MIINA HÄMÄLÄINEN

THE EFFECT OF BIPHASIC ELECTRIC CURRENT ON ADULT MESENCHYMAL STEM CELLS CULTURED ON POLYPYRROLE SURFACE

Master of Science Thesis

Examiner: Professor Minna Kellomäki,
Dr. Suvi Haimi
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ABSTRACT

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Electrical stimulation (ES) is a novel approach to osteogenic differentiation of mesenchymal stem cells (MSCs). Even though ES has been acknowledged as a treatment method for bone fractures already for two decades, it has gained more interest in the field of bone tissue engineering only for a few years. Therefore, also research of electroconductive scaffold materials is increasing in order to find an optimal osteogenic material to combine with the effect of ES. Polypyrrole (PPy) is a conductive biomaterial which is biocompatible with various cell lines and therefore a considerable option for mediating the electric current (EC) for MSCs. The combined effect of ES and PPy on MCSs could provide an alternative method for drugs and cytokines used in bone tissue engineering.

PPy was synthesized electrochemically on top of a gold-coated polyethylene terephtalate (PET) film. Adipose-derived MSCs, adipose stem cells (ASCs), were exposed to ES for 4 hours a day during 14 days at amplitude of ±0.2 V. There were 3 different stimulation groups to compare: a group with no stimulation (control) and groups with the frequency of 1 Hz and 100 Hz. In addition, two different PPy dopants were compared: hyaluronic acid (HA) and chondroitin sulphate (CS). Groups were also divided into osteogenic differentiation medium (OM) and basal medium (BM). In order to determine the viability, proliferation and differentiation of the cells, Live/Dead staining, DNA detection and alkaline phosphatase assays were used, respectively, at days 7 and 14 from the beginning of the stimulation.

PPy showed good biocompatibility with CS doped PPy (CS-PPy) in the viability examination in both media and in all of the stimulation groups, whereas ASCs on HA doped PPy (HA-PPy) formed clusters. Stimulation groups did not show any differences; however, a trend could be seen in the proliferation of CS-PPy in osteogenic medium (OM). CS-PPy showed significantly higher effect on proliferation, whereas HA-PPy supported on differentiation.

CS-PPy coatings seem to offer a suitable conductive surface for ASCs in osteogenic applications.

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Sähköstimulaatio on uusi lähestymistapa mesenkymaalisten kantasolujen luuerilaistuksen testaamiseen. Vaikka sähköstimulaatiota on käytetty luuvaurioiden hoidossa jo vuosikymmenten ajan, on kiinnostus noussut sitä kohtaan luun kudosteknologiassa vasta viime vuosien aikana. Myös sähköä johtavien materiaalien tutkimus on lisääntynyt sähköstimulaation myötä luusovelluksissa. Polypyrroli on sähköäjohtava biomateriaali, jonka on todettu olevan bioyhteensopiva useiden solulinjojen kanssa. Sen ja sähköstimulaation yhteisvaikutukset voisivat tarjota kasvutekijöiden ja lääkkeiden sijaan vaihtoehtoisen keinon mesenkymalisten kantasolujen luuerilaistukseen.

Polypyrroli syntetisoitiin sähkökemiallisesti kultapäällysteiseen polyeteeniteftalaattikalvoon. Rasvasta eristetyt mesenkymaaliset kantasolut altistettiin sähkövirralle 4 tuntia päivässä 14 päivän ajan amplitudilla ±0,2 V. Näytteet jaettiin 3 ryhmään: stimuloimattomiin (kontrolli) sekä 1 Hz ja 100 Hz taajuudella stimuloitaviin. Lisäksi verrattiin kahta eri polypyrrolin dopanttia: hyaluronihappoa ja kondroitiinisulfaattia. Ryhmien sisällä näytteet jaettiin vielä osteogeeniseen ja basaalimediumiin. Kokeessa tutkittiin solujen elinkykyä, proliferaatiota ja erilaistumista live/dead -värjäyksen, DNA- ja ALP-aktiivisuuden määrityksen avulla 7 ja 14 päivän kohdalla.

Kondroitiinisulfaattipitoinen polypyrroli osoittautui hyvin bioyhteensopivaksi rasvan kantasolujen kanssa, kun taas hyaluronihappopitoinen polypyrroli aiheutti solujen kasaantumisen ja irtoamisen. Eri stimulointiryhmien välillä ei nähty eroja, mutta DNA-määrityksen osalta osteogeenisessa mediumissa kondroitiinisulfaattipitoisissa polypyrrolinäytteissä oli havaittavissa trendi, joka osoitti 1 Hz taajuuden tukevan vähiten solujen proliferaatiota verattuna kontrolliin ja 100 Hz:n stimulaatioon. Kondroitiinisulfaattipitoinen polypyrroli tuki solujen proliferaatiota merkittävästi enemmän, kun taas hyaluronihappopitoinen tuki lievästi erilaistumista. Kondroitiinisulfaattipitoinen polypyrrolipnnoite sopii skaffoldin pinnoitemateriaaliksi luun kudosteknologiassa.

PREFACE

This study was conducted in the Adult Stem Cell Group, at the Institute of Biomedical Technology, University of Tampere. Financial support was provided by the Pirkanmaa Hospital District. This study was part of ElectroCell project supported by TEKES.

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TABLE OF CONTENTS

Abstract I
Tiivistelmä II
Preface III
Abbreviations and Notation VI
THEORETICAL PART IX
1. Introduction 1
2. Physiology of the Bone 3
2.1. Structure and Composition 3
2.2. Bone Cells and Bone Regeneration 5
3. Conductive Polymers in Bone Tissue Engineering 9
3.1. General Requirements for Osteogenic Scaffold Material 9
3.2. Conductive Polymers as Scaffold Materials 11
3.2.1. Dopants Determining the Final Conductivity 12
3.2.2. Hyaluronan and Chondroitin Sulphate as Bioactive Dopants 13
3.3. Polypyrrole Properties and Polymerization 15
4. Using Stem Cells in Tissue Engineering 19
4.1. Classification of Stem Cells 19
4.2. Adipose Stem Cells 21
4.2.1. Isolation and Characterization of Adipose Stem Cells 22
4.2.2. Osteogenic Differentiation of Adipose Stem Cells 24
4.3. Effect of External Stimulus on Osteogenic Differentiation of Adipose Stem Cells 25
4.3.1. The Effect of Osteogenic Medium and Growth Factors 25
4.3.2. Effect of Mechanical Stimulation on Mesenchymal Stem Cells 27
4.3.3. Effect of Electrical Stimulation on Mesenchymal Stem Cells 28
EXPERIMENTAL PART 33
5. Research Methods and Material 34
5.1. Polypyrrole Synthesis 34
5.2. Cell Culture Methods 35
5.2.1. Adipose Stem Cell Isolation and Cell Culture 35
5.2.2. Cell Attachment and Viability 36
5.2.3. Cell Proliferation 36
5.2.4. Osteogenic Differentiation 36
5.2.5. Mineralization 37
5.3. Electrical Stimulation 37
5.3.1. Statistical Analysis 38
6. Results 39
6.1. Cell Viability 39
6.2. Proliferation 42
6.3. Osteogenic Differentiation 44

7. Discussion of the Results **47**
7.1. The Effect of Material 47
7.2. The Effect of Electrical Stimulation 49
8. Conclusion **52**
9. References **53**

ABBREVIATIONS AND NOTATION

ρ	resistivity
σ	conductivity
2D	two dimension
3D	three dimension
A	area
AC	alternating current
ALP	alkaline phosphatase
ANOVA	analysis of variance
ASC	adipose stem cell
BEC	biphasic electric current
BM	basal medium
BMP	bone morphogenetic protein
BMSC	bone marrow-derived mesenchymal stem cell
CD	cluster of differentiation
CMFDA	5-chloromethylfuorescein diacetate
CP	conductive polymer
CS	chondroitin sulphate
DC	direct current
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's phosphate buffered saline
EC	electric current
ECM	extracellular matrix
ES	electrical stimulation
EthD-1	ethidium homodimer-1
EU	European Union
FBS	fetal bovine serum
FDA	Federal Drug Administration
FGF	fibroblast growth factor
GAG	glycosaminoglycan
HA	hyaluronic acid
IGF	insulin-like growth factor
in vitro	experiment that is performed in a controlled environment outside a living organism
in vivo	experiment takes place inside an organism
MSC	mesenchymal stem cell
OC	osteocalcin
OM	osteogenic medium
ON	osteonectin
OPN	osteopontin
Osteogenic	promotes or is involved in bone growth or repair

PANI	polyaniline
PEDOT	poly(3,4-ethylenedioxythiophene
PEMF	pulsed electromagnetic field
PLC	phospholipase C
PLLA	poly-L-lactide
PPy	polypyrrole
R	resistance
RANK	receptor activator of nucleator factor κB
SCV	stromal vascular fraction
TCB	tricalciumphosphate
TGF-β	transforming growth factor beta

THEORETICAL PART

1. INTRODUCTION

Tissue engineering is a field of regenerative medicine which exploits body's intrinsic healing processes by combining cells, biomaterials, and stimuli aiming to repair, regenerate and replace diseased or damaged tissues and even whole organs (Sundelacruz & Kaplan 2009). Despite inherent capacity of bone tissue to regenerate upon damage, there are mechanical and metabolic restrictions that often need augmentation especially in severe bone fractures, such as non-unions, substantial loss of bone tissue, or inability to heal because of disease. (Dawson & Oreffo 2008). Bone tissue engineering has become crucial field due to aging of the population followed by increased number of bone defects and diseases and even worse donor shortages (Dawson & Oreffo 2008, Stock & Vacanti 2001, Vacanti 2001).

