulated and autonomous, respectively. If external stimuli are able or indeed required to control the interfacial interactions, the interface is externally regulated. In contrast, autonomous interfaces are able to perform their function completely self-sustained and independent from external factors. Next generation of biomaterials should provide increased biomaterial functionality through a more seamless integration of the artificial materials with the biological surrounding. While this development has already begun for bulk materials, the design of biologically responsive surfaces able to provide an interactive cell–material interface (interactive biomaterials) will present major challenges and opportunities **[70]**.

Chapter 4: *In vitro* osteoblasts cultures – biocompatibility test

4.1. *In vitro* culture

Human body is structured following a hierarchical scale and we can distinguish "components" working in such a way that you can call the whole team "a unit". For example within a cell there are lots of different organelles, working in such a way that we can call the whole company "a cell". The structure of the system is the description of the system as a special arrangement of matter whereas the organization of the team is the way it works following always the same rules. In our researches, we can study "units" into which there are lots of "components" working following always the same rules, which "units" are at the same time, a "component" of a more complex team. For example teams of cells ("units") of different types may work following a higher level of organization, as "components" of a new, more complex "unit", i.e. the "tissue" level **[77].**

All living beings are organized respecting the same growing scale of "levels of complexity", molecular, macro-molecular, subcellular, cellular, tissue, organic, systemic, individual, population, communitary, ecosystemic. Also in all living beings, when there is an action into a given level of complexity, there is always a reaction manifested or expressed at a higher level that tends to neutralize the change that took place at the lower level, depending on the magnitude of the initial change and the ability of the "system" to react against it. So if we limit our investigation within just one of the lowest levels of complexity (e.g., cellular), artificially blocking any possibility to affect the organization of the components of units structured at higher levels of complexity (e.g., tissue), then we are forcing our system to work following a totally different organization compared with the natural behaviour of the same elements when they are integrated into a whole living being. The "big question" involved here is the classical fight between "reductionists" who try to discover the "first causes" of natural phenomena, and the "holists" who prefer to investigate the "real" behaviour of integrated living beings. Theoretical Physicists are adding lots of pepper to this salad currently **[77]**.

The application of *in vitro* models in evaluating the toxicity of toxicants has enhanced our limited understanding of drugs and chemical-induced toxicity. The advantages of the *in vitro* models for consistent, rapid, cost-effective toxicity testing and no animal-testing are of major

importance. Moreover, *in vitro* tests are valuable for screening of chemicals, drugs, toxicants, pesticides, etc. In the recent decades, *in vitro* methods have been routinely used as these can be correlated with the *in vivo* studies and can help in understanding a specific *in vivo* response in any given species. Optimization of cell culture is the primary concern to *"in vitro"* studies as it includes handling of cells and tissues outside the body under controlled conditions. *In vitro* cytotoxicity testing, biotransformation, and mechanistic investigations based studies can be effectively used as a cost-effective method for determination of toxicity **[78]**.

In vitro test systems are extremely simplified form of very complex *in vivo* systems. Therefore, there is always a limitation with extrapolating the *in vitro* data with *in vivo* studies in toxicological situations. The biggest problem with in vitro systems is the lack of biotransformation studies **[79]**.

As compared to the real world, the culture condition used in *in vitro* test systems are not homeostatic, i.e., accumulation of waste products, depletion of nutrients and limited dissolved oxygen supply, which sometimes results in anaerobic culture. As compared with the actual tissue situation, the cell density in *in vitro* culture is less than 1%, which disrupts intracellular signaling and the simulated non-physiological conditions like relatively different body temperature as in animals, different blood electrolyte concentration, and the extracellular matrix. Special consideration should be given to characterize *in vitro* cell-based assays so as to develop a possible understanding of the reaction in each cell of a particular assay **[80]**.

4.2. Definition of a Cell Culture

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established **[81]**.

4.3. Primary Cell Culture and Passages

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be sub-cultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for

continued growth. Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; these cell lines are known as finite **[81]**.

4.3.1. Culture Conditions

Culture conditions vary widely for each cell type, but the man-made environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physicochemical environment (pH, osmotic pressure, temperature). Most cells are anchorage dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture). In this study we deal with osteoblasts that are attached in the solid substrate when cultured **[81]**.

4.3.2. Applications of Cell Culture

The major advantage of using cell culture for any of the applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. More specifically, cell culture provides a great model system for studying the biochemistry of cells (e.g., metabolic studies, aging) and normal physiology, the effects of toxic compounds and drugs on the cells, mutagenesis and carcinogenesis. It is also used in drug screening, development, and large-scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins) **[81]**.

4.3.3. Media

The culture medium is the major component of the culture environment. It provides the necessary nutrients and growth factors, as well as regulating the osmotic pressure and the pH of the culture. Initial cell culture experiments were performed using natural media obtained from body fluids and tissue extracts, but the need for standardization, media quality, and increased demand led to the development of defined media. The three basic classes of media are serum-free media, reduced-serum media, and basal media, which differ in their

requirement for supplementation with serum. Serum is important as a source of growth and adhesion factors, lipids, hormones and minerals for the culture of cells in basal media. Serum also regulates cell membrane permeability, trace elements into the cell and serves as a carrier for lipids, enzymes, micronutrients. However, disadvantages of serum in the media are: high cost, problems with specificity, standardization, variability, and unwanted effects such as inhibition or stimulation of growth and/or cellular function on certain cell cultures. Moreover, to avoid the risk of contamination, all products, including sera, should be obtained from reputable source, tested for contamination and guaranteed for their consistency, quality, safety, and regulatory compliance **[81]**.

4.3.4. Exhaustion of Medium

A drop in the pH of the growth medium usually indicates an increase of lactic acid, which is a by-product of cellular metabolism. Lactic acid can be toxic to the cells, and the decreased pH can be sub-optimal for cell growth. The rate of change of pH is generally dependent on the cell concentration so cultures at a high cell concentration exhaust medium faster. We should subculture cells in a rapid drop in pH (> 0.1–0.2 pH units) with an increase in cell concentration **[81]**.

4.3.5. CO_2

The growth medium buffers the cells in culture against changes in the pH. Usually, this buffering is achieved by including a CO_2-bicarbonate based buffer. Because the pH of the medium is dependent on the delicate balance of dissolved carbon dioxide (CO_2) and bicarbonate (HCO_3 –), changes in the atmospheric CO_2 can alter the pH of the medium. Therefore, it is necessary to use exogenous CO_2 when using media buffered with a CO_2-bicarbonate based buffer, especially if the cells are cultured in open dishes or transformed cell lines are cultured at high concentrations. While most researchers usually use 5–7% CO_2 in air, 4–10% CO_2 is common for most cell culture experiments. However, each medium has a recommended CO_2 tension and bicarbonate concentration to achieve the correct pH and osmolality; refer to the media manufacturer's instructions for more information **[81]**.

4.3.6. Temperature

The optimal temperature for cell culture depends on the body temperature of the host from whom the cells were isolated, and to a lesser degree on the anatomical variation in temperature (e.g., temperature of the skin may be lower than the temperature of skeletal muscle). Overheating is a more serious problem than underheating for cell cultures, so the temperature in the incubator is set slightly lower than the optimal temperature. Most human and mammalian cells are maintained at 36°C to 37°C for optimal growth **[81]**.

4.3.7. Subculturing

Subculturing, or passaging, is the removal of the medium and transfer of cells from a previous culture medium into a fresh one, a procedure that enables the further propagation of cells. Adherent cultures should be passaged when they are in the log phase, before they reach confluence. Normal cells stop growing when they reach confluence (contact inhibition), and it takes them longer to recover when reseeded **[81]**.

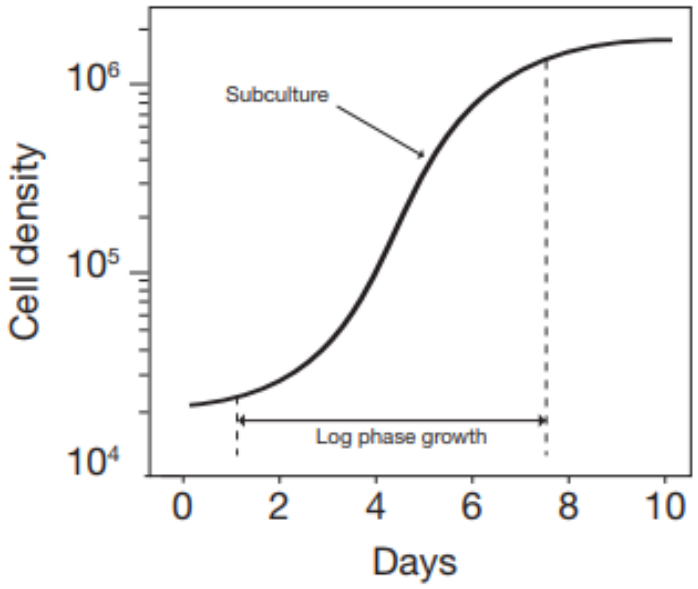


Figure 4-1 Characteristic Growth Pattern of Cultured Cells **[81]**.

4.3.8. Characteristic Growth Pattern of Cultured Cells

Cells in culture usually proliferate following a standard growth pattern (Figure 4-1). The first phase is the lag phase, after the culture is seeded it is a period of slow growth when the cells are adapting to the culture environment, preparing for fast growth. The second phase is the log phase (i.e., logarithmic phase), where the cells proliferate exponentially consuming the nutrients in the growth medium. When the cells occupy all of the available substrate or when all the growth medium is spent, the cells enter the stationary phase (i.e., plateau phase), where the proliferation is greatly reduced or ceased. When the cells in adherent cultures occupy all the available substrate and have no room left for expansion, or when the cells in suspension cultures exceed the capacity of the medium to support further growth, cell proliferation is greatly reduced or ceases entirely (Figure 4-1). To keep them at an optimal density for continued growth and to stimulate further proliferation, the cells have to be subcultured and fresh medium should be supplied **[81]**.

4.4. Cell Counting

The Neubauer counting chamber is a reliable way to calculate the concentration of cells in the medium overall, by observing a small percentage of fluid. More specifically, the Neubauer counting chamber is a type of hemocytometer, consisting of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. By observing a defined area of the grid, it is therefore possible to count the number of cells **[82]**. The ruled area is 3mm2 divided into 9 large squares each with a 1 mm2 area. The large central square (which can be seen in its entirely with the 10X objective), is divided into 25 medium squares with double or triple lines. Each of these 25 squares is again divided into 16 small squares with single lines, so that each of the smallest squares has an area of 1/400 mm2 **[83]**.

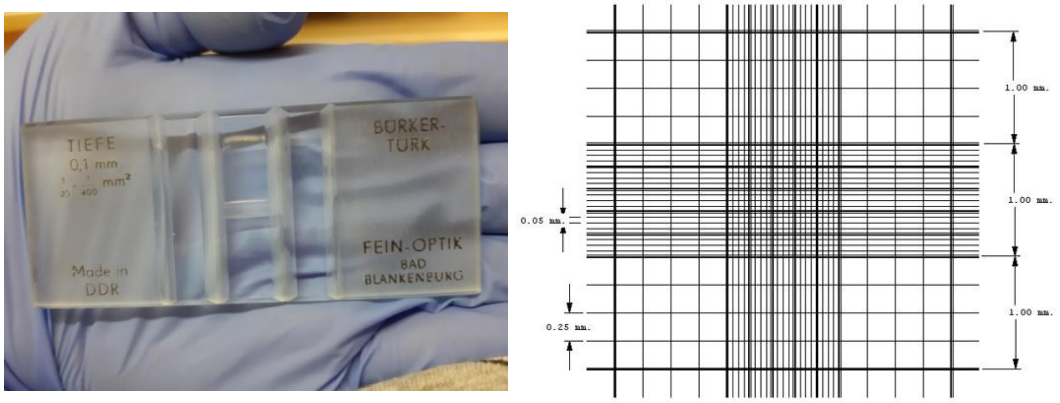


Figure 6-10 *(a) Neubauer used for cell counting. (b) The ruled area inside the Neubauer chamber.*

4.5. Osteoblasts

Osteoblasts are derived from mesenchymal stem cells. The biocompatibility of implantable materials has been evaluated using different methods and models, in particular *in vitro* studies using osteoblast cell cultures. During the process of differentiation, these cells pass through a number of distinct stages that can be characterized by the pattern of expression of osteoblastic marker proteins **[84]**. The isolation of osteoblast cells from residual bone obtained during an orthopedic surgery may be difficult and needs repeated trials. The earliest report to describe the isolation of viable cells from adult human bone is that of Bard et al **[85]**. The isolation procedure they employed involved extensive demineralization of the tissue in a solution of ethylenediaminetetraacetic acid (EDTA) followed by digestion with collagenase. The cells obtained expressed low levels of alkaline phosphatase and remained viable for periods of up to 2 weeks, but failed to proliferate leading the authors to conclude that osteocytes were the predominant cell type present **[86]**.

Osteoblasts and their precursors are known as resistant cells, with strong adaptation capacity, which is one of the reasons of their use in biocompatibility studies. However, standard procedures to test the substrates are not yet available. Cells development, reproduction and homogeneous spreading on the entire tested surface of the substrate are clearly influenced by the initially deposited number of cells. Cells kinetics is saddled *in vitro*, comparing to *in vivo* where physiological body fluids as well as other factors enhance all their functions and their communication with similar or different bone tissue component cells. Some aspects related to cells deposition on substrates, especially on rough materials such as orthopedic metals and

alloys should be adjusted. Moreover, various numbers of osteoblasts ranging from 5.000 to 10^5/cm2 or even more are deposited on materials in different studies, while constant values should be maintained to assure comparability. Thus, Uggeri et al **[87]** studied the adhesion of human osteoblasts to titanium and used for all experiments osteoblasts at a density of ~ 5.000 cells/ cm2. On the other hand, more complex studies involving various cell numbers in populations were performed by Martin et al much earlier **[88]**, in a study on the effect of titanium surface roughness on the proliferation, differentiation and protein synthesis of osteoblasts-like cells. Populations containing from 104 until 6x104 cells were used in his study; in several other studies similar cell numbers were seeded **[89][90]**. In other investigations, much higher cell populations were deposited on the tested substrates **[91]**. In such cases, a comparative study between different literature results is difficult.

Chapter 5: Electricity in cell culture

5.1. Introduction

When a load is moving it creates both an electric and a magnetic field, called electromagnetic field. At low frequencies (e.g., 50Hz) there are two independent fields and so no practical coupling between the electric and magnetic field. Electric fields are created by voltage differences: the higher the voltage, the larger the field created, whereas magnetic fields are generated when there is an electric current flow: the higher the current flow, the greater the magnetic field strength. Maxwell's equations and Lorenz's law describe charges and current interaction. Static and low frequency fields may interfere with biological systems after observing some ion redistribution and weak current generation. The biological effects of low frequency magnetic fields have been extensively studied due to deeper penetration into the tissues **[92]**.

5.2. Internal electricity in human body

Electricity is everywhere, even in the human body. Human cells are specialized to conduct electrical currents. Electricity is required for the nervous system to send signals throughout the body and to the brain, making it possible for us to move, think and feel **[93]**. Electricity is simply the movement of an electrical charge, to move and as a result use the potential charge we need an energy source. Some of the most common sources of electricity are hydroelectric, nuclear, solar and wind. In human cells electricity is created by chemical energy. This energy created by chemicals is due to the reactions of the atoms and molecules present all of the elements we take in our bodies like oxygen (O), sodium (Na), potassium(K) and calcium(Ca) have a specific electrical charge meaning they have a specific number of electrons, protons and neutrons. Those specific charges, whether positive or negative react of adjacent molecules. This reaction is what creates the energy needed to create electricity **[94]**.

The contents of the cell are protected from the outside environment by a cell membrane. This cell membrane is made up of lipids that create a barrier that only certain substances can cross to reach the cell interior. Not only does the cell membrane function as a barrier to molecules, it also acts as a way for the cell to generate electrical currents. Resting cells are negatively charged on the inside, while the outside environment is more positively charged. This is due

to a slight imbalance between positive and negative ions inside and outside the cell. Cells can achieve this charge separation by allowing charged ions to flow in and out through the membrane. The flow of charges across the cell membrane is what generates electrical currents **[93]**.

Cells control the flow of specific charged elements across the membrane with proteins that sit on the cell surface and create an opening for certain ions to pass through. These proteins are called ion channels. When a cell is stimulated, it allows positive charges to enter the cell through open ion channels. The inside of the cell then becomes more positively charged, which triggers further electrical currents that can turn into electrical pulses, called action potentials. Our bodies use certain patterns of action potentials to initiate the correct movements, thoughts and behaviours **[93]**.

A disruption in electrical currents can lead to illness. For example, in order for the heart to pump, cells must generate electrical currents that allow the heart muscle to contract at the right time. Doctors can even observe these electrical pulses in the heart using a machine, called an electrocardiogram or ECG. Irregular electrical currents can prevent heart muscles from contracting correctly, leading to a heart attack. This is just one example showing the important role of electricity in health and disease **[93]**.

5.3. External Electricity in Human Body - Till Now Applications

Electrical stimulation is the application of electrical current through electrodes placed directly within the wound or on the skin near the wound. Bone tissue, unlike most other tissues of the human body, has the ability to completely regenerate after lesion. Bone regeneration can be supported by biophysical stimuli and bone grafting like electric and magnetic stimulation in large defects, though large bone defects (resulted from osteomyelitis, osteoporosis, joint arthroplasty, tumor resection, trauma, and avascular necrosis) are a challenge to the complex and well-coordinated bone regeneration. If the repair mechanisms are overstrained, then it can result in fibrous tissue with impaired bone healing. Application of electric currents is a promising approach to enhance bone regeneration. This approach is based on observations that mechanical stress leads to bone formation **[95]**. Yasuda and Fukada proved that mechanical forces generate electric currents and potentials in bone tissue **[96]**. These potentials were contributed to piezoelectricity and have recently been confirmed at the nanoscale level **[97]**. Furthermore, potentials in vital bone tissue during fractures, bone

remodeling, bone growth, and fracture healing, as well as streaming potentials, occur **[98-100]**.

5.3.1. Strength Training

The use of electrical stimulation is used to increase voluntary strength. Electrical stimulation has long been advocated as a way of inducing hypertrophy in paralyzed muscles and increasing stimulated strength. Increasing 'strength' in fully paralyzed muscles may be beneficial if electrical stimulation can subsequently be used for purposeful tasks. For example, electrical stimulation can be used to provide crude hand function to patients with tetraplegia. Similarly, electrical stimulation continues to be used to develop sophisticated ways of enabling people with full lower limb paralysis to walk and cycle. Electrical stimulation is sometimes used to induce hypertrophy and improve blood flow. Improved blood flow, particularly in the gluteal area, may reduce the incidence of pressure ulcers. Electrical stimulation is also used as a means of increasing voluntary strength of partially paralyzed muscles. Despite the widespread use of electrical stimulation for this purpose, few clinical trials have been directed at ascertaining whether electrical stimulation alone or in combination with any type of progressive resistance training increases voluntary strength of partially paralyzed muscles more than voluntary exercise alone. A recent systematic review identified just three clinical trials in people with spinal cord injury but none were of sufficient quality to guide clinical practice. A few clinical trials in people following stroke demonstrate small increases in strength with electrical stimulation but it is unclear whether these results can be extrapolated to people with spinal cord injury. In the absence of clear evidence, and given the time-consuming nature of administering electrical stimulation, it is probably prudent to concentrate therapeutic effort on voluntary strength training interventions alone. If electrical stimulation is to be used, then it is probably best if it is applied in conjunction with voluntary strength training and appropriate resistance to ensure patients adhere to the principles of progressive resistance training **[101]**.

5.3.2. Pain Procedures in Clinical Practice

Electrical stimulation remains a crucial tool in the treatment of numerous acute and chronic pain conditions. Electricity throughout history has been regarded as a possible method of

analgesia. Although modern medicine has not embraced it wholeheartedly, growing technologic advances have pioneered three distinct methods of the use of electrical stimulation. These widely different methods lend more weight to this growing field. And although the exact role and mechanism of how TENS, NMES, and IFC applied to the human body for analgesia has not been thoroughly established, further research may prove its benefits **[102]**.

Because of its wide variability, conflicting research remains to haunt this sector of medicine. Proponents of this method of pain analgesia believe that conflicting research about the extent of it efficacy in pain management is lacks consensus when it comes to the basic issues of its prescription, such as electrode placement, treatment length, and the best waveform and stimulation parameters. Clinical trials suggest that adequate dosing, particularly intensity, is critical to obtaining pain relief with electrical stimulation. Electrical stimulation is growing as a modality for pain management but continued research is needed to ensure that this technology is maximally utilized. And with this in mind, continued advancements in technology, a better understanding of optimal stimulation parameters, and a better understanding of physiologic changes induced by this technology are required.

Studies will continue to explore these issues in the hopes of creating a universal basic protocol for electrical stimulation in modern medicine. Evidence continues to emerge from basic science and clinical trials supporting the use of electrical stimulation for the treatment of a variety of painful conditions, while identifying strategies to increase the efficacy of electrical stimulation **[102]**.

5.3.3. The Skin and Wound Healing

Electrical stimulation is the application of electrical current through electrodes placed on the skin near the wound or directly within the wound. It allows clinicians to deliver exogenous electrical signals into wound tissue, which mimics the underlying natural bioelectrical response to injury. It has been found that within 1 mm of the wound edge, there is a natural steady electrical field that can be manipulated through therapeutic electrical stimulation to accelerate the wound-healing process. The resulting electrical fields can cause the stimulation of biomolecular signaling pathways and cell migration. Electrical stimulation has been found to promote cellular activity in nearly all phases of experimental wound healing **[103]**.

Current research focuses in the area of electrical stimulation are to develop new technologies, including dressings with embedded batteries, which will make the electrical stimulation devices more tractable for clinical use, and the design of devices for use in the community, which may be a more cost-effective therapy than current standard therapies **[103]**.

5.3.4. Upper Extremity Function and Management

Electrical stimulation can be triggered by voluntary movement or non-triggered. EMG-triggered stimulation detects underlying muscle activity when it reaches a threshold level prior to providing the stimulation. The stroke survivor must voluntarily activate the correct muscles prior to the stimulation facilitating the motor response. This type of stimulation assures that the intervention is not passive in nature. Triggered electrical stimulation may be more effective than non-triggered electrical stimulation in facilitating upper extremity motor recovery following stroke. This intervention has been shown to be effective at improving wrist extension, a key movement to be considered a candidate for some task-oriented approaches such as CIMT **[104]**.

5.3.5. Lower Urinary Tract Disorders in Postmenopausal Women

Electrical stimulation therapy is another adjunction to pelvic floor exercises. This technique involves the use of vaginal or rectal probes to deliver a low-level electrical current to the muscles and nerves of the pelvic floor. Stimulation of the afferent fibers of the pudendal nerve can produce contractions of the pelvic floor and periurethral skeletal muscles, which augment their tone in women with SUI. Simultaneously, the electrical current also causes a reflex inhibition of detrusor activity and improves UUI. Electrical stimulation can be used to increase pelvic floor muscle strength or to identify these muscles for voluntary control in Kegel exercises. Improvement rates of 60% to 90% and cure rates of 10% to 30% were reported in patients with stress, urge, or mixed incontinence. Although this mode of therapy remains an option for the treatment of urinary incontinence, patient acceptance of the technique is often poor. Furthermore, the effectiveness of electrical stimulation therapy in SUI has not been formally evaluated **[105]**.

5.3.6. Tissue Engineering of the Peripheral Nerve (Schwann cells)

Electrical stimulation, when applied directly to the nerve at levels proximal to the nerve suture, is sufficient to increase neurotrophic factor levels in the distal nerve as well (Wang et al., 2009). Partly, the increased amount of neurotrophic factors is of neural origin and subsequently stimulates the biological activity of Schwann cells in the distal nerve (Wang et al., 2009).

As downstream effector of neurotrophin signaling in Schwann cells of motor pathways, the HNK-1 carbohydrate has been discussed. Its expression has been shown to contribute to the preferential motor reinnervation (PMR) phenomenon in the femoral nerve model, and this specific expression pattern is significantly enhanced by short-term electrical stimulation of acutely injured and reconnected nerves (Eberhardt et al., 2006). Again, the importance of BDNF signaling for the translation of electrical stimulation into increased axonal regeneration has been evidenced by the abolishment of increased HNK-1 expression after electrical stimulation in the distal nerve stump in trkB-deficient mice (Eberhardt et al., 2006).

With regard to PMR, it is of high importance to recognize that the guiding Schwann cells and regenerating axons can influence each other reciprocally. Polysialic acid (PSA) linked to the neural cell-adhesion molecule (NCAM) has been shown to crucially contribute to the PMR phenomenon (Franz, Rutishauser, & Rafuse, 2005). Subsets of femoral motoneurons, which regenerate preferentially into the motor branch instead of the sensory branch of the femoral nerve, show an upregulation of PSA–NCAM (Franz et al., 2005). Electrical stimulation increases the upregulation of PSA and thereby further improves preferential motor axonal regeneration into motor target tissue (Franz, Rutishauser, & Rafuse, 2008). However, it has to be remembered that if appropriate and inappropriate motor targets are located distal to the lesioned nerve, electrical stimulation does not positively influence appropriate reinnervation of original motor targets (Hamilton et al., 2011).

BDNF-secretion from regenerating axons after electrical stimulationalso advances the myelination of the regrown axons (Wan, Xia, & Ding, 2010). This has been demonstrated *in vitro* and *in vivo* where increased BDNF levels after electrical stimulation were identified to accelerate myelination by advancing Schwann cell polarization and initiation of myelination resulting in a higher degree of myelination at earlier times points as in nonstimulated controls (Wan et al., 2010).

Electrical stimulation also has a high potential to directly influence Schwann cell behavior. *In vitro* electrical stimulation resulted in increased proliferation of Schwann cells cultured on conductive membranes (Huang, Hu, et al., 2010). Furthermore, the expression of NGF and BDNF was significantly increased in Schwann cells due to electrical stimulation as was the secretion of both factors over a period of 36 h after stimulation (Huang, Hu, et al., 2010). That application of electric fields of varying intensities and frequencies to cultured neonatal rat Schwann cells significantly increased their production and release of NGF in an intensity- and frequency-dependent manner was also shown in a second *in vitro* study (Huang, Ye, Hu, Lu, & Luo, 2010). Again, as also described for neuronal cells before, an increase in Schwann cell intracellular calcium levels related to electrical stimulation demonstrated to be involved in elevated expression and exocytosis levels of NGF (Huang, Ye, et al., 2010).

That the effect of electrical stimulation on Schwann cells has a regeneration-promoting character, additional to its stimulation of neurons, has been demonstrated with another *in vitro* study (Koppes, Seggio, & Thompson, 2011). Neurite outgrowth from sensory dorsal root ganglion neurons was significantly stronger when electrical stimulation was applied to neurons cocultured with Schwann cells in comparison to stimulated neurons cultured alone or unstimulated neurons in cocultures with Schwann cells (Koppes et al., 2011).

Over the past decade, plenty knowledge has been achieved on the effects of electrical stimulation which qualified the treatment as promising cotherapy for the clinic **[106]**.

5.3.7. Network Experimental Approaches

Electrical stimulation has been widely used to study synaptic plasticity. Brief high-frequency electrical stimulation can lead to long-term potentiation, and prolonged low-frequency stimulation results in long-term depression. Electrical stimuli can also be used to simulate a disease process and, under certain conditions, can deactivate a brain structure or change its pattern of firing. Kindling is a widely used animal model to simulate epileptogenesis, and it can be produced by repetitive and intermittent electrical or chemical stimulation of limbic structures of the brain, including the amygdala. Electrically-kindled animals exhibit stimulus-evoked generalized motor seizures as well as spontaneous seizures, which are similar to temporal lobe epilepsy in humans. Another example of the use of this technique can be found in exploring neuronal networks in AGS. Thus, low-current electrical stimulation of the inferior colliculus (IC) induces seizures in normal rats, including wild

running, that resemble seizure behaviors seen in AGS-susceptible rodents as described here. Thus, electrical stimulation in the IC of normal rats evoked the wild-running seizure behavior, closely paralleling the initial behavior seen in AGS, which provided additional evidence of the involvement of this structure in the neuronal network for AGS. This finding is consistent with studies using microinjection that found that the IC is the initiation site for AGS (discussed in the "Chemical Stimulation via Microinjection" section). Acute electrical stimulation of the IC induces seizure activity that does not involve forebrain structures, such as the amygdala, in normal rats. However, repeated electrical stimulation of the IC generated postictal epileptiform electrographic activity in the amygdala, similar to that seen in amygdala kindling. With twice-daily repetition of the electrical stimulus, the duration of the wild running increased and the more severe seizure behaviors, including limb extension, were evoked, resembling the severity increase seen with rats genetically susceptible to AGS. This is an example of how electrical stimulation within the putative seizure network provided further evidence of the involvement of a specific structure in the AGS network. These results are consistent with the repetitive induction of AGS (audiogenic kindling), which causes the neuronal network for AGS to expand from brainstem to forebrain structures, including the amygdala **[107]**.

5.3.8. Mechanisms Activated by Electrical Stimulation of the Stomach

Electrical stimulation of gastric strips (Smits and Lefebvre, 1996), or of the gastric wall in *in vivo* studies (), results in ACh release from enteric neurons, which in turn triggers contraction. While current pulses shorter than 10 ms seem to activate muscles through neural activation exclusively (Sakai and Daniel, 1984;), it is likely that current pulses longer than 10 ms, besides activating enteric neurons, directly depolarize muscle cells.

Electrical stimulation of gastric muscles can also induce relaxation, which is mediated through NO transmission (; ; Xing and Chen, 2006). In addition to directly acting on the muscle membranes, NO also activates adrenergic fibers, which consequently amplifies muscle inhibition and gastric relaxation (Sotirov and Papasova, 2000).

Whether the stomach reacts by contraction or relaxation to electrical stimulation seems to depend on stimulation frequencies used. Yoneda and Suzuki (2001) stimulated muscle strips dissected from guinea-pig stomach and evoked cholinergic excitatory junction potentials

when using 0.5 Hz pulses. Increasing the stimulation rate to higher than 1 Hz resulted in the activation of NO-releasing fibers, which, in turn, inhibited the excitatory junction potentials.