Adipose stem cells (ASCs) originating from adipose tissue, are an interesting source of cells for bone tissue engineering since they are easy to isolate, culture and differentiate into osteogenic cell lines. In addition, ASC isolation is smaller operation for the patient than when isolating mesenchymal stem cells (MSCs) from bone marrow. (Locke *et al.* 2009)

Most commonly used growth-directive cues used for stem cells include drugs, cytokines and growth factors (GFs) (Porter *et al.* 2009). Minimal use of these bioactive molecules is supported by substantial institutions such as European Union (EU) and Federal Drug Administration (FDA) (FDA 2009). This is because their use may contain a risk of the introduction, transmission or spread of communicable diseases for the patient due to their allo- or xenogenous, or other origin. In addition, GFs are expensive and possess relatively short half-life. Despite of the extensive research, novel approaches concerning GFs do not compensate the fact that these approaches are risky and costly. (Park *et al.* 2011)

To overcome problems related to GF's, research has been focusing more and more on physical stimulation, such as electrical stimulation (ES). ES has arisen to hasten bone regeneration and has become an accepted treatment method to assist with bone healing (Ramanujam *et al.* 2009). Despite the good results of this treatment method, the underlying mechanism in the bone tissue is still poorly understood (Huang *et al.* 2008). However, MSCs are reported to respond to ES similarly to osteoblasts under mechanical strain. (Hammerick *et al.* 2010).

ES is a fairly novel approach in bone tissue engineering and there are only few studies concerning electrically stimulated ASCs (Hammerick *et al.* 2010, McCullen *et al.* 2010, Kim *et al.* 2009, Sun *et al.* 2007, Tandon *et al.* 2009, Hronik-Tupaj *et al.* 2011). The studies concentrated on intracellular reactions, mainly intracellular calcium levels, and cell proliferation, viability, differentiation, orientation and migration under ES. As in the case of mechanical stimulation, the main challenge is to find optimal ES parameters. Even though several studies have reported on osteogenic effects of ES, the

results vary strongly between experiment configurations. Therefore, the main challenges lie in adjusting various parameters (electroconductivity, voltage, frequency, cell line variation, culture medium etc.) to serve for bone tissue engineering. Optimal conditions have not yet been found.

The advantage of ES compared to the traditional mechanical stimulation is the possibility to direct cell migration and growth by exploiting conductive materials in the scaffold. It is also possible to use non-conductive materials since the growth media contains several different electrolytes.

Polypyrrole (PPy) is a conductive polymer (CP) and potential biomaterial for bone tissue engineering, because it can transmit electrical current (EC) in addition to providing physical support to cells (Ateh *et al.* 2006). It also allows incorporation of various biomolecules which enables tailoring of PPy-based scaffolds for different tissue applications. EC applied through PPy has been shown to enhance growth of various cell lines, such as fibroblasts and nerve cells (Ateh *et al.* 2006, Wang *et al.* 2004, Schmidt *et al.* 1997)

The aims of this study were; firstly, to compare two different PPys doped with negatively charged molecules; secondly, to evaluate the effect of ES on ASCs with two different frequency parameters (high and low); and thirdly, to compare the effect of osteogenic culture medium with BM under ES. These three different factors were studied in terms of cell viability, proliferation and osteogenic differentiation.

2. PHYSIOLOGY OF THE BONE

Bone serves a variety of functions. It protects tissues, provides structural foundation for the body and serves as the attachment of the muscles for locomotion and other movements (Shea & Miller 2005, Clarke 2008). In addition, it provides maintenance of mineral homeostasis storing mainly calcium and phosphate and balances the ratio of acid and base, serves as a reservoir of GFs and cytokines as well as provides an environment for hematopoiesis within the marrow spaces (Shea & Miller 2005, Taichman 2005). Bone is highly vascularised and metabolically active tissue. It has particular ability to regenerate without scarring and respond to external and internal cues by remodelling its structure. (Sommerfeldt & Rubin 2001)

2.1. Structure and Composition

There are two types of bone tissue in the adult skeleton: cortical bone and trabecular bone. Human cortical bone is dense having degree of porosity smaller than 5 % and it is usually found in the outer shell of long bones. Trabecular bone is more porous having a honeycomb-like network or trabecular plates and rods which are distributed among the bone marrow compartment. (Shea & Miller 2005) The ratio of the cortical and trabecular bone matrix varies between different bones. Both trabecular and cortical bone are composed of osteons, microsized lamellae of collagenous fibres. Lamellae of cortical bone form osteons that are arranged around a vascular canal, called Haversian canal. It also contains nerves, lymphatics and connective tissue (Datta *et al.* 2008). The structure of the long bone is presented in Figure 1.

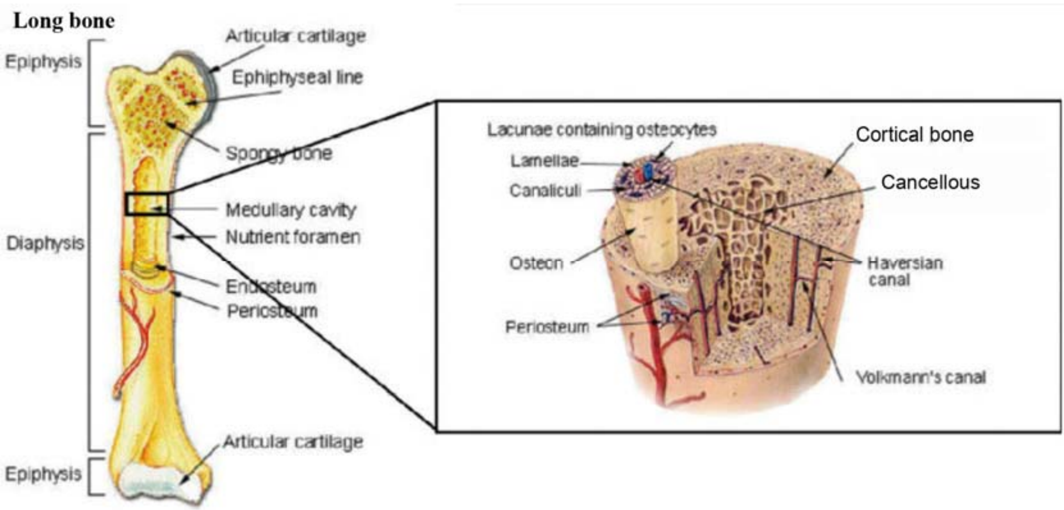

Figure 1. The structure of the long bone (Flint *et al.* 2007).

In long bones, cortical bone has two surfaces: an outer periosteal surface (periosteum) and inner endosteal surface (endosteum). The periosteal surface has two layers: a fibrous outer layer and inner one which contains vessels, nerve fibers, osteoblasts and osteoclasts and it is important for bone growth and fracture repair. The endosteum covers the inner surface of cortical bone and trabecular bone and the blood vessel canals (Volkman's canals) of present in bone. (Clarke 2008, Datta *et al.* 2008) Bone has a tendency to undergo resorption from the endosteal surface (Datta *et al.* 2008).

The organic matrix of the bone consists mainly of Type I collagen which is a triple helical molecule composed of two identical alpha-1 chains and a single alpha-2 chain (Gelse *et al.* 2003) Also collagen type III, V and Fibril Associated Collagens with Interrupted Triple helices which are important for organization and stabilization of extracellular matrix (ECM), are present in small amounts (Clarke 2008). In addition to providing tensile strength for bone, collagen contains peptides which cue bone cells (McCann *et al.* 1997). Other proteins are derived from exogenous and endogenous sources. Exogenous proteins, such as albumin, are mainly derived from serum and may help regulate matrix mineralization. (Clarke 2008)

Endogenous proteins include large variety of GFs as well as other non-collagenous proteins, namely alkaline phosphatase (ALP), osteonectin (ON), osteocalcin (OC), osteopontin (OPN) and bone sialoprotein, which have an important role in the calcification of bone mineral (Shea & Miller 2005, Bonucci 1995, Robey 1996). From the non-collagenous proteins, ON is the most abundant protein. It has multiple Ca^{2+} and collagen binding sites and has shown to be a potential nucleator of hydroxyapatite. OC is a typical biomarker for osteoblastic differentiation with OPN taking part into inhibition of mineralization (Porter *et al.* 2009, Tambasco de Oliveira & Nanci 2004, Marcus *et al.* 2009). In bone, ALP participates in the nucleation of hydroxyapatite and mineralization. (Storrie & Stupp 2005, Beertsen & van den Bos 1992, Anderson 1995). It is considered as an early marker of osteogenic differentiation

whereas molecules affecting on mineralization and secretion, such as OC, are related to the final phase of differentiation. Organic matrix contains also glycosaminoglycans (GAGs), namely chondroitin sulphate (CS) and keratan sulphate (Palmer *et al.* 2008). In general, GAGs are reported to inhibit mineralization; however, CS is suggested to take part into mineral deposition and crystal morphology (Marcus *et al.* 2009, Palmer *et al.* 2008). Composition of organic matrix is presented in Table 1.

Table 1. Organic composition of bone matrix (Shea & Miller 2005, Clarke 2008, Gelse *et al.* 2003, Bonucci 1995, Robey 1996, Marcus *et al.* 2009)

<table>
<tr><td rowspan="2">Collagenous proteins 85–90 %</td><td>Collagen type I</td><td rowspan="2"></td></tr>
<tr><td>Collagen type III, V and FACIT collagens</td></tr>
<tr><td rowspan="2">Noncollagenous proteins 10–15 %</td><td>Exogenous</td><td>Albumin
α2-HS-glycoprotein
Growth factors
Other molecules affecting cell activity</td></tr>
<tr><td>Endogenous</td><td>Proteoglycans: chondroitin sulphate, keratin sulphate
Glycoproteins: ALP, ON, bone sialoprotein, OP, fibronectin
γ-carboxylated (gla) proteins: OC
Growth factors</td></tr>
</table>

The main mineral component, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], consists of very small crystals and contains many impurities, such as carbonate and magnesium, which tend to lower the crystallinity of the bone matrix. These impurities might be important in rendering apatite more soluble permitting it to release ions when it is needed for homeostasis. (Shea & Miller 2005) Some molecules, such as albumin, can bind to hydroxypatite because of their acidic properties (Clarke 2008).

2.2. Bone Cells and Bone Regeneration

There are four distinct cells that enable the bone tissue to respond to external cues and maintain the basic functions on bone: osteoblasts, osteoclasts, osteocytes and bone lining cells (Shea & Miller 2005). A typical character for bone cells is their high number of adhesion receptors belonging to four basic families: the integrins, cadherins, selectins and members of the immunoglobins (Ig) (García & Reyes 2005). Osteoblasts synthetize the bone matrix by depositing unmineralized bone matrix (osteoid) which encompasses endogenous proteins as well as calcium and phosphorus that are excreted in membrane-bound matrix vessels together with ALP (Storrie & Stupp 2005, Beertsen & van den Bos 1992, Anderson 1995). Mature osteoblast has cuboidal shape, extensively rough

endoplasmic reticulum and well-developed Golgi apparatus due to its active role in protein synthesis and secretion (Shea & Miller 2005).

Osteocytes are formed from osteoblasts that are entrapped and buried by the bone matrix. They are often connected with other osteocytes, osteoblasts and bone lining cells by small channels called canaliculi. Osteocytes have several roles, such as the maintenance of mineral homeostasis by permitting the diffusion of the fluids and mineral. (Shea & Miller 2005) They may also serve as a mechanical or damage sensor, hence initiate bone remodelling or repair (Bonewald 2006, Mori & Burr 1993).

Osteoclasts are responsible for the resorption of bone. They are multinucleated giant cells originated from the monocyte/macrophage haematopoietic lineage that become polarized and adhere to the bone matrix. (Boyle *et al.* 2003, Schindeler *et al.* 2008) Osteoclast activation is induced by signals that lead to polarization, and as a result of RANK signalling pathway activation, the cell undergoes structural changes in its actin skeleton that prepares it to resorb bone (Boyle *et al.* 2003, Burgess *et al.* 1999). The activation requires that the osteoclasts attach to the bone surface, where actin and integrins play an important role (Teitelbaum 2000). After resorption, osteoclasts either return to inactive state or die by apoptosis (Shea & Miller 2005)

Despite the dynamic character of the bone, the majority of the adult bone surface is not undergoing any bone formation or resorption (Shea & Miller 2005). These surfaces are called inactive or resting surfaces and a characteristic cell for this area is a bone lining cell which is formed by the inactivation of osteoblasts and perhaps mesenchymal cells (Miller *et al.* 1980) Bone lining cells are capable of proliferation (Bowman & Miller 1986) and several roles have been suggested for them including the initiation of osteoclast resorption (Everts *et al.* 2002).

Bone development can follow two different pathways: through intramembranous or endochondral ossification. In intramembranous ossification bone arises directly from MSCs. This is typical for embryonic development and is also involved in the development of flat bones, such as bones in the cranium and several facial bones. Bone in load-bearing joint undergoes endochondral ossification during their growth and formation of the skeleton having a cartilage intermediate before ossification (Figure 2). (Kanczler & Oreffo 2008)

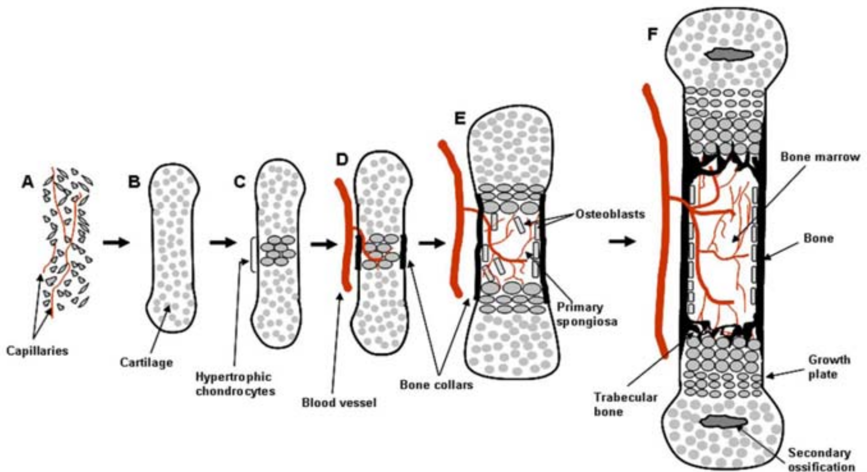

Figure 2. Bone development by endochodral ossification (Kanczler & Oreffo 2008).

Bone fracture healing proceeds through endochondral ossification; however, in some cases intramembranous hard callus formation may dominate. Fracture healing can be roughly divided into four stages that can in practice significantly overlap each other:

1) Inflammation
2) Soft callus formation
3) Hard callus formation
4) Bone remodelling

Bone fracture typically interrupts the integrity of soft tissue, normal vascular function and bone marrow structure, which leads to activation of non-specific wound healing pathways. (Schindeler *et al.* 2008) For instance, hypoxia and acute necrosis caused by the vasculature breakdown induce infiltration of inflammatory cells that fight against infection, secrete cytokines and GFs, and promote clotting into a fibrous thrombus (Schindeler *et al.* 2008, Kanczler & Oreffo 2008, Gerstenfeld *et al.* 2003, Einhorn 1998, Glowacki 1998) Capillaries grow into the clot over the time and reorganize granulation tissue. Macrophages and other phagocytic cells clear the dead cells and other debris (Schindeler *et al.* 2008).