As shown above, the tonic activity of gastric muscles is driven by slow depolarization waves, and several studies investigated the effects of electrical stimulation on gastric slow wave activity. In 1974, Kelly was the first who reported that stimulation of the stomach in dogs entrained gastric slow waves (Kelly, 1974). Since then, several authors observed that improvement of gastric emptying or symptoms in response to electrical stimulation in both animals and humans was accompanied by changes in gastric slow wave activity. Since slow waves are generated by the ICC, at least some of the motor effects induced by electrical stimulation of the stomach could be mediated by changes in the ICC pacemaker activity.

Besides activating enteric neurons, ICC or muscle cells directly, electrical stimulation of the stomach may also activate sympathetic and parasympathetic terminals within the gastric wall. report that stimulation of periarterial nerves in the isolated rat stomach induces noradrenaline release from sympathetic fibers. This observation was later confirmed by studies in dogs, which showed that gastric stimulation inhibits postprandial gastric contractions (Zhu and Chen, 2005) and antral motility through sympathetic mechanisms. On the other hand, showed that gastric electrical stimulation increases vagal activity, and observed that an increased gastric contractility in response to gastric stimulation is accompanied by increased vagal tone. By contrast, electrical stimulation to inhibit myoelectrical activity results in inhibition of parasympathetic activity. Regarding central effects of gastric stimulation, studies have shown that stomach stimulation with parameters used for treating obesity activates hypothalamic neurons involved in controlling gastric motility and the feeding behavior in rats **[108]**.

5.3.9. Deep Brain and Peripheral Nerve Stimulation as Potential Therapies for Epilepsy

Electrical stimulation delivered directly into the brain or to specific peripheral nerves is being actively investigated as a new therapy for patients with epilepsy whose seizures are uncontrolled by, or who are at risk for adverse side effects from currently available pharmacologic treatments. In many ways, the clinical use of electrical stimulation has outpaced the basic science required to provide a solid foundation for these treatments. The fundamental questions of where and how to stimulate remain the focus of many laboratories.

There is evidence that stimulation frequency can be an important factor in determining whether a given stimulation paradigm will be successful. Our studies have focused on the use of low-frequency stimulation as a therapy for epilepsy in the kindling seizure model **[109]**.

5.3.10. Vasoactive Intestinal Peptide

Electrical stimulation of the vagus nerve trunks stimulates pancreatic enzyme and bicarbonate secretion. This stimulation achieves pancreatic secretory levels approximately 50% of the response seen with exogenous secretin. In addition to effects on secretion, electrical stimulation of the vagus nerve also increases pancreatic blood flow and local vasodilation. A body of work has now implicated VIP as the transmitter most likely responsible for fluid and bicarbonate secretion and vasodilation.

Data to support that VIP as a neural peptide involved in regulating pancreatic fluid and bicarbonate secretion came from two primary observations. First VIP-containing neurons are found within the pancreas. These fibers surround intrapancreatic ganglia and pancreatic ducts. Second, VIP can directly stimulate pancreatic bicarbonate secretion from the isolated vascularly perfused the pancreas. Subsequent studies demonstrated that electrical stimulation of the vagus nerve following atropine treatment resulted in an increase in bicarbonate secretion and an increase in VIP in the venous effluent from the pancreas. In addition, the pattern of VIP release paralleled the secretion of fluid and bicarbonate and the amount of VIP in pancreatic venous effluent was similar to that required to stimulate pancreatic secretion with intra-arterial injection. Nevertheless, it is difficult to conclude that VIP plays a major role in regulating pancreatic fluid and bicarbonate secretion *in vivo*, and it is most likely that it serves as a neurotransmitter that is involved in mediating pancreatic vasodilation and in increasing blood flow. Further studies indicated that multiple mediators are involved in the pancreatic secretory response to electrical stimulation of the vagus nerve **[110]**.

Chapter 6: Experimental part

6.1. Materials and Methods

6.1.1. Equipment

- Laminar Flow Clean Bench – Gold Model Labtech, providing an aseptic work area
- Forma Scientific Model 3546 Water Jacketed Incubator to grow and maintain cell cultures (37^{o}C, 5% CO_2)
- Prestige Medical Series 2100 Clinical Autoclave
- Hermle Z 300 K Centrifuge
- Refrigerator and freezer (–20°C)
- Nikon contrast phase microscopy and Scanning Electron Microscopy

6.1.2. Additional Supplies

- Cell culture vessels (e.g., flasks, tubes, multi-well plates)
- Syringes, needles
- Pipettors, pipettes
- Media, serum, reagents
- Waste containers

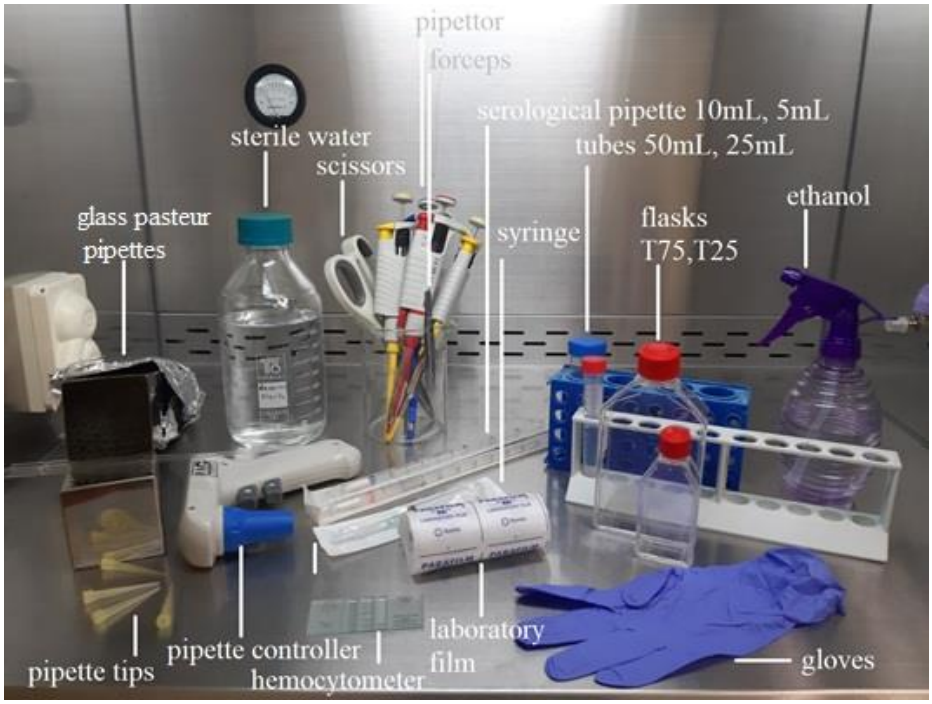


Figure 6-1 *Laboratory consumables*

6.1.3. Substrates Preparation

- TCP (Tissue Culture Polystyrene)

A hot-wire cutter was used to cut the polystyrene flask (Figure 6-2). The device consists of a thin, taut alloy wire, named Kanthal D. Kanthal D is a ferritic iron-chromium-aluminium alloy (FeCrAl alloy) for use at temperatures up to 1300°C (2370°F). The alloy is characterized by high resistivity and good oxidation resistance. The wire is fixed in the two ends in a wooden handle which is connected via Cu wire electrodes to the power supply. The power supply is fixed in 3A, so that the heat from the wire vaporises the material just in advance of contact, as the wire is passed through the material to be cut. The depth of the cut is limited only by the wire length. The length of the wire was fixed so that the handle can be easily managed. Longer wires require higher tension. We have to set properly the wire temperature and the cutting speed in order to have a uniform kerf thickness and a balanced surface finish of the cut material.

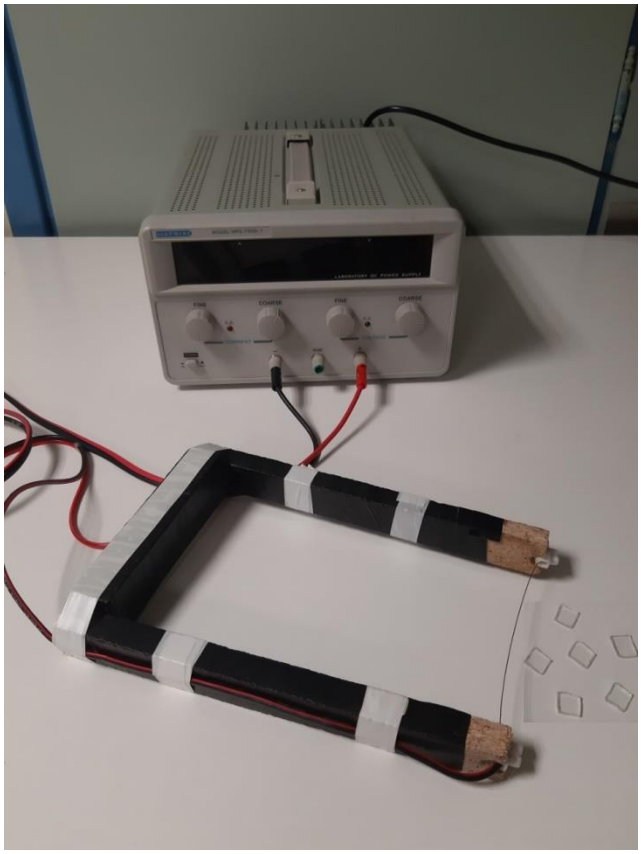
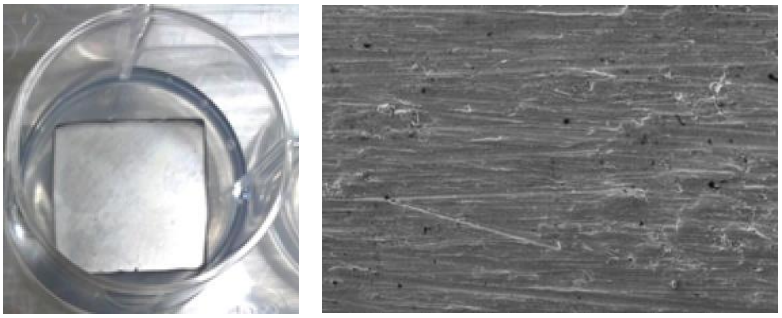


Figure 6-2 *Hot-wire cutter*

- Pure titanium

Titanium by Alfa Aesar S.A. Massachusetts was supplied, in the form of foils. The material was 99.6% pure, with thickness of 1 mm and dimensions 1 x 1 cm^2.

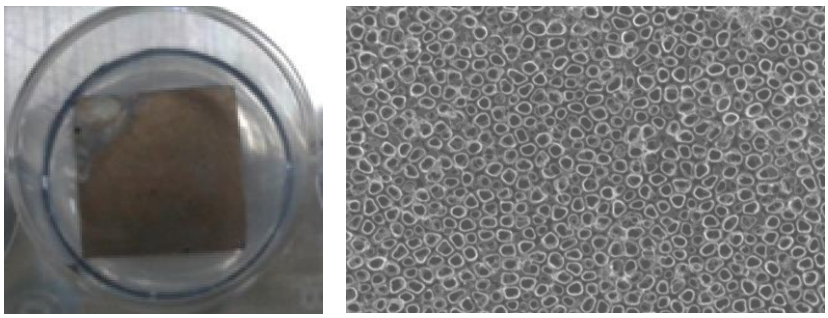


(a) (b)

Figure 6-3 *(a) Pure titanium (b) SEM image*

- TiO_2 nanotubes

The titanium surfaces were processed in the lab, in a previous research **[111]**, using the electrochemical method of anodization so that vertically grown layers of nanotubes on titanium's surface resulted. Through this process, the two electrodes configuration was assembled having as cathode the graphite electrode and as anode the titanium sample. Nanotubes are created in a nano-layer and providing greater cell adhesion than anodized titanium. The titanium samples were anodized in an optimum combination of current-electrolyte and time of anodization (0.5% HF).

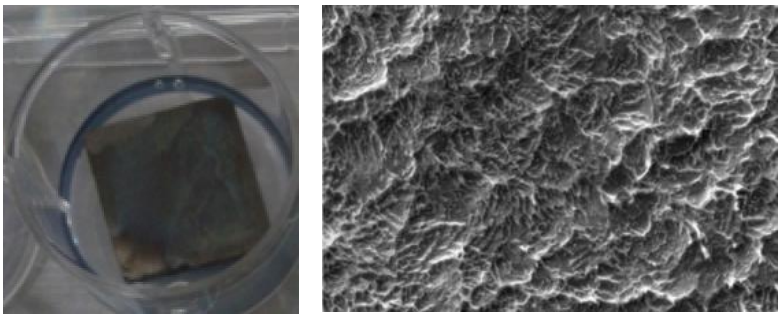


(a) (b)

Figure 6-4 *(a) TiO_2 nanotubes (b) SEM image*

- Rough Titanium (Anodized)

The titanium surfaces were processed through anodization **[111]**, in order to form a rough surface but not through the optimum parameters' combination as in TiO_2 nanotubes.



(a) (b)

Figure 6-5 *(a) Rough titanium (b) SEM image*

All the substrates were sterilized using ethanol and UV radiation for 10 hours.

6.1.4. The choice of the materials

The substrates used in this thesis are tissue culture polystyrene, pure titanium and substrate modified titanium. TCP is widely used in cell culture studies as control substrate. Cell culture consumables (e.g., flasks, petri plates) are made of TCP. Untreated polystyrene surface is not suitable for cell attachment due to its chemistry, so the hydrophobic plastic surface is made more hydrophilic via exposure to a plasma gas. The resulting surface carries a net negative charge due to the presence of oxygen-containing functional groups (e.g., hydroxyl and carboxyl) that will lead to increased cell attachment.
Titanium is the metal of choice for inner body devices, instrumentation, internal fixation and prosthetics due to its strength and biocompatibility. There is though the need of improvement of this material already used, through surface modifications; bioactive surface.

6.2. Osteoblasts Gathering

6.2.1. Surgery Experience

Osteoblasts were isolated from residual bone obtained after total hip arthroplasty and total knee replacement surgery. For this purpose we visited the Panarcadic Tripolis' Hospital where we entered the surgery room. To enter the surgery room the nurses informed us to follow some rules because contamination field could lead to an infection:

- Wear hospital scrubs, masks, hair and shoe covers. No jewellery is permitted.
- Stay at least 2 feet away from anything blue (draped tables, patients and people).
- Don't walk between two sterile areas.
- Always face the sterile field, not to risk contaminating it accidentally.



Figure 6-6 *Femoral Head*

6.2.2. Ethics Protocol

For approval of any clinical study or trial, a full dossier request, found in the hospital site was submitted to the department of the hospital protocol. This ethics approval process or ethics clearance is something that allows us to reflect upon what we are doing, how we are engaging it in our project and all patients gave informed consent. It is found in the hospital site, and after its submission at the hospital is being submitted for approval to the Committee on Research, Ethics and Deontology (CRED). The ethic application CRED is an independent body made up of health professionals and non-medical members whose task is to protect the rights, safety and health of people involved in a study and to provide public protection for this protection by, inter alia the opinion on the study protocol, the suitability of the researchers and the adequacy of facilities, as well as the methods and documents used to inform the participants in order to obtain the post- updating their consent.