In soft callus formation, chondrocytes derived from MSCs and fibroblasts proliferate and synthesize cartilaginous matrix until the fibrous clot is replaced by cartilage. Soft callus is able to provide support for the fracture when simultaneously acting as a template for bony callus. Chondrocytes turn into hyperthropic cells in the final stage of soft callus formation and undergo apoptosis after mineralization of the matrix. Angiogenesis is promoted by invasion of vascular endothelial cells and capillary in-growth. (Schindeler *et al.* 2008)

Hard callus formation is also known as primary bone formation. Osteoblasts migrate into the callus and produce osteoid (Schindeler *et al.* 2008). Mineralization begins 10 to 15 days after osteoid deposition as the matrix becomes supersaturated with respect to hydroxyapatite (Sommerfeldt & Rubin 2001, Marcus *et al.* 2009). Hypertrophic chondrocytes and osteoblasts deposit matrix vesicles that participate in

nucleation. Initial formation of hydroxyapatite within matrix vesicles is followed by the propagation phase in the matrix. (Golub 2009) Concomitant with revascularisation, the soft callus is gradually removed resulting in woven bone which has disorganized weak structure. Intramembranous bone formation can also occur in the formation of the hard callus in some cases; however, often some level of endochondral ossification is present. (Schindeler *et al.* 2008, Kanczler & Oreffo 2008). Vasculature formation and increased oxygen tension is critical for hard callus and osteoblast differentiation.

Bone remodelling is the final stage of fracture healing and encompasses remodelling of woven bone to lamellar bone. It can be referred to as secondary bone formation. Remodelling includes the resorption of the hard callus followed by the formation of lamellar bone, hence osteoclasts play an essential role. (Schindeler *et al.* 2008).

There are a large variety of GFs that regulate bone regeneration through the stages. These involve transforming growth factor-β (TGF-β), insulin growth factor (IGF), bone morphogenic proteins (BMPs) vascular endothelial growth factor (VEGF) (Schindeler *et al.* 2008).

Common proangiogenic factors of vasculature formation are fibroblast growth factor-1 (FGF-1) and VEGF. In the later stage of vascularisation angiopoietin I and II play an important role. (Gerstenfeld *et al.* 2003, Ai-Aql *et al.* 2008).

3. CONDUCTIVE POLYMERS IN BONE TISSUE ENGINEERING

Biomaterials used in tissue engineering are so called third generation biomaterials which exhibit both: bioactivity and biodegradation properties (Navarro *et al.* 2008). Commonly used materials in bone applications are ceramics and polymers or their composites. (Porter *et al.* 2009, Navarro *et al.* 2008) Biomaterials can be divided according to their origin into natural, synthetic materials and natural modified materials. Natural polymers used in bone scaffolds include collagen, GAGs, fibrin, silk and many others. This origin includes some significant drawbacks in their properties such as risk of infection, fixed degradation rates and immunogenicity. (Porter *et al.* 2009)

Ceramics are attributed bioactive because of their similarity with the mineral phase of bone (El-Ghannam 2005). Commonly used bioceramics are hydroxyapatite, tricalciumphosphate (TCP), biphasic calciumphosphates and multiphasic bioactive glasses. (Navarro *et al.* 2008)

Most of the polymers are not inherently bioactive and can degrade through hydrolysis or enzymatic pathways. The degradation rate can be tailored by copolymerization and by tuning the degree of crystallization. Commonly investigated polymers for scaffold materials include polyesters, polydioxanone, poly(propylene fumarate), poly(ethylene glycol), poly(orthoesters), polyanhydrides and polyuretanes. Polyesters are most commonly studied polymers due to several FDA approved polyesters and easy copolymerization with variable constituent percentages. From polymer composites, micro and nanoscale hydroxyapatite is mostly used with polymers. (Porter *et al.* 2009)

3.1. General Requirements for Osteogenic Scaffold Material

In general, the most important aim of the scaffold is to mimic ECM of the target tissue. The other essential requirement is the biodegradability of the scaffold, which allows body's own healing process to replace the scaffold with its own ECM. (Dawson & Oreffo 2008) According to the other reguirements, material should be biocompatible, not awake any inflammatory or toxic response and the bioactivity of the material should be maintained after sterilization (Porter *et al.* 2009).

The specific characteristics of the scaffolds vary according to the field of tissue engineering e.g. bone has its own specific requirements presented in Table 2. In bone

applications, material needs to promote osteogenesis. In other words, it needs to be osteogenic. Ceramic materials can be, in theory, divided into osteoconductive and osteoinductive materials (Habibovic & de Groot 2007) Osteoconduction allows bone formation onto the surface of the material whereas osteoinductive materials can trigger bone formation even in soft tissues. This is due to their ability to recruit and differentiate MSCs into osteoblasts (Laurencin & Khan 2009). Practically, no osteoinductive material has been discovered; nevertheless, tissue engineered construct or biomolecule-incorporated material can be included under the definition.

Most common osteogenic bioceramics used for bone applications consist of similar components as inorganic ECM of bone. Therefore, calcium phosphate-based bioceramics, such as hydroxyapatite, β-tricalcium phosphate, are widely applied (Rahaman *et al.* 2011). In addition, bioactive glasses and glass-ceramics have gained more interest after Hench *et al.* invented Bioglass® in the early 1970s (Hench *et al.* 1972). Bioactive glasses are attractive for producing bone scaffolds due to their versatile properties. Also, their osteogenic property can be enhanced by addition of some ions, such as zinc and magnesium (Oki *et al.* 2004, Balamurugan *et al.* 2007, Saboori *et al.* 2009). The newest bioactive glasses are based on borate and borosilicate compositions. The disadvantage of the bioceramics and bioactive glasses and glass ceramics is their brittleness. (Rahaman *et al.* 2011)

Since adhesion is essential for bone cells due to their high number of adhesion receptors, surface properties of the material play an important role in cell regulation. (García & Reyes 2005)

Table 2. Scaffold property and its desired effect in bone tissue engineering. [modified (Sundelacruz & Kaplan 2009, Dawson & Oreffo 2008, Porter et al. 2009, Navarro et al. 2008)

Porosity	Cell recruitment, attachment and vascularisation
Pore size	Cell infiltration, migration, proliferation, distribution and nutrient and oxygen exchange
Pore interconnectivity	Determines geometry of resulting tissue and allows the cell, ECM and vascular penetration trough the scaffold as well as liquid flow perfusion, which assures nutrient supply.
Degradation	Allows deposition of native matrix by growing tissue, allows the transfer of structural support to native matrix when it is deposited. Mechanical properties should be maintained at least 1-3 months after implantation. By products need to be non-toxic.
Mechanical strength	Similar mechanical properties with native tissue and retain three dimensional structure and space for growing tissue.
Incorporation of biochemical signaling	Provides stimuli for cell adhesion, proliferation, differentiation and vascularisation.
Topography	Wettability, cell adhesion and migration

Scaffold properties are greatly affected by material property and fabrication methods. For instance, wettability is inherently defined by chemical composition of the material but, in the end, surface roughness determines the wettability of the material surface. (Serra Moreno *et al.* 2008)

3.2. Conductive Polymers as Scaffold Materials

Conductivity (σ) describes material's ability to transmit an EC (I). It is reciprocal to resistivity (ρ) and can be written as follows:

$$\sigma = 1/\rho = l / (R \times A) \quad (1)$$

where l is the distance between two points in the measured resistance, A is the cross-sectional area perpendicular to the direction of the current and R is the resistance of the material. Unit for conductivity is S/cm. Alternating current (AC) requires more complex equations to define the conductivity, namely impedance which is the ratio of the amplitude of the voltage across the circuit to the current amplitude in the circuit. In addition to resistance, it includes capacitive and inductive reactance which vary according to frequency used (Young & Freedman 2000, Callister 2003)

Good conductors are typically having a value of greater than 10^3 S/cm, semiconductors between 10^{-8} and 10^3 S/cm and insulators less than 10^{-8} S/cm (Guimard *et al.* 2007). Most polymers are poor conductors because of unavailability of abundant free electrons to participate in the conduction. (Callister 2003) However, there is a special group of polymers that exhibits electrically active structure, called conductive polymers (CPs) that can have conductivity values ranging between $1–10^3$ S/cm (Ravichandran *et al.* 2010, Guimard *et al.* 2007).