6.3. Osteoblasts Isolation and Differentiation

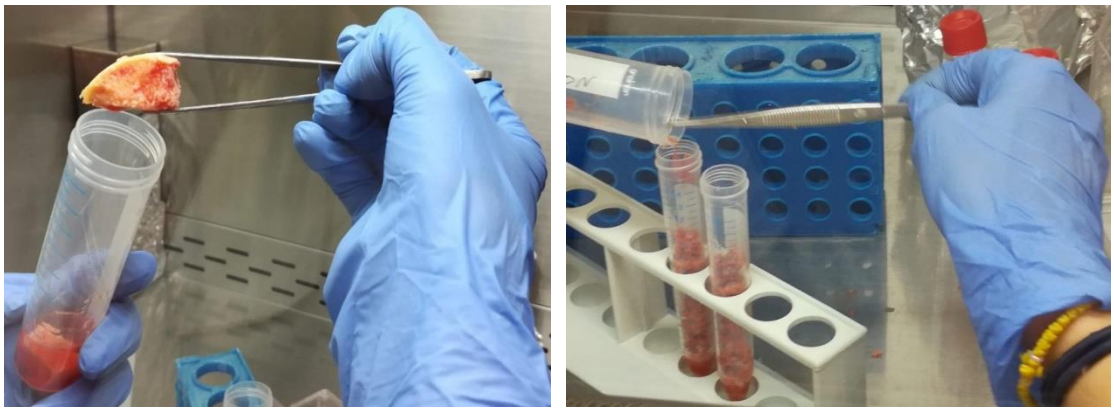
Bone fragments from a hip arthroplasty surgery of a 60 years old woman were transported between the surgery room and the cytology laboratory using 50ml tubes containing appropriate medium for osteoblast differentiation and proliferation. A single-cell suspension was prepared by aspirating the cells successively through 19 and 21-gauge needles. To induce osteogenic differentiation, cells were cultured in alpha-Minimal Essential Medium (a-LEL,

Biochrom KG, seromed, Berlin), with 10% fetal bovine serum (FBS, Biochrom), supplemented with 2.5 lg/mL amphotericin B (Biochrom), 50 lg/mL gentamycin (Biochrom), 108M dexamethasone (Sigma Aldrich), 1.5 mM b-glycerophosphate (Sigma) and 50 lg/mL ascorbic acid (Sigma). In the laboratory, the tubes (Figure 6-7) were centrifuged at 2000 rpm for 10 minutes.



Figure 6-7 *Bone fragments after centrifugation*

If the fragments are still large after centrifugation, we use a surgery scalpel under the laminar flow cabinet to shrink them. Then using glass pipettes connected to a pump we remove the residual fat that is accumulated in the medium's surface, the big bone fragments and the medium, carefully not to absorb the cells left in the bottom of the tube. Then the remnant medium with the small bone fragments from the bottom of the conical centrifuge tubes are transferred to 50ml T25 flask, containing 7ml of medium each (Figure 6-8).



(a) (b)

Figure 6-8 *Bone fragments before (a) and after (b) scalpel*

The flasks were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2, leaving the lids of the flasks slightly open in order to allow to the incubator environment (CO_2, humidity, temperature) to pass inside. After 4 days, mesenchymal stem cells were observed under the optical microscope and after 7 days, residual bones were thrown away and the specimens were washed with PBS to eliminate the unattached cells and fed again with medium. At an 80-90% confluence, cells were trypsinated, transferred to a 50 ml conical centrifuge tube and further centrifuged with the purpose to deposit subcultures in new flasks. Cells of second and third passaging were used in our study.

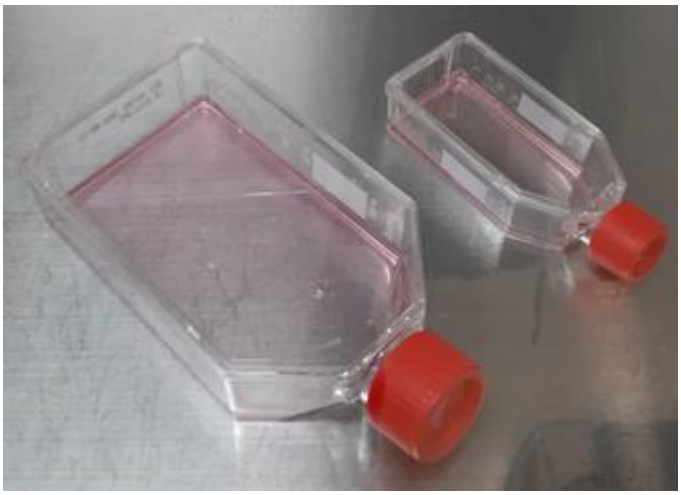


Figure 6-9 *Flasks after 7 days of incubation*

6.4. Dissociating Adherent Cells

The first step in subculturing adherent cells is to detach them from the surface of the culture vessel using trypsin because osteoblasts are strongly adherent cells.

6.4.1. Protocol for Passaging Adherent Cells

All solutions and equipment that come in contact with the cells are sterile. Always use proper sterile technique and work in a laminar flow hood.

- Absorb and discard the spent cell culture media from the culture vessel using glass pipettes and a pump.

- Wash cells using PBS, which is a balanced salt solution without calcium and magnesium. This removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent. More specifically, we add PBS solution to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer. Then we rock the vessel back and forth several times. Note: The wash step 3. Remove and discard the wash solution from the culture vessel
- Add enough pre-warmed dissociation reagent (trypsin) to the side of the flask, to cover the cell layer (approximately 0.5 mL/10 cm^2) and gently rock the container to get complete coverage of the cell layer.
- Incubate the culture vessel at room temperature for approximately 2 minutes.
- Observe the cells under the microscope for detachment. If cells are less than 90% detached we increase the incubation time a few more minutes, checking for dissociation every 30 seconds. We also tap the vessel to expedite cell detachment.
- When ≥ 90% of the cells have detached, we tilt the vessel for a minimal length of time to allow the cells to drain. After that, we add twice the volume, used for the dissociation reagent, of pre-warmed complete growth medium and we disperse the medium by pipetting over the cell layer surface several times.
- Transfer the cells to a 15-mL conical tube and centrifuge at 2000 rpm for 10 minutes.
- Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and remove a sample for counting.
- Determine the total number of cells and percent viability using the neubauer. If necessary, add growth media to the cells to achieve the desired cell concentration and recount the cells.
- Dilute cell suspension to 10mL for 75mL flasks and 4mL for 25ml flasks, pipet the appropriate volume into new cell culture vessels, and return the cells to the incubator, loosening the caps to allow proper gas exchange.

6.5. Cell Counting

In order to calculate the overall concentration of cells in the medium the Neubauer counting chamber has been used to count the cells, always under the laminar flow. First of all, we cleaned the Neubauer chamber and the cover slip with 70% ethanol and put the glass cover on the Neubauer chamber central area. Using a pipette, we slowly expelled around 10 mL of the

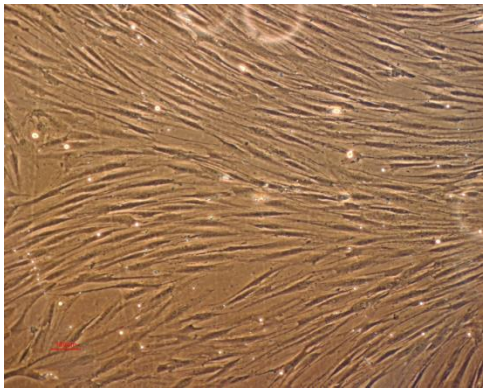
mixture cells-medium until the counting chamber fills. Then we observed the Neubauer chamber under the microscope (10x objective), counting the total number of cells found in 4 large corner squares. For cells touching the 4 perimeter sides of a square, we counted only the up and left side. A number of 30.000 cells/cm^2 have been calculated using the formula:

$$\text{Concentration} = \frac{\text{No.of cells x } 10.000}{\text{No.of squares x dilution}}$$

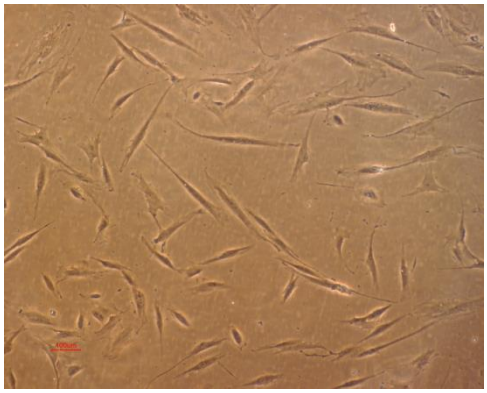
It was observed through previous experiments in the laboratory that the optimum concentration of cells is between 10.000 and 50.000 cells/cm^2. This is because if the concentration is less than 10.000 cells/cm^2, cell culture proliferation on titanium and modified titanium with an increased roughness is slow, whereas if the concentration is more than 50.000 cells/cm^2 cells will overflown on TCP substrates. Therefore, for the better comparative study of the cell culture proliferation on the different substrates, a concentration of 30.000 cells/cm^2 has been used.

6.6. Development of Osteoblast Populations on Tissue Culture Polystyrene (TCP) flasks

Osteoblasts are anchorage dependent cells, so they were cultured while attached to a solid substrate, more specifically to TCP flasks. Generally, osteoblasts are firstly isolated in small colonies, more likely in the 4 edges of the flask and so did in our culture. After their intense proliferation begins, they started migrating on the entire surface of the flask bottom to find new fields to populate. The multiplication process of physiologic osteoblasts started at the end of the first week of incubation, while two or three weeks of incubation were needed to have a confluent flask. The first ten days of osteoblasts growth are very important for the faith of the entire population. Cells multiplication and progress is influenced on a great extent by the initial number of the population. It is well known that osteoblasts self-adjust their dimensions (length, width). This is because if cells number in a population is not high enough and distance between them is too long, apoptosis takes place, especially when exposed to shear and mechanical stress. On the other hand, if cells are overpopulated, apoptosis takes place again. In confluent areas osteoblasts are arranging themselves from the beginning in such a way to save space, taking a spindle-shaped form, so the self-adjustment is not that obvious in confluent areas.



(a)



(b)

Figure 6-10 (a) *Osteoblasts are interconnected and form a confluent region.* ***(b)*** *Osteoblasts are developing by extending their actin filaments in a circular/ oval shape around their nuclei, forming a relaxed region.*

6.7. Development of Osteoblast Populations on Different Substrates - Biocompatibility Testing

Osteoblasts from the second and third passage were detached using trypsin from the TCP flasks and deposited on different substrates.

6.7.1. Cell Proliferation Indices

Alkaline Phosphatase (ALP) is a biochemical marker for osteoblast early activity. ALP enzyme activity (Sigma Aldrich, P7998) was measured after 1, 3 and 7 days of cell culture. The ALP levels were detected using an Infinite F200PRO UV/Visible Spectrometer at 405nm wavelength.

Total Protein (TP) is an indication of cells proliferation. The intracellular quantity for TP synthesis was assessed after 1, 3 and 7 days of cell culture by Total Protein Kit (Sigma TP0400). The TP levels were detected using the same Infinite F200PRO UV/Visible Spectrometer at 600nm wavelength.

Three experiments were conducted, expressed as mean values 6SD and analysed using SPSS 14.0 software (SPSS, USA). The statistical significance was assumed at the 95% confidence limit or greater ($p<0.05$) using Student's test for single comparisons and ANOVA for multiple comparisons.

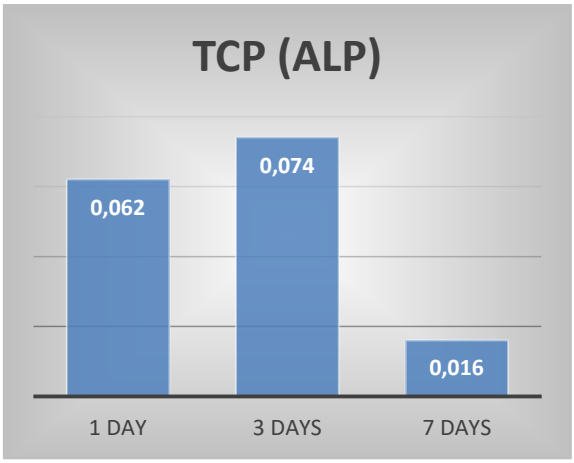
6.7.2. TCP substrate

A low quantity of detached cells, diluted with medium in a concentration of 10.000 to 30.000 cells/cm^2, was released carefully on the TCP specimen (Figure 6-11). Osteoblasts of different concentrations were cultured on the substrate for 1, 3, 7 and 10 days. Well plates were left for 5 hours in the incubator to induce fast adhesion of osteoblasts to the substrates. Afterwards, medium was added until it covered the entire surface of each substrate.

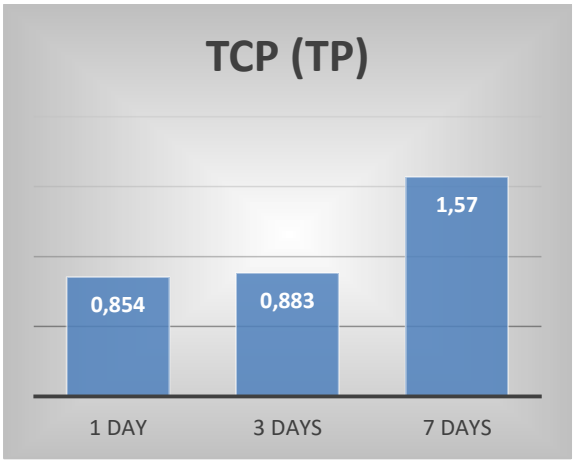


Figure 6-11 *Cell suspension on TCP specimen*

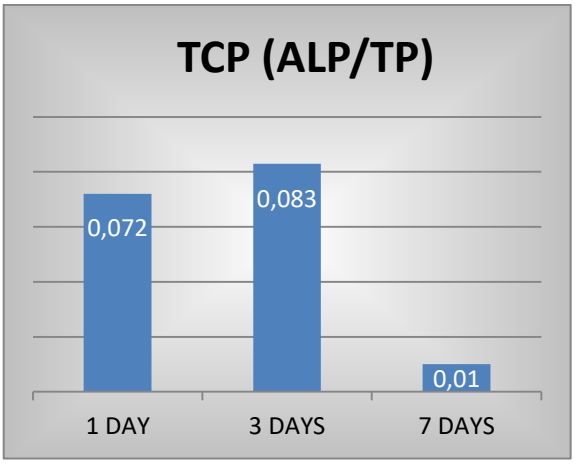
The cell concentration on the medium has been chosen after literature consulting **[112]**. TCP's smooth surface assures fast migration and proliferation.



(a)



(b)



(c)

Figure 6-12 *Measured Cell Values of (a) ALP (b) TP (c) ALP/TP on TCP substrates after 1,3 and 7 days of incubation.*

6.7.3. Titanium Substrate

As we expected due to the surface of the metal, that is less smooth than polystyrene, osteoblasts expand easier on polystyrene substrate.