The electronic and ionic conductivity of CPs is based on conjugated double bonds along the polymer backbone, where bonds between carbon atoms are alternatively single and double. These bonds are generated by an electron cloud overlap of p-orbitals which form π molecular orbitals. This leads to delocalization of electrons in the polymer and hence enables their movement along the chain. The bonds are referred as conjugated system. Planar conformation of the conjugated system maximizes sideways the overlap between the π molecular orbitals, which is critical to conductivity. This creates metal-like and semiconductive properties for polymers. (Guimard *et al.* 2007)

CPs are attractive candidates for scaffold material having several advantages for use in tissue engineering such as conductivity, reversible oxidation, redox stability, biocompatibility, hydrophilicity (40−70° water contact angle promotes cells adhesion) and 3D geometry (Guimard *et al.* 2007). They are inexpensive, easy to synthesize and their properties can be easily modulated by different surface functionalization techniques and by use of wide range of molecules. (Ravichandran *et al.* 2010) There are

numerous biomedical applications besides scaffolds that CPs are considered including drug release, artificial muscles, also termed as bioactuators, and biosensors (Otero & Sansinena 1998, Wallace *et al.* 2009). The molecular structure of some CPs, used in regenerative medicine, is presented in Figure 3.

polyacetylene (PA)
polypyrrole (PPy)
polythiophene (PT)
poly(3,4-ethylene dioxythiophene) (PEDOT)
polyaniline (PANI)

Figure 3. Chemical structure of various conducting polymers [modified (Guimard et al. 2007)].

From commonly used CPs, PPy and polythiophene (PT) have been considered as scaffold material. PPy is widely studied for neural and cardiac applications (Thompson *et al.* 2010, Prabhakaran *et al.* 2011). Poly(3,4-ethylenedioxythiophene) (PEDOT) have been studied for neural prosthesis and polyaniline (PANI) for cardiovascular applications. (Ravichandran *et al.* 2010)

3.2.1. Dopants Determining the Final Conductivity

CPs would be insulators without dopant ions that oxidize or reduce the neutral polymer during the polymerization depending on their charge. In this process, referred as doping, the polymer becomes polarized, and thus a charge carrier. The dopant is incorporated in the structure by electrical forces as shown in Figure 4. However, large molecular dopants, such as high-molecular-weight HA can be, to some extent, physically trapped into CP, and hence, more stably integrated in the structure (Guimard *et al.* 2007). Attraction of electrons yields charge mobility along the chains and between the chains. Upon doping, the net charge is zero because of the close association of the counter ions with the charged backbone (Guimard *et al.* 2007), which stabilizes the polymer structure (Ravichandran *et al.* 2010). Dopant ion affects on the band gap (the distance between conducting band and valence band), and thus determines the conductivity: the smaller the band gap the more conductive is the polymer. Besides the choice and percentage of the dopant, also temperature and the synthesis method have an influence on conductivity. (Guimard *et al.* 2007)

The size of the dopant determines its mobility within the polymer structure. Large molecules are usually immobile and organized into the same phase with individual polymer chains keeping their location during redox cycling. The size has also an effect on expansion and contraction of the polymer during redox reactions. For instance, polymers containing large dopant anions are observed to expand upon reduction, whereas small anions, which are free to move within the structure and thus are involved in the exchange with the ions of the surrounding electrolyte, induce contraction of the material. (Pelto *et al.* 2010) In order to improve the biocompatibility of the material, bioactive molecules can be used as dopants (Cui *et al.* 2003). Most bioactive molecules are not capable of redox chemistry and therefore they need to be doped electrochemically. Otherwise biomolecule can only be entrapped into PPy in the presence of other dopant. (Guimard *et al.* 2007)

3.2.2. Hyaluronan and Chondroitin Sulphate as Bioactive Dopants

Both hyaluronic acid (HA) and chondroitin sulphate (CS) are disaccharides of glucuronic acid and N-acetylglucosamine (Lodish *et al.* 2000.). They are highly negatively charged molecules and primarily located in the ECM and on the surface of the cells. (Calabro *et al.* 2000) The common ability of GAGs is to bind water and form hydrated matrices in order to fill in the space between the cells. Therefore, HA and CS are essential components in cartilage supporting weight bearing by resisting compression (Hardingham & Fosang 1992, Nair & Laurencin 2007). In addition, in the ECM, they bind cytokines, GFs, morphogens and chemokines to protect them for proteolysis (Esko *et al.* 2009). This binding ability has also believed to be important in cell proliferation due to their role in co-receptors for GFs of the FGF family. GAGs possess viscous and lubricating properties (Gandhi & Mancera 2008). Other essential properties are presented in Table 3.

Table 3. Properties and molecule structure of HA and CS [modified (Gandhi & Mancera 2008)*]*.

Glycosamino-glycan	**Disaccharide units**	**Features**
Hyaluronic acid	D-GlcA- $\beta(1\rightarrow 4)$-D-GlcNAc-$\alpha(1\rightarrow 4)$	Molecular weight 4−8,000 kDA Non-sulphated, non-covalently attached to proteins in the ECM; also found in bacteria Excellent lubricators and shock absorbers Short half life (few minutes to weeks)
Chondroitin sulphate	D-GlcA-$\beta(1\rightarrow 3)$-D-GalNAc4S-$\beta(1\rightarrow 4)$	Molecular weight 5−50 kDA Most abundant GAG in the body: found in cartilage, tendin, ligament and aorta Bind to proteins (for instance collagen) to form proteoglycan aggragates

HA is the only GAG that is not added covalently to a protein core by glycosyltranferases in the Golgi apparatus to yield proteoglycans. (Calabro *et al.* 2000, Lodish *et al.* 2000). Traditionally, HA can be isolated from bacteria, rooster combs and bovine vitreous humor (Nair & Laurencin 2007, Shiedlin *et al.* 2004). It is actively involved in various biological processes such as cell migration and differentiation during embryogenesis, wound healing and inflammation (Weigel *et al.* 1997). HA can be modified by cross-linking with variety of chemical and physical methods, which has been exploited for wound healing applications (Nair & Laurencin 2007).

CS is often modified by sulphate groups replacing one or more of the OH-groups. (Lauder 2009, Sugahara *et al.* 2003). The degree of sulphation depends on source organism, tissue (Lauder *et al.* 2000), location within a tissue and age (Bayliss *et al.* 1999). Chain size of CS is heterogeneous even from a single tissue source (Lauder 2009).

CS plays an important role in central nervous system development, wound repair, infection, GF signalling, morphogenesis and cell division (Sugahara *et al.* 2003). The active functional groups of CS are reported to interact with mineral structures, such as hydroxyapatite in bone (Bali *et al.* 2001). Also, CS has been found to enhance bone remodelling and new bone formation (Schneiders *et al.* 2008). According to the clinical studies CS is well tolerated and there are rare adverse reactions (Lauder 2009) CS-PPy has been assessed for biocompatibility with human fibroblast cells showing good spreading and adhesion (Serra Moreno *et al.* 2008).

Both HA-PPy and CS-PPy have been reported to be compatible substrates for supporting osteoblast growth (Serra Moreno *et al.* 2009)

3.3. Polypyrrole Properties and Polymerization

PPy is used in various applications in biomedical science. Due to its biocompatibility properties it can be exploited as a conductive scaffold material allowing also use of chemical cues. The conductivity of PPy varies between 40–100 S/cm. Other applications include neural probes, biosensors, drug delivery devices and bioactuators. (Guimard *et al.* 2007) The main advantages and disadvantages of PPy are presented in Table 4.

Table 4. Correlation of PPy properties to tissue engineering requirements [modified (Guimard et al. 2007)*].*

Advantages of PPy	Limitations of PPy
• Biocompatible • Good conductivity • Modification possible for including chemical cues	• Poorly biodegrading • Not highly porous • hydrophobic

PPy is generally synthetized chemically or electrochemically by oxidation of pyrrole (Py) (Ravichandran *et al.* 2010). Chemical synthesis is normally used when large quantities of material are wanted. It requires mixing of strong oxidizing agent with a monomer solution (Ravichandran *et al.* 2010, Armes 1987, Duchet *et al.* 1998) Electrochemical synthesis is preferred for research purposes because of the simplicity of the technique and easy control over material properties. The polymerization process presented in simplified form in the Figure 4 proceeds through application of an external potential by using 3 electrodes: working, counter and reference electrodes. When current passes through the solution, condensation reaction proceeds from the bulk solution phase of monomer units resulting in a thin solid film deposited on the anode. (Ravichandran *et al.* 2010, Guimard *et al.* 2007).