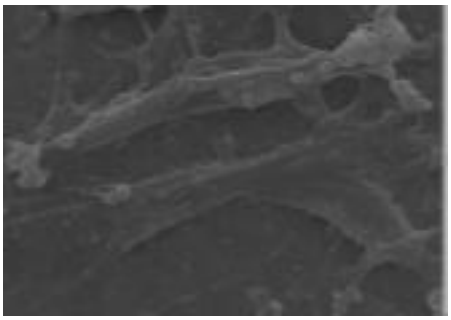
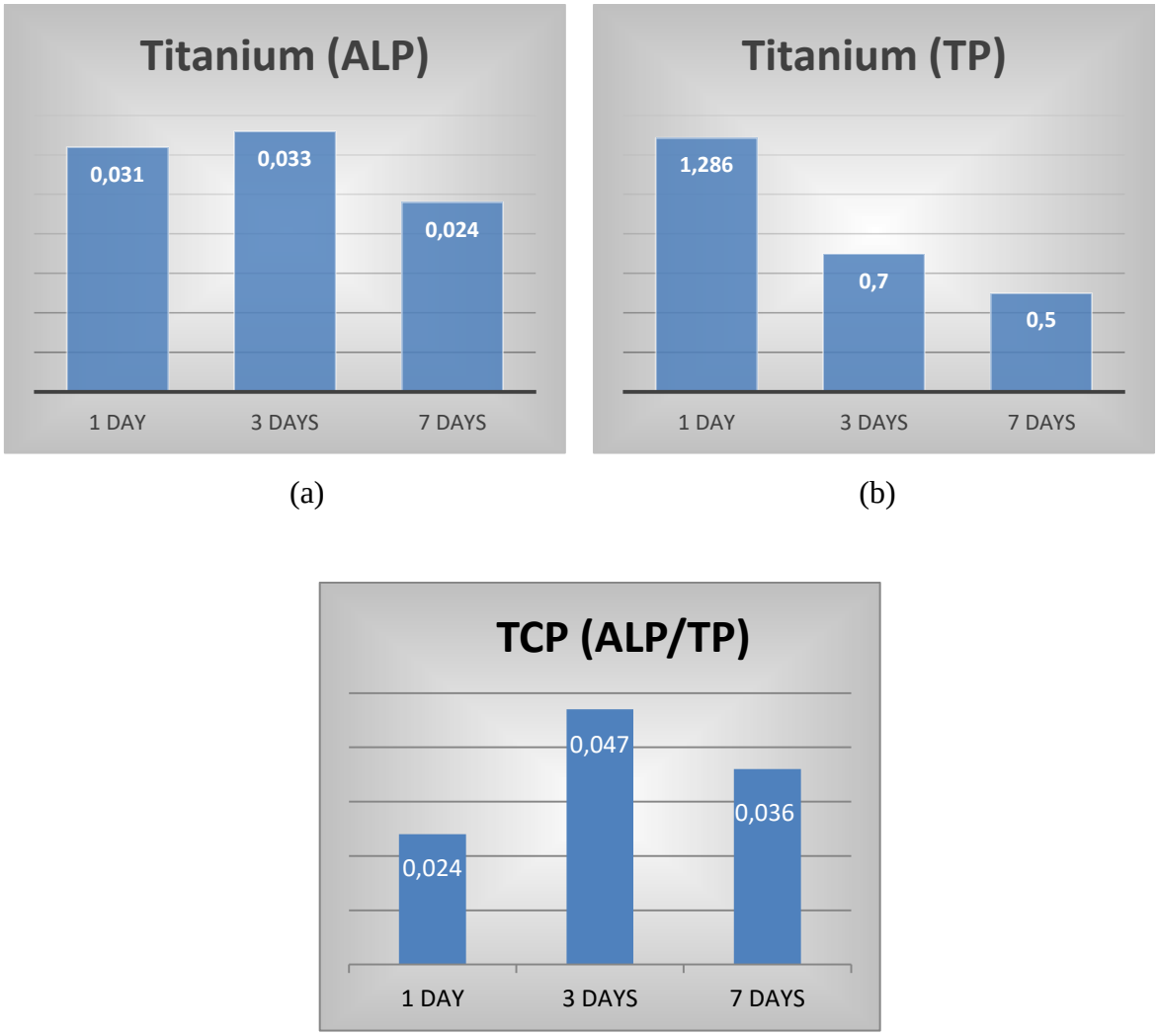


Figure 6-13 *SEM image of cells attached on pure titanium (1 week incubation).*



(a) (b)

(c)

Figure 6-14 *Measured Cell Values of (a) ALP (b) TP (c) ALP/TP on titanium substrates after 1,3 and 7 days of incubation.*

6.7.4. TiO_2 Nanotubes Substrate

TiO_2 nanotubes layers are characterized by discontinuities. There are some areas of the substrate that cells spread adequately and others that cells are not at all expanded.

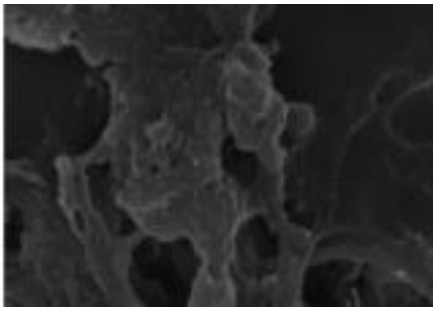
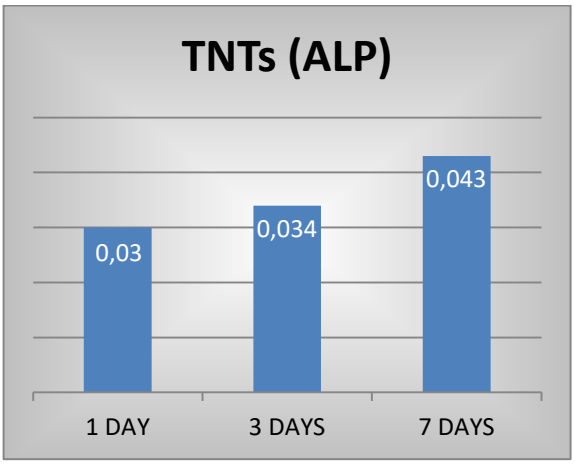
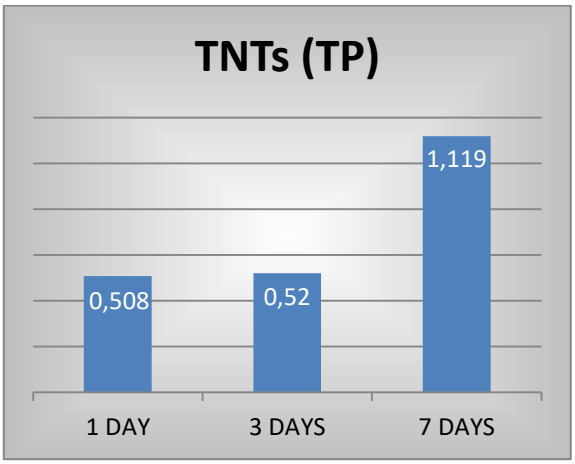


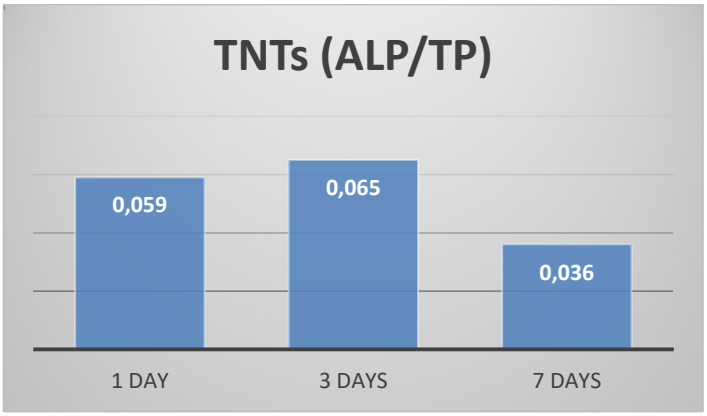
Figure 6-15 *SEM image of cells attached on titania nanotube layer (1 week incubation).*



(a)



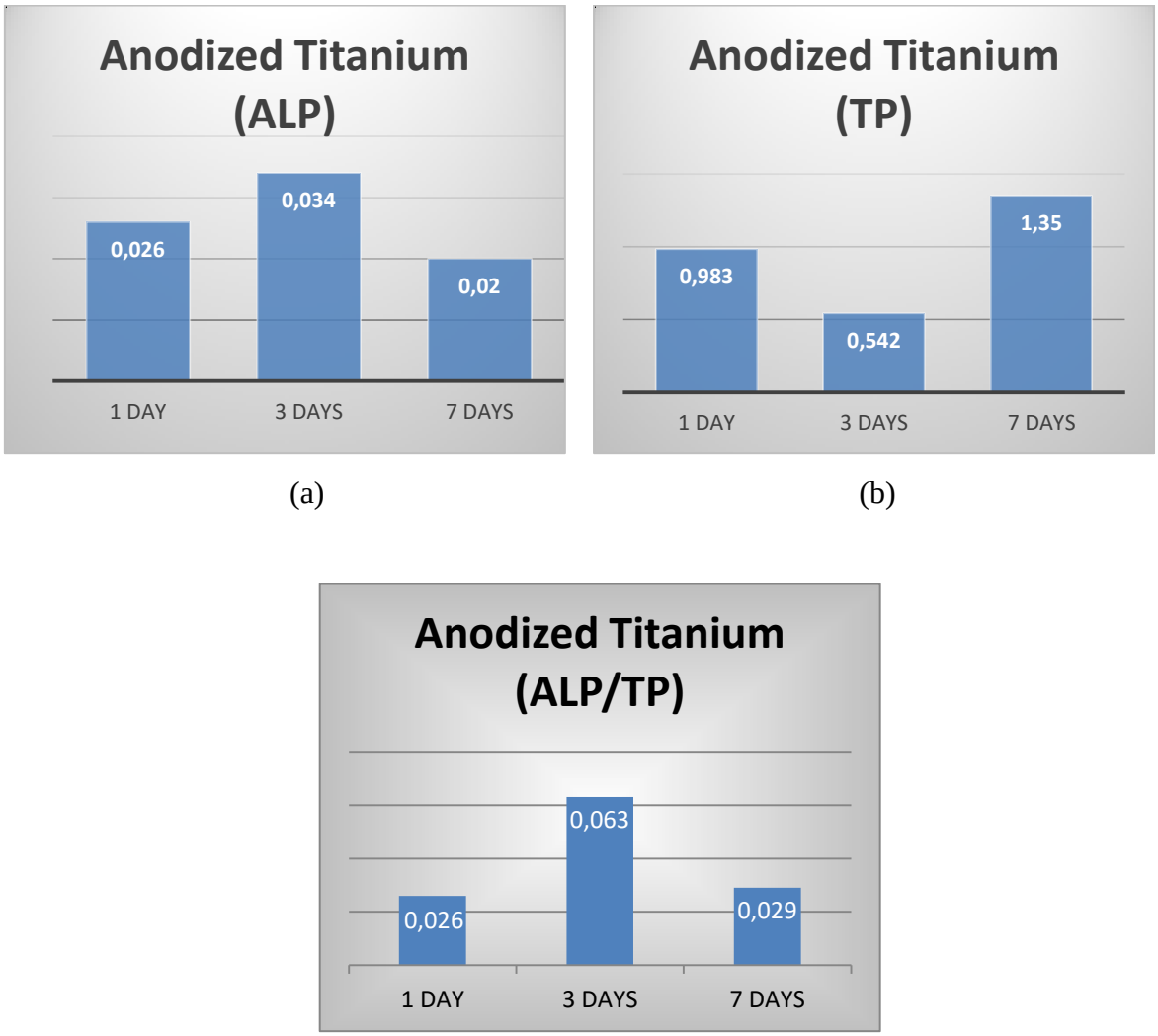
(b)



(c)

Figure 6-16 *Measured Cell Values of (a) ALP (b) TP (c) ALP/TP on titanium dioxide nanotubes after 1,3 and 7 days of incubation.*

6.7.5. Rough Titanium Substrate



(a) (b)

(c)

Figure 6-18 *Measured Cell Values of (a) ALP (b) TP (c) ALP/TP on rough titanium substrates after 1,3 and 7 days of incubation.*

TABLES OF VALUES MEASURED FOR ALL SUBSTRATES

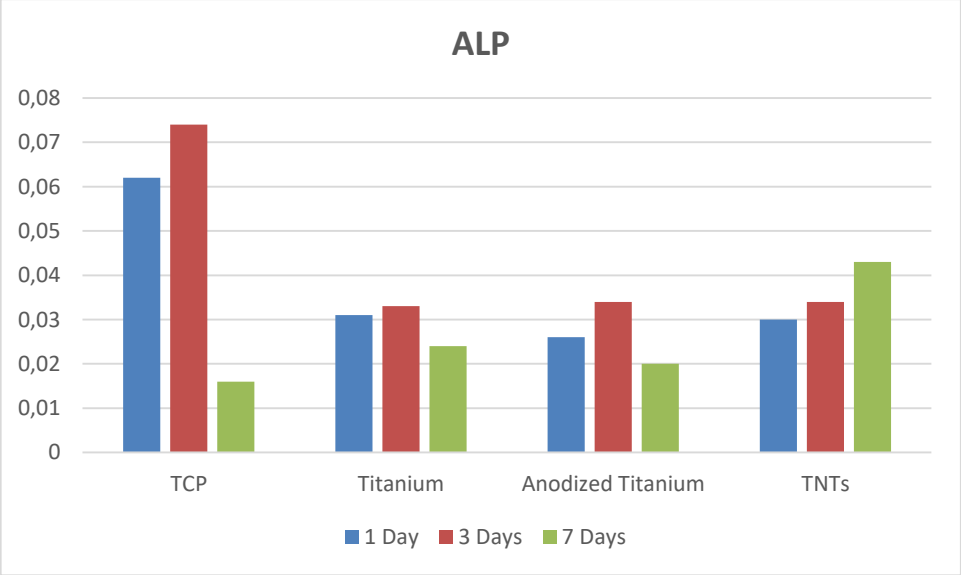
Alkaline Phosphatase (ALP)			
Material	**1 Day**	**3 Days**	**7 Days**
TCP	0.062	0.074	0.016
Titanium	0.031	0.033	0.024

Anodized Titanium	0.026	0.034	0.02
TNTs	0.03	0.034	0.043

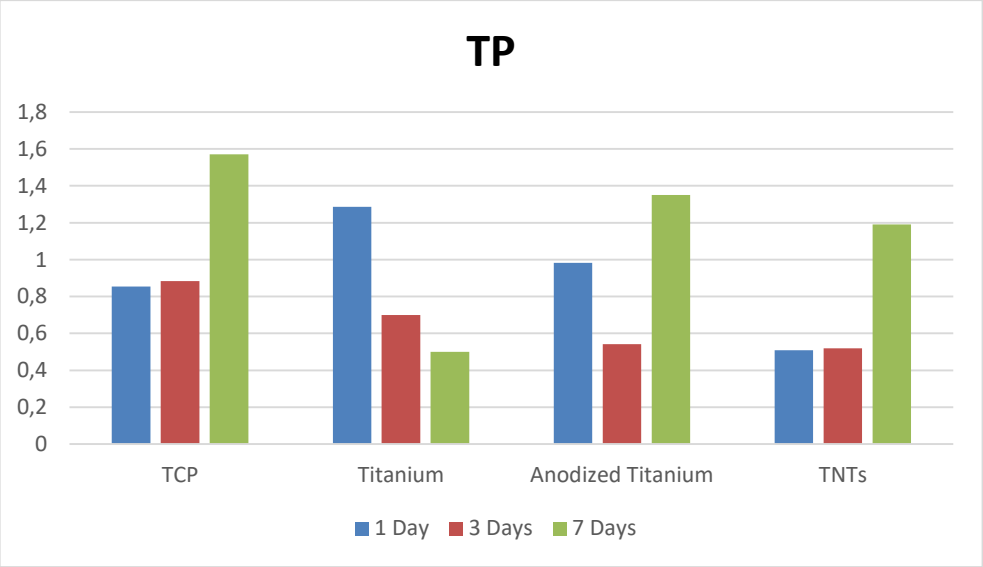
Total Protein (TP)			
Material	**1 Day**	**3 Days**	**7 Days**
TCP	0.854	0.883	1.57
Titanium	1.286	0.7	0.5
Anodized Titanium	0.983	0.542	1.35
TNTs	0.508	0.52	1.191

ALP/ TP			
Materials	**1 Day**	**3 Days**	**7 Days**
TCP	0.072	0.083	0.01
Titanium	0.024	0.047	0.036
Anodized Titanium	0.026	0.063	0.029
TNTs	0.059	0.065	0.036

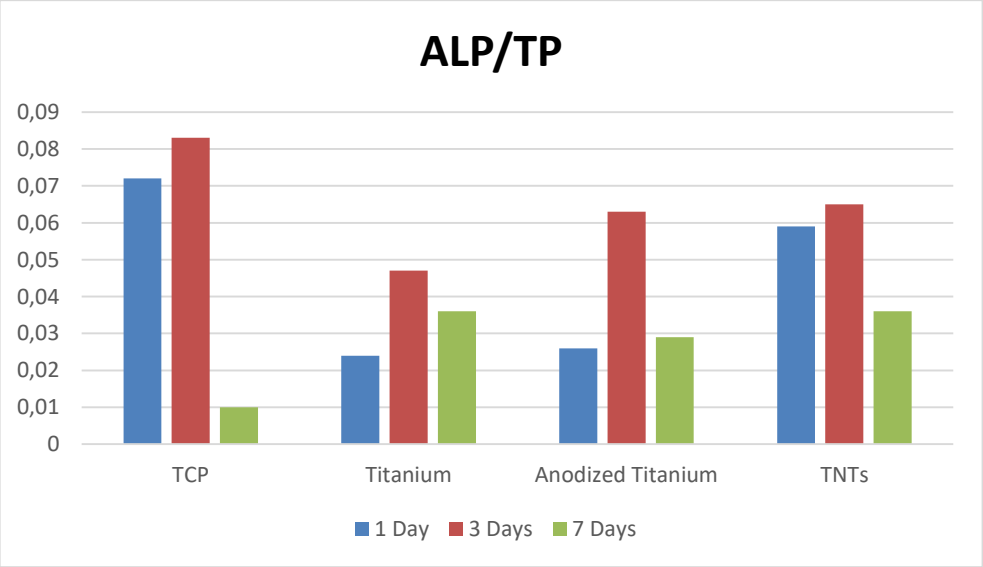
Diagrams - Comparative study of several substrates for (a) ALP values, (b) TP values and (c) ALP/TP values – Figure 6-19



(a)



(b)



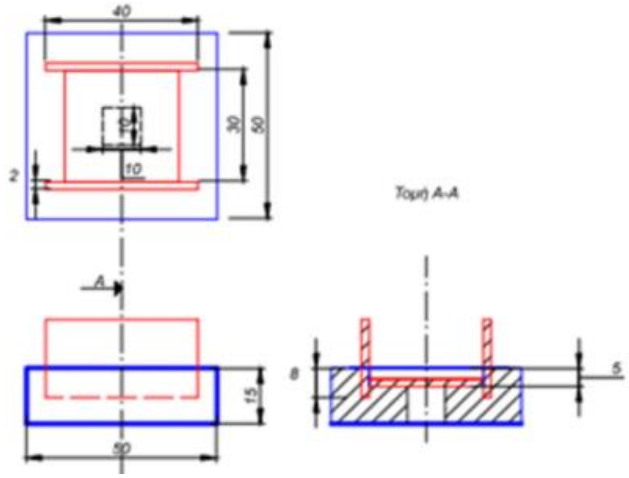
(c)

6.8. Development of Osteoblast Populations on Different Substrates - Biocompatibility Testing (electrical stimulation)

6.8.1. Stimulation Device

An electricity stimulation device has been designed and manufactured, in dimensions such as to fit four 1x1cm2 substrates. The device is consisted of 3 titanium plates and a Teflon base

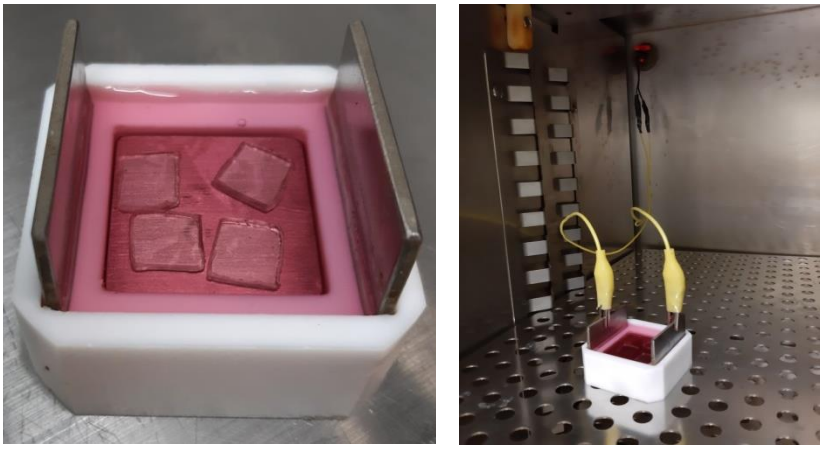
(Figure 6-20). The two vertical titanium electrodes, connected to the power supply, come in contact with the culture medium. Substrates were placed on the titanium bottom and were covered by medium so that cell populations may be maintained for several hours in the device, under electrical stimulation. The materials were chosen since they can be easy sterilized in an autoclave (high temperature and pressure resistance) and they are non-toxic and generally biological compatible when in contact with human cells. The stimulation device was installed in the incubator (Figure 6-20 (c)) and electrically stimulated the human bone cells through both the substrate and the medium.



(a)



(b)

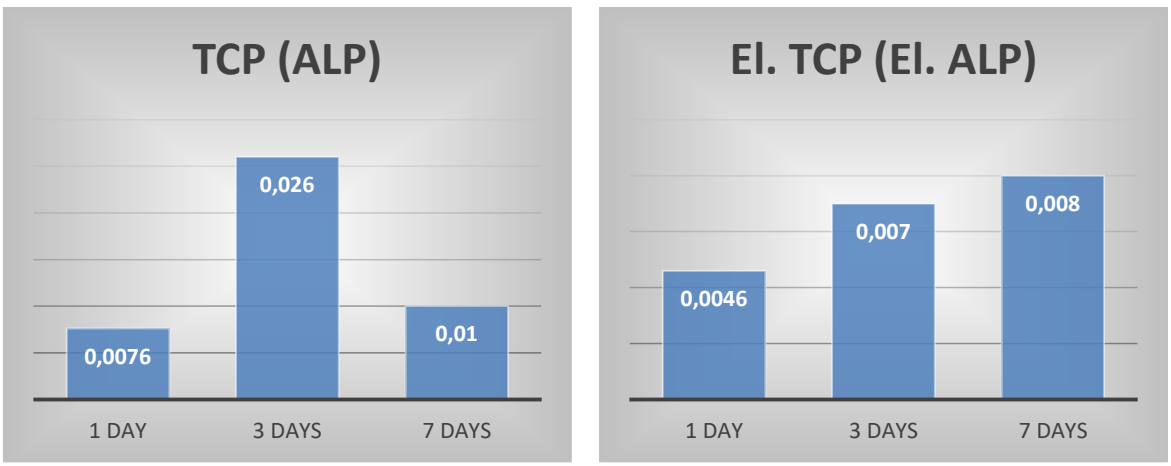


(c)

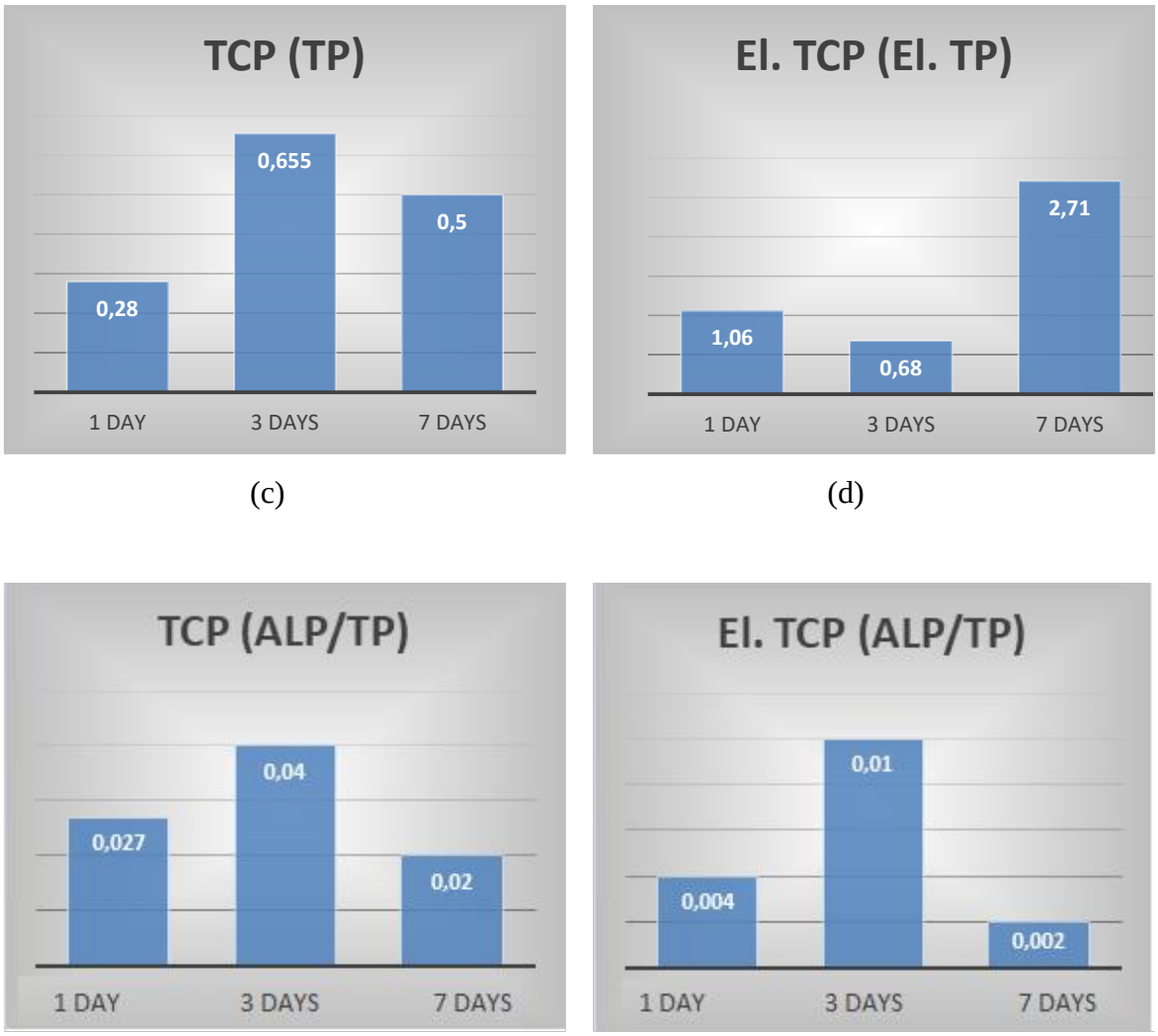
Figure 6-20 *(a) Technical Design of the Electrical Stimulation Device (b) Physical Implementation (c) Implementation in Practice*

The osteoblasts were placed on the different substrates and maintained in the incubator overnight. The day after, osteoblasts were exposed to an electric field with potential between 0.3 and 1 V for periods of 20 minutes to maximum 2 hours. At first, the osteoblasts were exposed to 1V electrical stimulation for an hour and did not survive. After that, as a second experiment cells were electrical stimulated at 0.7V for 2 hours and were necrosed, too. Finally, osteoblasts were exposed to electrical stimulation at 0.3V for 3 hours and they survived. The biochemical differentiation markers, alkaline phosphatase and total protein, were measured after 1, 3 and 7 days of incubation.

6.8.2. TCP Substrate



(a) (b)

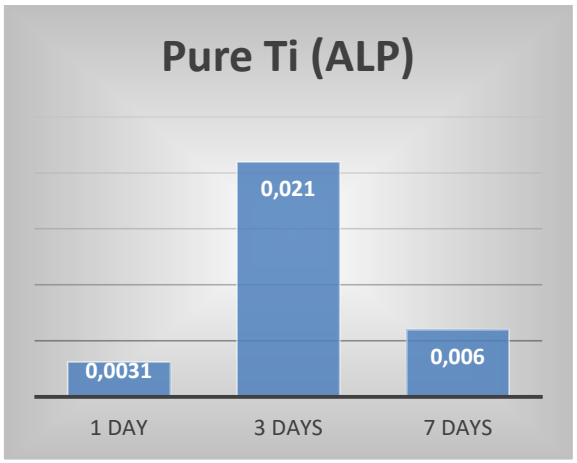


(c) (d)

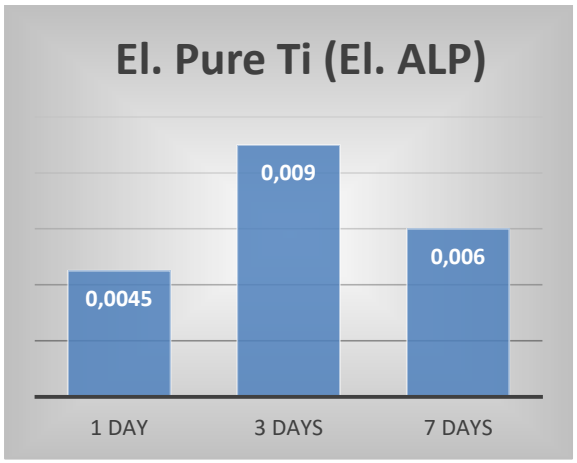
(e) (f)

Figure 6-21 *(a) ALP value without Electrical Stimulation (b) ALP value with Electrical Stimulation (c) TP value without Electrical Stimulation (d) TP value with Electrical Stimulation (e) ALP/TP value without Electrical Stimulation (f) ALP/TP value with Electrical Stimulation on TCP substrates.*

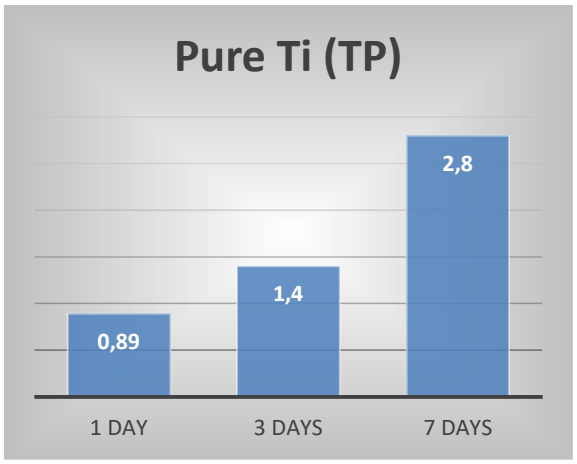
6.8.3. Titanium Substrate



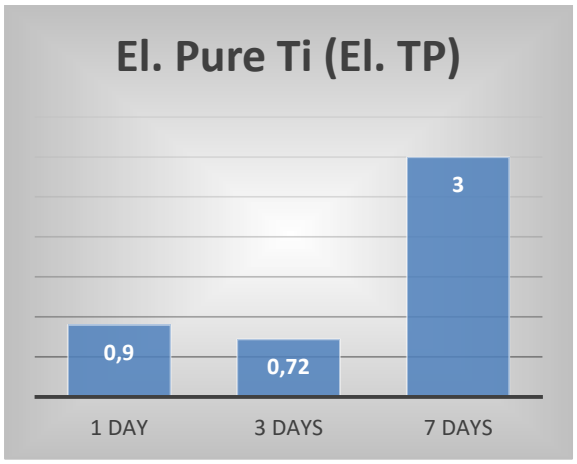
(a)