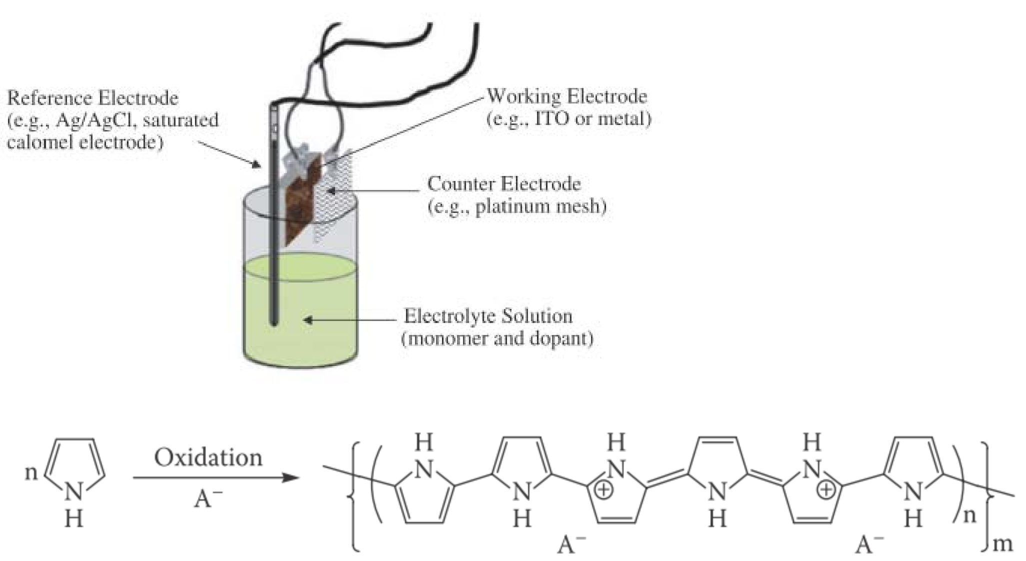

Figure 4. Electrochemical polymerization of PPy. Ions (A^-), such as HA or CS, can enter the PPy in the oxidized state [modified (Wallace et al. 2009, Guimard et al. 2007)].

Monomers undergo oxidation at the working electrode and from radical cations which subsequently react with other monomers or radical cations forming insoluble polymer chains. Oxidation reaction induces also conductivity to the polymer structure when forming radical cations. Radical cation constitutes a polaron due to the coupling with the local deformation. Further oxidation induces pairs of polarons which combine to form bipolarons since they are energetically more favourable. Bipolarons are able to migrate along the conjugated polymer chain, which provides the main charge transport mechanism for the conducting polymer chain. (Ateh *et al.* 2006) Process is affected by several variables including deposition time, temperature, solvent system, electrolyte-electrode system and deposition charge. Parameters have an effect on film morphology (thickness and topography), mechanics and conductivity. (Guimard *et al.* 2007)

As a drawback, PPy's weak mechanical properties do not make PPy ideal for scaffold materials. PPy has oxidation potential and thus is susceptible for breakdown due to overoxidation. In addition, there is α-β coupling which leads to structure disorder, disruption of electroactivity, and is the primary site of polymer breakdown. PPy is crystalline and brittle which makes it susceptible to delamination in coatings. (Guimard *et al.* 2007)

Even though, polypyrrole is not inherently biodegradable, the ability of the material to erode serves the same purpose (Guimard *et al.* 2007). Biodegradation can be modified by adding ionizable or hydrolyzable small anion (e.g., Cl^- ion) as its dopant; however, it does not trigger bioactivity of PPy (Guimard *et al.* 2007).

PPy has been reported to support growth and cell adhesion of various cell lines: endothelial cells (Wong *et al.* 1994), rat pheochromocytoma (PC12) cells (Schmidt *et al.* 1997), neurons and support cells associated with dorsal root ganglia (Song *et al.*

2006), primary neurons (Gomez *et al.* 2007), keratinocytes (Ateh *et al.* 2006), and MSCs (Castano *et al.* 2004).

Biocompatibility of the simplest form of PPy, PPy-Cl, has been evaluated *in vitro* and *in vivo* by Wang *et al.* (Wang *et al.* 2004). The study encompassed surface morphology of PPy membrane and toxicity tests in accordance of ISO 10993 and ASTM F1748-82. The results suggested that chemically prepared extraction solution did not have acute or subacute toxicity to tested animals. In addition, the study reported PPy coated silicon tubes to be useful to growth of nerve cells *in vitro* and *in vivo*. (Cui *et al.* 2003, Chen *et al.* 2000, Brett Runge *et al.* 2010, Yow *et al.* 2011).

Electrochemically fabricated CS-PPy and HA-PPy surfaces have been previously characterized with AFM, and as a result, HA-PPy was reported to have rougher surface than CS-PPy (Figure 5) (Gelmi *et al.* 2010). Recently Gilmore *et al.* showed that HA-PPy exhibits poorer myoblast adhesion compared to CS-PPy. It was also reported that HA-PPy gave a good support for myoblast proliferation but was poor in terms of differentiation, whereas CS-PPy was an effective substrate for supporting, proliferating and differentiating cells. (Gilmore *et al.* 2009)

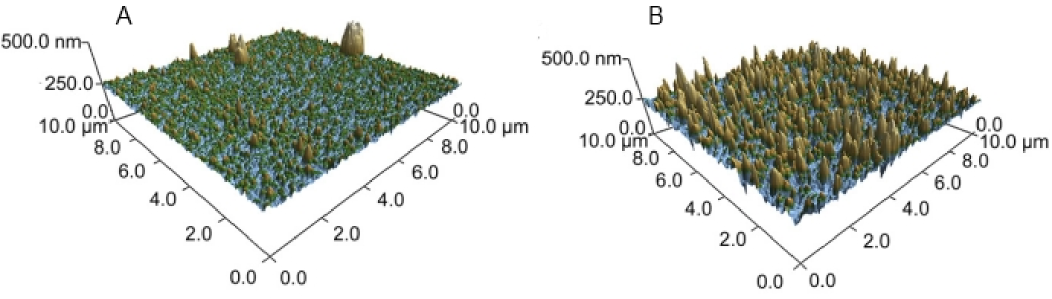

Figure 5. *A) Surface topography of CS-PPy and B) HA-PPy* (Gelmi et al. 2010)

The choice and amount of dopant affects significantly on surface topography. Generally, large dopants decrease conductivity and have more effect on topography than small molecules. Also the concentration of the dopant is relevant. For instance CS doped PPy (PPy-CS) shows good cells adhesion with fibroblasts in the smooth surface where the CS concentration is 2.0 gm/ml; whereas, that of 5.0 mg/ml produces rough surface morphology and little points of adhesion for the cells (Serra Moreno *et al.* 2008).

Also, film thickness has been reported to affect on surface topography. Castano *et al*. cultured MSCs on HA doped PPy (PPy-HA) films that were synthesized from different monomer concentrations. They reported that those of having the lowest concentration, and thus, being thickest, showed the best cell attachment. (Castano *et al.* 2004)

Properties can be modified by various methods; however, since there is a strong relation between chemical, mechanical and conductive properties, modifying of one

property will have an effect on others. Besides doping, following methods are used to modify PPy:

- entrapping the molecules during electrochemical synthesis
- binding molecules covalently to the PPy
- physically adsorbing molecules
- using nano or micro patterning techniques to modify PPy topographically

Modification methods are illustrated in Figure 6. (Guimard *et al.* 2007)

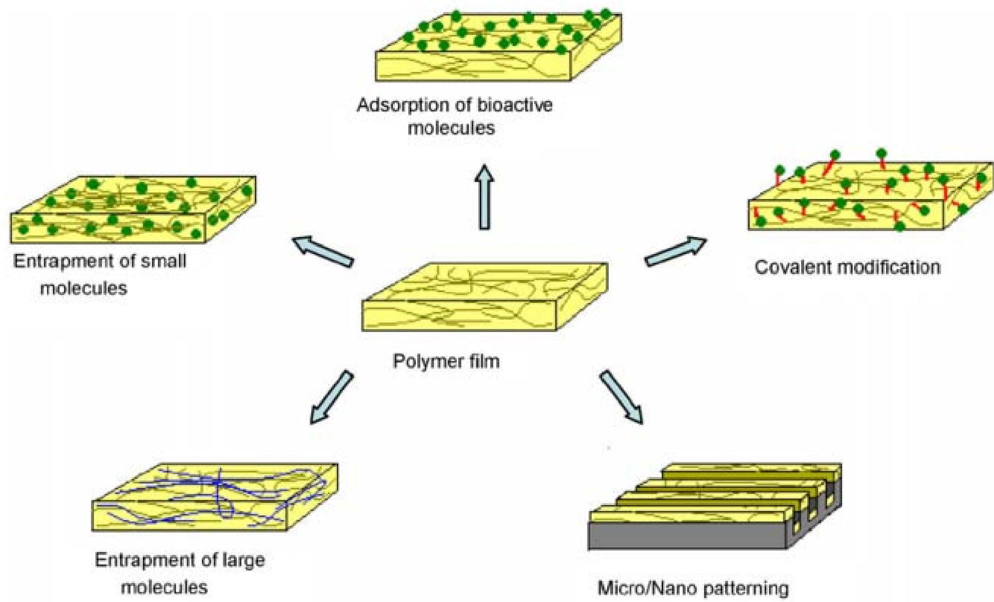

Figure 6. Modification techniques of PPy to modify the biological properties (Guimard et al. 2007)

Also, the ES has an effect on PPy's properties. For instance, reducting potential causes expulsion of small negative ions in the case of small dopants or, causes the uptake of positive ions from the medium in the case of large entrapped dopants. (Guimard *et al.* 2007) Shi *et al.* demonstrated that fibroblast viability was multiplied when subjecting PPy/PLLA membranes to ES. The same phenomenon did not occur when the same experiment was repeated on gold-coated petri dish. (Shi *et al.* 2004)

PPy actuation can occur during ES due to the movement of the dopants in and out of PPy. Moreover, Gelmi *et al.* (2010) reported that dopant content and thickness of the PPy film affects on strain caused by actuation under BEC, where CS-PPy showed higher strain compared to HA-PPy (Gelmi *et al.* 2010).

4. USING STEM CELLS IN TISSUE ENGINEERING

Stem cells provide a unique opportunity for regenerative medicine to cure a broad number of diseases and tissue defects. A stem cell is defined as a cell type which is able to self-renew while maintaining the capacity to differentiate into diverse cell types. This provides several advantages for research purposes since cells can be expanded *in vitro* while maintaining native properties. Biggest challenges are to define standards and assays for the differentiation potential of stem cells. When human-derived stem cells are concerned, also ethical issues are involved. (Teo & Vallier 2010)

4.1. Classification of Stem Cells

Stem cells can be classified according to their potential to differentiate (Figure 7). Embryonic stem cells possess the widest differentiation potential which decreases along the embryo development. Embryonic stem cells of 1 to 3 days old are totipotent being capable of differentiating into all adult and embryonic tissues. (Teo & Vallier 2010, Seydoux & Braun 2006, Wobus & Boheler 2005). The main issues considering *in vitro* use of totipotent human stem cells are to define assays and standards which allow the definition of totipotency. This, however, involves ethical considerations and very advanced assays to demonstrate that cells can differentiate into all the cell types existing in human body. (Teo & Vallier 2010)

Pluripotent stem cells can be derived from the inner cell mass of embryos at the blastocyst stage (Thomson *et al.* 1998). They are able to proliferate indefinitely while maintaining the capacity to differentiate into derivatives of all the 3 germ layers ectoderm, mesoderm and endoderm, from which adult organs are derived (Teo & Vallier 2010). Pluripotent stem cells can also be found from the adult germline. It has been recently reported that pluripotent spermatogonial stem cells can be found from a neonatal and even adult mice (Payne & Braun 2008, Conrad *et al.* 2008). Pluripotent stem cell can also be generated from somatic cells by reprogramming them using either somatic cell nuclear transfer or overexpression of pluripotency factors which is commonly performed with human somatic cells. These cells are called induced pluripotent stem cells and various cell lines can be reprogrammed including fibroplasts, keratinocytes and neural stem cells. Since the procedure can be done from large number of patients, it is possible to avoid use of immunosuppressive treatment during the cell-

based therapy. One advantage is also to provide *in vitro* models from patient's own cells by inducing pluripotency to easily accessible cells and differentiating them into the cells targeted by a disease. (Teo & Vallier 2010) Pluripotency of human cells is usually determined by their capacity to form teratomas. However, since this method is not quantitative, the current issue is to develop properly functioning assays (Teo & Vallier 2010)

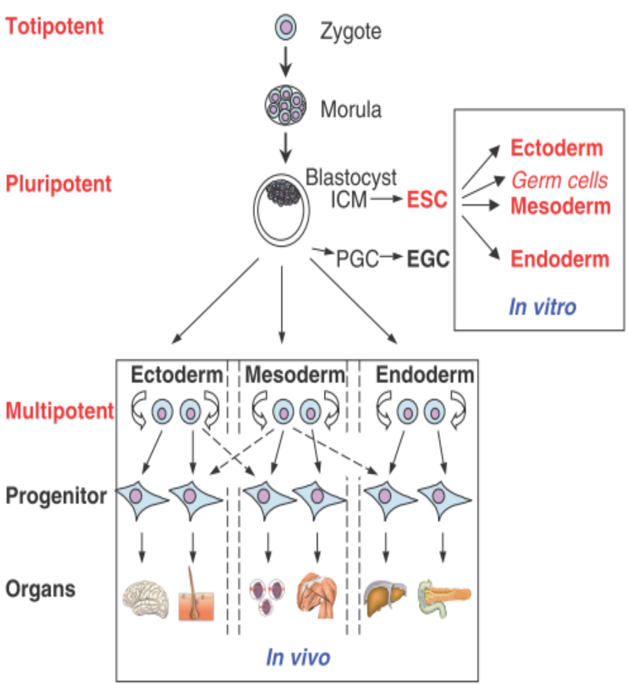

Figure 7. Classification of stem cells by their ability to differentiate (Wobus & Boheler 2005).

In general, multipotent stem cells are able to differentiate into all the tissues needed for an organ. (Teo & Vallier 2010) Multipotent stem cells are defined to differentiate only into its germ layer of origin (Hodgkinson *et al.* 2009). However, in practice, some multipotent cell lineages can extent the differentiation beyond the original definition (Hodgkinson *et al.* 2009). Stem cell sources have been traced to all kind of adult tissues and are termed as somatic stem cells. Furthermore, they are usually classified into endogenous and exogenous sources. Endogenous sources include cells which cannot be used *in vitro* at the moment. This is due to their challenging location in adult tissues, which is why also lineage tracing or reconstruction experiments are impossible. (Teo & Vallier 2010) Endogenous somatic stem cells have been reported to found in the muscle (Shi & Garry 2006), lung (Neuringer & Randell 2006), intestine (Casali & Batlle 2009), liver (Kung & Forbes 2009), heamatopoietic system (Teo & Vallier 2010), skin (Bickenbach & Grinnell 2004), heart (Beltrami *et al.* 2003) and brain (Lederer & Santama 2008).

Exogenous somatic stem cells can be derived *in vitro* from diverse tissues and have a high capacity of proliferation. Also their maintenance *in vitro* for a prolonged period of time makes them ideal source for tissue engineering. In addition, their capacity to differentiate concerns wide number of tissues unrelated to their origin, which is why their pluripotential properties have been discussed. (Teo & Vallier 2010) MSCs are commonly used exogenous stem cells in regenerative medicine (Taupin 2006).

MSCs can be isolated from various sources such as adipose tissue (Zuk *et al.* 2002), bone marrow (Friedenstein *et al.* 1968), amniotic fluid (De Coppi *et al.* 2007), placenta (Chien *et al.* 2006) and umbilical cord (Bieback *et al.* 2004). MSCs can be identified by their adherent property, differentiation potential, and cell surface markers (Bernardo *et al.* 2009, Karp & Leng Teo 2009). MSCs isolated from different tissues may exhibit slightly different propensities and capacities to differentiate into certain cell types. The differentiation is limited into approximately 20 population doublings (DiGirolamo *et al.* 1999). Another, more severe disadvantage is their tendency to generate abnormal karyotypes *in vitro* (Li *et al.* 2007). Advantage for using MSCs in tissue engineering is their weak immunogenicity, which suggests that immunosuppressive treatment might not be needed (Teo & Vallier 2010). Also, they have shown to limit inflammation and modulate immune response of cells *in vitro* (Spitkovsky & Hescheler 2008).