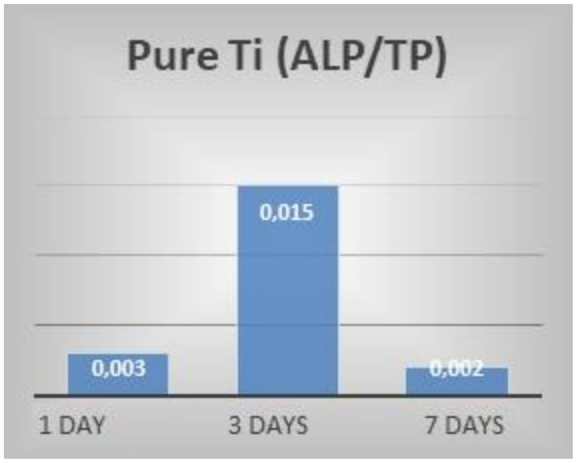
(b)

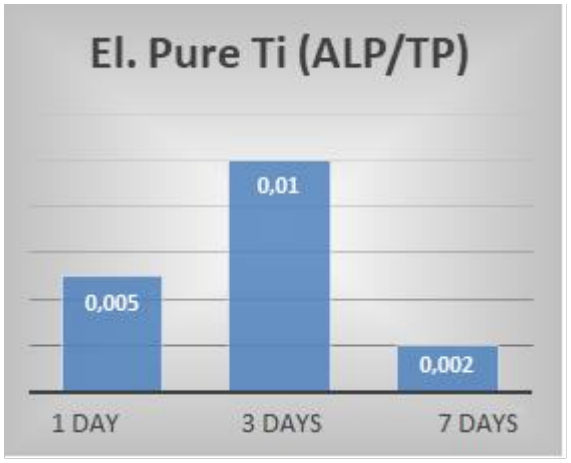


(c)



(d)

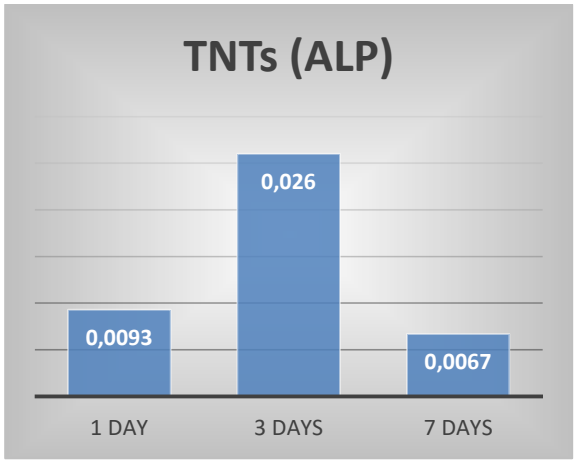




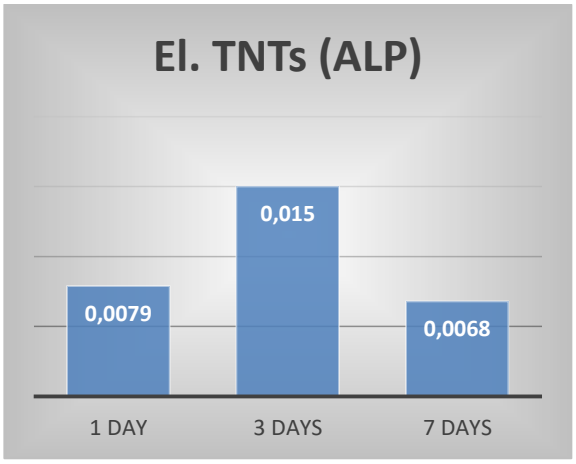
(e) (f)

Figure 6-22 *(a) ALP value without Electrical Stimulation (b) ALP value with Electrical Stimulation (c) TP value without Electrical Stimulation (d) TP value with Electrical Stimulation (e) ALP/TP value without Electrical Stimulation (f) ALP/TP value with Electrical Stimulation on titanium substrates.*

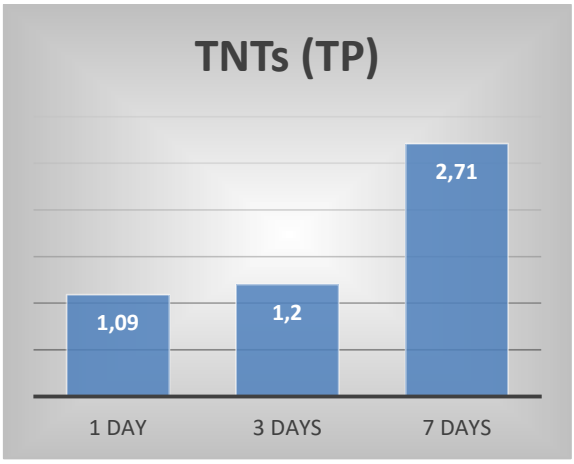
6.8.4. TiO_2 Substrate



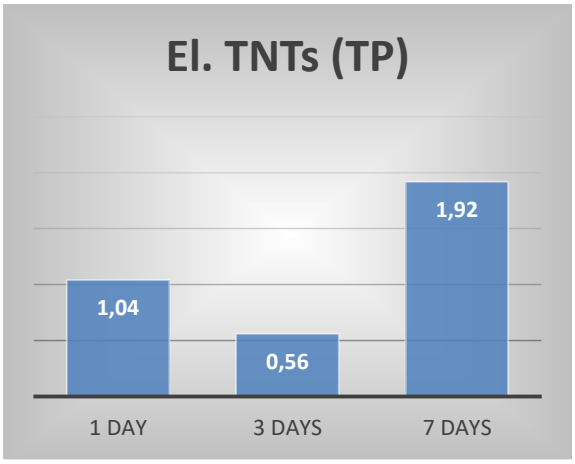
(a)



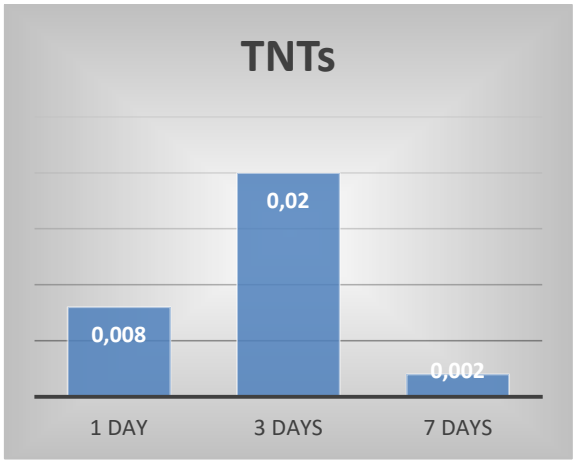
(b)



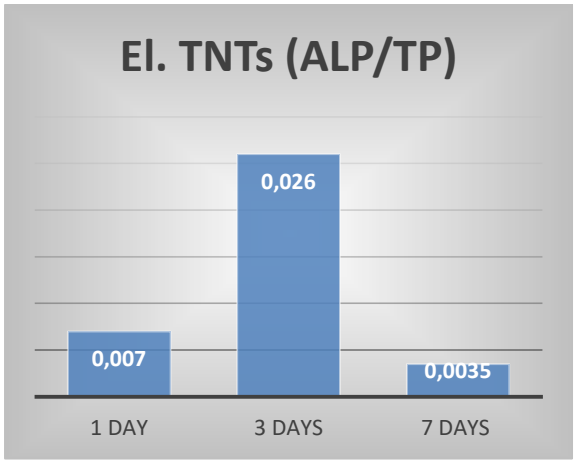
(c)



(d)



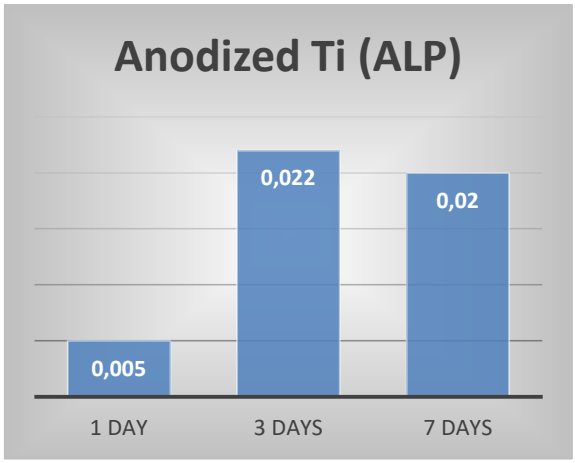
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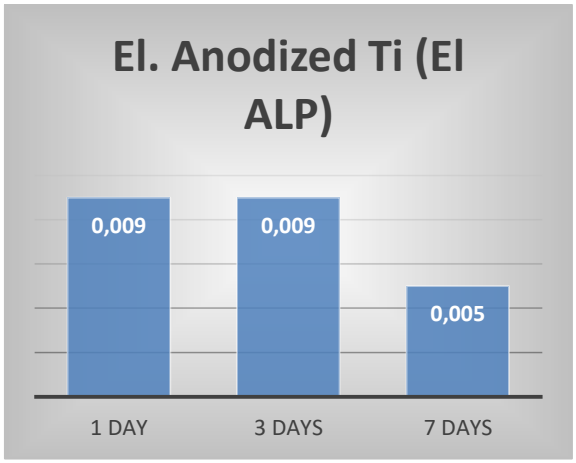
(f)

Figure 6-23 *(a) ALP value without Electrical Stimulation (b) ALP value with Electrical Stimulation (c) TP value without Electrical Stimulation (d) TP value with Electrical Stimulation (e) ALP/TP value without Electrical Stimulation (f) ALP/TP value with Electrical Stimulation on titania nanotubes substrates.*

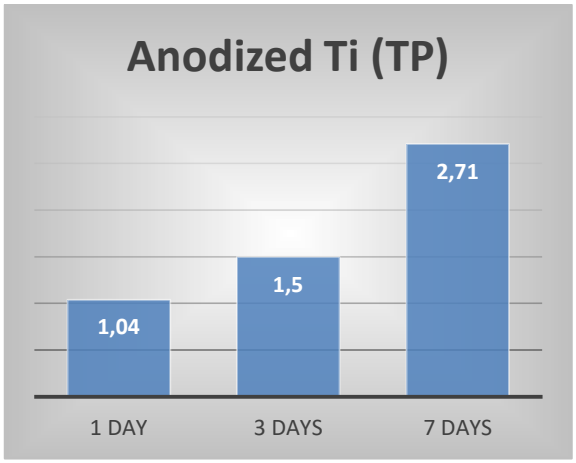
6.8.5. Rough Titanium Substrate



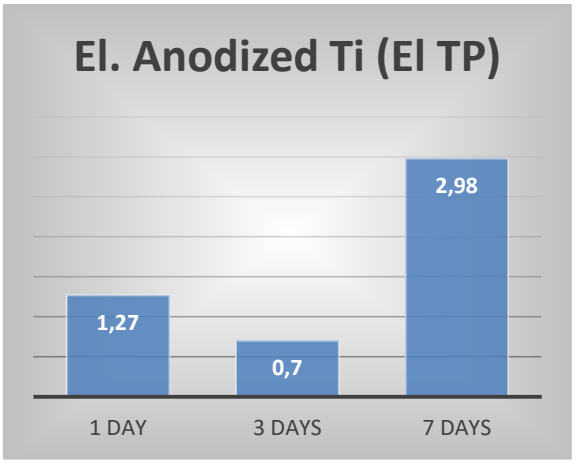
(a)



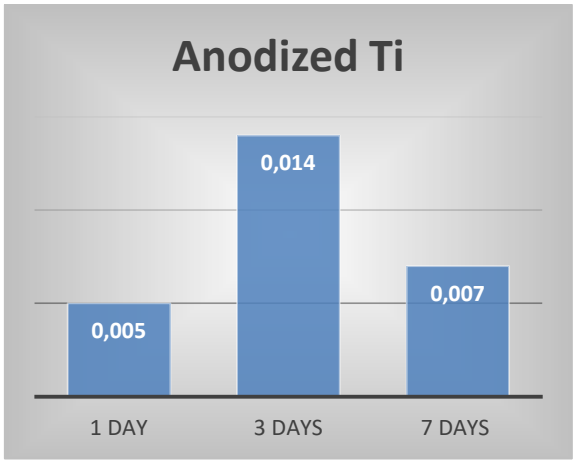
(b)



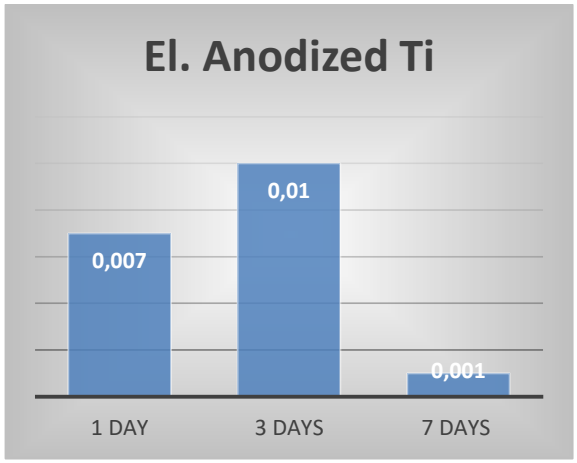
(c)



(d)



(e)



(f)

Figure 6-23 *(a) ALP value without Electrical Stimulation (b) ALP value with Electrical Stimulation (c) TP value without Electrical Stimulation (d) TP value with Electrical Stimulation (e) ALP/TP value without Electrical Stimulation (f) ALP/TP value with Electrical Stimulation on rough titanium substrates.*

TABLES OF VALUES MEASURED FOR ALL SUBSTRATES

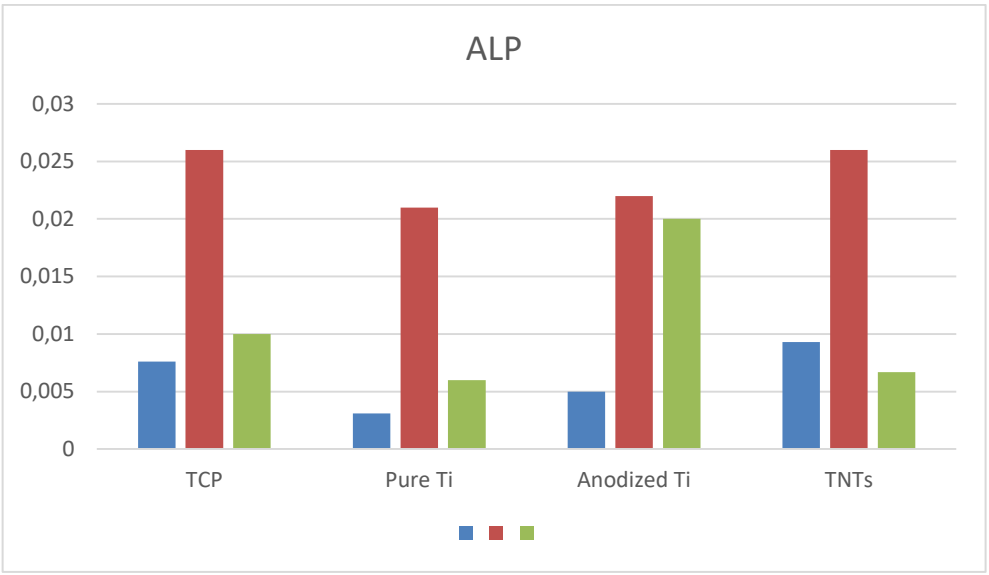
Alkaline Phosphatase (ALP)			
Material	**1 Day**	**3 Days**	**7 Days**
TCP	0.0076	0.026	0.01
Pure Ti	0.0031	0.021	0.006
Anodized Ti	0.005	0.022	0.02

TNTs	0.0093	0.026	0.0067
El. TCP	0.0046	0.007	0.008
El. Pure Ti	0.0045	0.009	0.006
El. Anodized Ti	0.009	0.009	0.005
El. TNTs	0.0079	0.015	0.0068

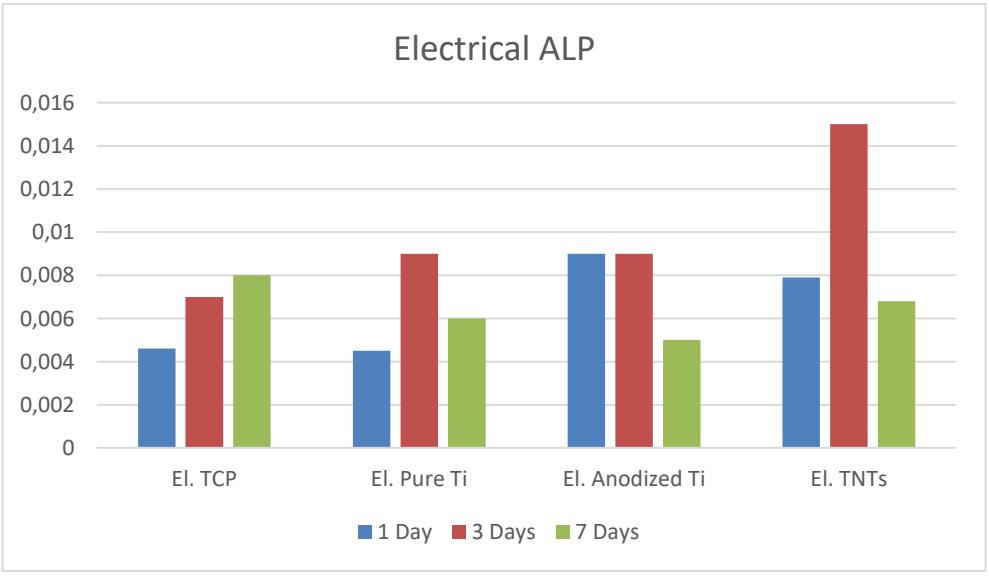
Total Protein (TP)			
Material	**1 Day**	**3 Days**	**7 Days**
TCP	0.28	0.655	0.5
Pure Ti	0.89	1.4	2.8
Anodized Ti	1.04	1.5	2.71
TNTs	1.09	1.2	2.71
El. TCP	1.06	0.68	2.71
El. Pure Ti	0.9	0.72	3
El. Anodized Ti	1.27	0.7	2.98
El. TNTs	1.04	0.56	1.92

ALP/TP			
Material	**1 Day**	**3 Days**	**7 Days**
TCP	0.027	0.04	0.02
Pure Ti	0.003	0.015	0.002
Anodized Ti	0.005	0.014	0.007
TNTs	0.008	0.02	0.002
El. TCP	0.004	0.01	0.002
El. Pure Ti	0.005	0.01	0.002
El. Anodized Ti	0.007	0.01	0.001
El. TNTs	0.007	0.026	0.0035

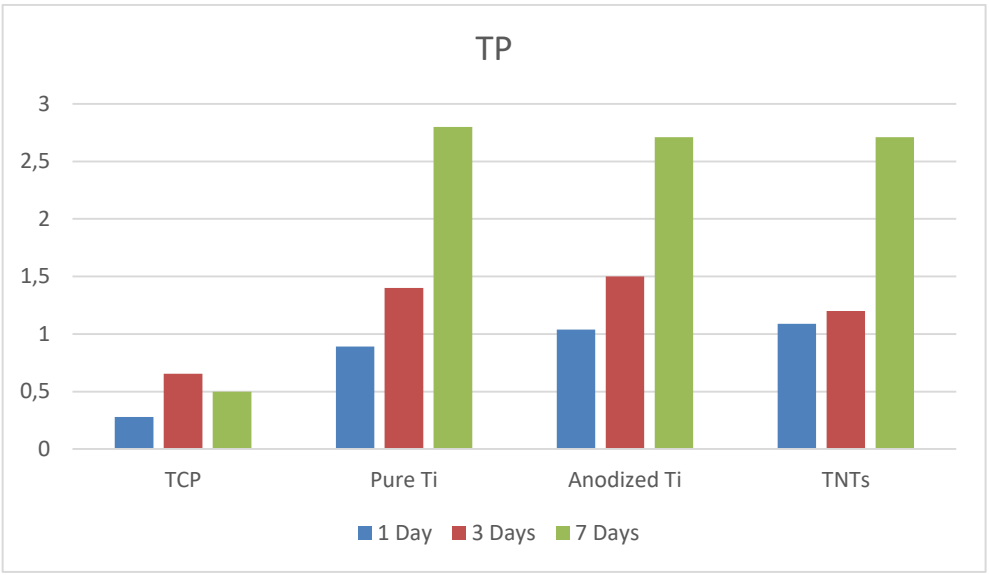
Diagrams - Comparative study of several substrates for (a) ALP values, (b) ALP values with electric stimulation (c) TP values (d) TP values with electric stimulation (e) ALP/TP values (f) ALP/TP values with electric stimulation – Figure 6-24


ALP
0,03
0,025
0,02
0,015
0,01
0,005
0
TCP
Pure Ti
Anodized Ti
TNTs

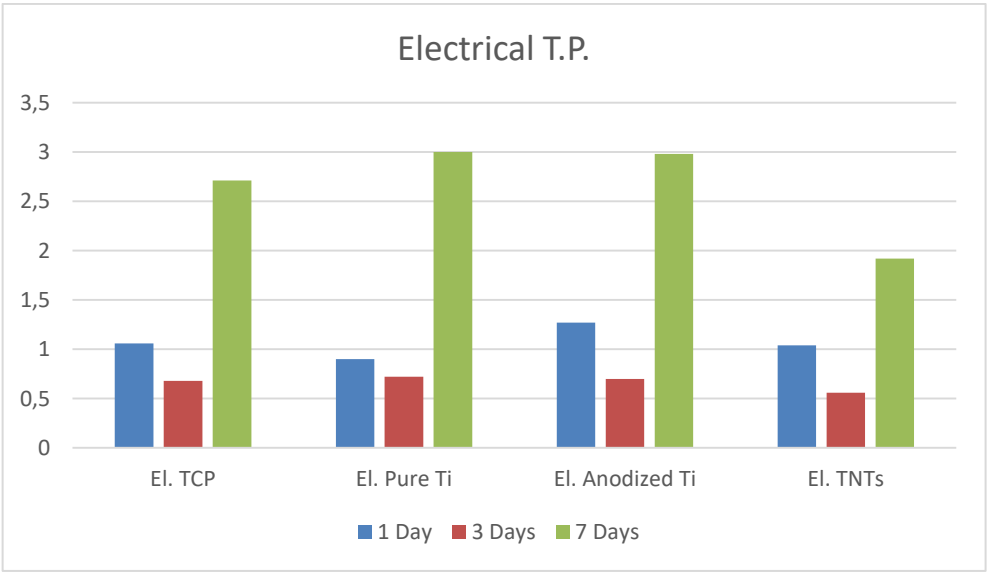
(a)


Electrical ALP
0,016
0,014
0,012
0,01
0,008
0,006
0,004
0,002
0
El. TCP
El. Pure Ti
El. Anodized Ti
El. TNTs
1 Day
3 Days
7 Days

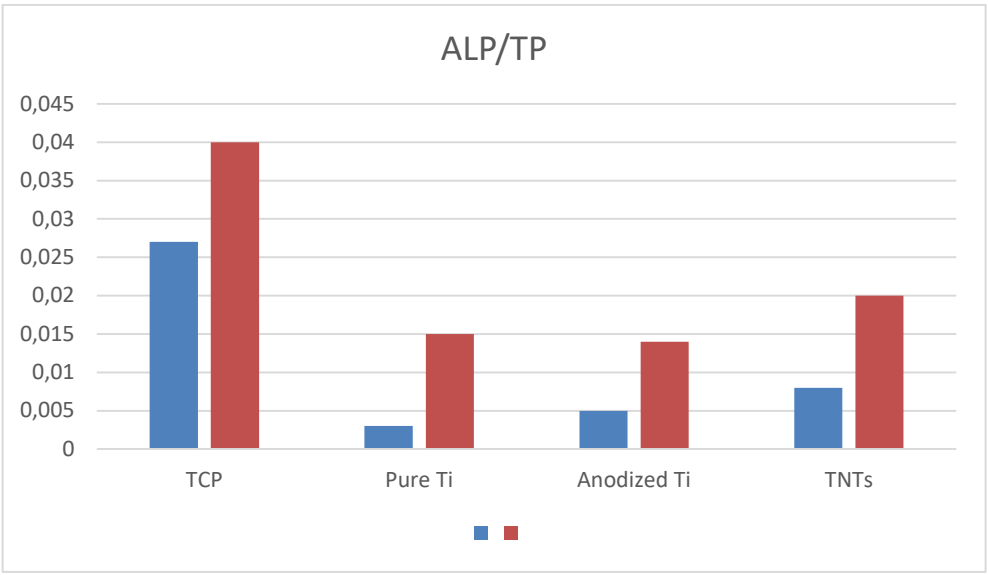
(b)


TP
3
2,5
2
1,5
1
0,5
0
TCP
Pure Ti
Anodized Ti
TNTs
1 Day
3 Days
7 Days

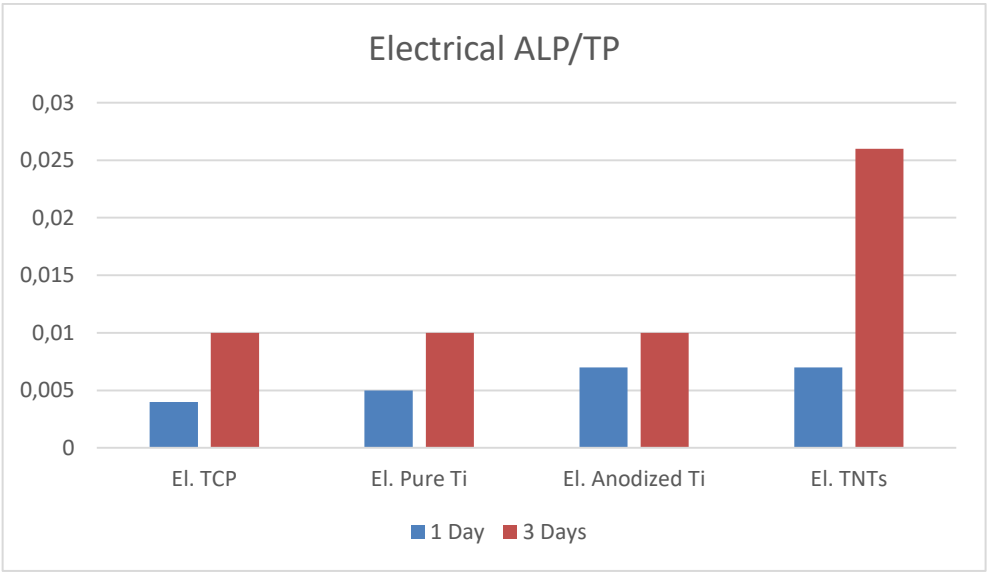
(c)


Electrical T.P.
3,5
3
2,5
2
1,5
1
0,5
0
El. TCP
El. Pure Ti
El. Anodized Ti
El. TNTs
1 Day
3 Days
7 Days

(d)


ALP/TP
0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
TCP
Pure Ti
Anodized Ti
TNTs

(e)


Electrical ALP/TP
0,03
0,025
0,02
0,015
0,01
0,005
0
El. TCP
El. Pure Ti
El. Anodized Ti
El. TNTs
1 Day
3 Days

(f)

Conclusions and Future Work

In the present research, several aspects related to the isolation and *in vitro* behaviour of osteoblast cells have been clarified. The isolation of bone tissues that contain mesenchymal cells during a hip or knee surgery is a complex procedure that involves several sterilization and maintenance steps. The isolation protocol of mesenchymal cells has been improved with respect to the sterilization of the working conditions. Mesenchymal cells from bone marrow were differentiated into osteoblast cells by the addition of specific substances (dexamethasone) in their medium. Osteoblasts behaviour is complex and under the control of many signaling molecules. Their functioning mechanisms are strongly substrate-dependent and have not yet been fully described. The present research aimed to clarify some aspects related to this issue. For this purpose, the development and behaviour, as well as specific biomarkers (alkaline phosphatase and total protein) of the osteoblast cells have been measured on the different substrates, in the presence and without an electrical field. The number of seeded cells was ~30.000 cells/ cm^2. The investigated substrates were tissue culture polystyrene (TCP) as a control material, pure titanium, anodized titanium with an increased surface roughness and titanium dioxide nanotubes layer (TNTs). It was found that the topography of biomaterials is critical to guide cellular behaviour like adhesion, spreading and cells development.

The present investigation aimed to find an appropriate combination of parameters related to substrate characteristics (nature and topography) and an induced electrical field that leads to the proliferation and enhanced activity of osteoblast bone cells. With this purpose, a cell carrying device has been designed and used for the electrical stimulation of the human osteoblast cells that were isolated from osseous tissue obtained during orthopaedic surgery. A direct current field was applied in the culture medium and a series of protocols for the electrical stimulation of the osteoblasts have been tested to find the appropriate combination of parameters (voltage, time, substrate) which enhance their activity. It was found that a combined effect of both applied electrical field (0.3V, 3 hours) and specific substrate nature and topography considerably increase the ration ‘alkaline phosphatase / total protein’ (ALP/TP), which has been translated as the degree of biocompatibility. When no electric field is applied, the cells tend to increase ALP levels associated to their multiplication and proliferation on soft surfaces, while a light decrease is observed when they are seeded on rough surfaces. On the opposite, the total protein level is increased cells seeded on rough

substrates, since this is related to their adhesion process. The ALP/TP is decisive for the faith osteoblasts population. Generally, a light increase in proliferation on TNTs compared to other substrates is associated with the highly organized nature of the TNTs. Results agree with already reported studies and confirm that substrate nature which includes its chemical composition, its mechanical properties, and surface topography are very important factors, decisive for osteoblasts well-functioning. When an electric field is applied, cells functions are improved. Titanium dioxide nanotubes, due to their nature and highly organized architecture, contribute to the uniform distribution of the electric field in the medium and under the bone cell population; finally, the effect of an adjusted intensity of the electric field and the appropriate topography of the substrate on cells behaviour demonstrate the importance of these two *in vitro* parameters for the activity of bone forming cells and may be considered crucial in the manufacturing of advanced biomaterials for orthopaedic applications.

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