4.2. Adipose Stem Cells

ASCs are considered potential for several tissue engineering applications. Due to their easy accessibility, minimal ethical issues and also abundance of the cells assists *in vitro* culturing. (Hodgkinson *et al.* 2009) The differentiation potential of ASCs expands beyond the traditional mesenchymal lineages (Figure 8). For instance, ASCs have differentiation potential towards adipogenic, osteogenic, chondrogenic, myogenic, cardiac, neurogenic, and endothelial lineages (Hodgkinson *et al.* 2009, Bunnell *et al.* 2008, Strem *et al.* 2005, Mizuno 2009). However, some lineages, such as neurons, are very difficult to differentiate.

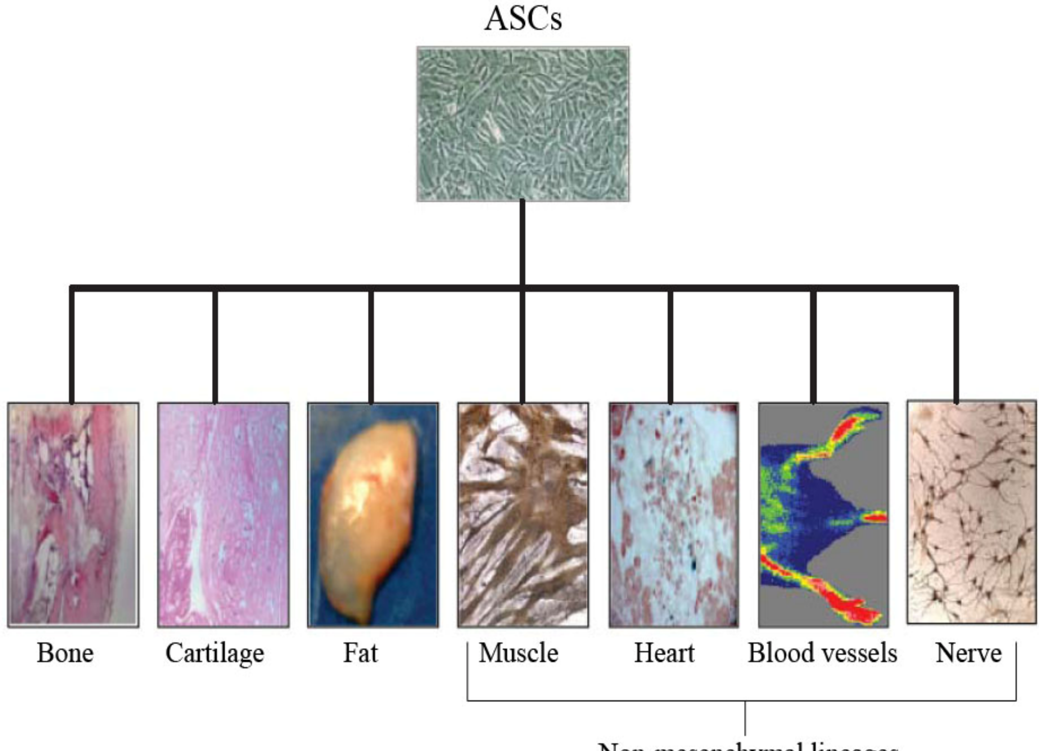

Figure 8. The differentiation potential ASCs extends beyond the traditional lineages. [modified (Strem et al. 2005)*]*

Potential to differentiate into endothelial cells provides angiogenic properties for ASCs (Miranville *et al.* 2004, Rehman *et al.* 2004). In addition, current research has reported ASCs being potential for tendon regeneration and support hepatic and haematopoietic regeneration (Ishikawa *et al.* 2010, Uysal & Mizuno 2010, Gimble & Guilak 2003a, Gimble & Guilak 2003b).

4.2.1. Isolation and Characterization of Adipose Stem Cells

ASCs can be acquired from surgeries, namely from liposuction or other operations where fat tissue is removed from the patient. They can be distinguished from other cells by their plastic-adherent property after digesting the fat tissue by collagenase and centrifuging to separate the stromal vascular fraction (SVF) pellet (Figure 9) (Bunnell *et al.* 2008).

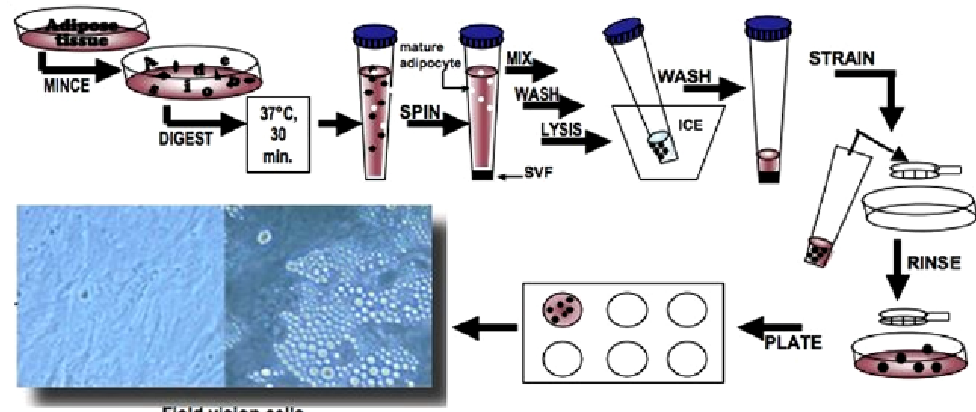

Figure 9. The isolation procedure of ASCs (Bunnell *et al.* 2008).

The standard characterization of ASCs is done by studying their capability to differentiate at least into bone, cartilage and fat and by studying cell surface immunophenotypes, namely cluster of differentiation (CD) markers (Strem *et al.* 2005, Mizuno 2009, Katz *et al.* 2005, Dominici *et al.* 2006). Expression changes during their passages (Rada 2009). The main surface proteins reported on ASCs are listed in Table 5. ASCs and bone marrow derived stem cells (BMSCs) express very similar surface receptors and adhesion molecules. (Hodgkinson *et al.* 2009, Strem *et al.* 2005, Romanov *et al.* 2005).

Table 5. Surface markers of ASCs (Locke et al. 2009, Strem et al. 2005, Gimble & Guilak 2003a, Katz et al. 2005, Rada 2009, Mitchell et al. 2006)

Type of the molecule	Marker	Specification of the marker
Adhesion molecules	CD29	Integrin β1
	CD49d	Integrin α4
	CD105	Vascular cell adhesion molecule
Receptor molecules	CD44	Hyaluronate
	CD73	Ecto 5’ nucleotidase
Surface enzymes	CD10	Neutral endopeptidase
	CD13	aminopeptidase
ECM proteins and glycoproteins	CD90	Thy-1
Complement regulatory proteins	CD55	Decay accelerating factor
	CD59	Complement protein

The appearance and the potential to differentiate may depend on the age of the patient and the type and location of the adipose tissue. (Mizuno 2009) ASCs secrete potent GFs for tissue regeneration, such as VEGF, hepatocyte growth factor (HGF, FGF-2, and IGF-1 (Mizuno 2009).

4.2.2. Osteogenic Differentiation of Adipose Stem Cells

There are various *in vivo* studies for osteogenic differentiation conducted with ASCs. Animal studies have been mostly performed by using different GFs and scaffold materials, such as poly(DL-lactide-co-glycolide) (PLGA) (Cowan *et al.* 2004), β-TCP (Hattori *et al.* 2006), collagen (Kakudo *et al.* 2008) and hydroxyapatite (Scherberich *et al.* 2007). These tissue engineering constructs showed enhanced bone regeneration at the defect site when cells were integrated into the structure.

It has been studied that osteogenic differentiation of ASCs occurs within 2–4 weeks when culturing the cells in the similar osteogenic conditions as BMSCs *in vitro* (Rada 2009). Although the mechanisms behind the differentiation are still unclear, some transcription factors have been suggested to induce transformation into bipotent stem cells with the capacity to differentiate towards osteoblastic or adipocyte phenotype (Gimble *et al.* 2006).

Osteogenic differentiation proceeds at two developmental states. Osteopoiesis includes differentiation process of MSCs into osteoblasts. First, they differentiate into osteoprogenitor cells, which lack self-renewal capacity but are responsible for expansion of osteoblast numbers via proliferation (Figure 10). Osteoprogenitors further differentiate into preosteoblasts, which is transitional state between osteoprogenitor and osteoblasts, and finally to osteoblasts. Osteogenesis is known as deposition of bone matrix. (Long 2001)

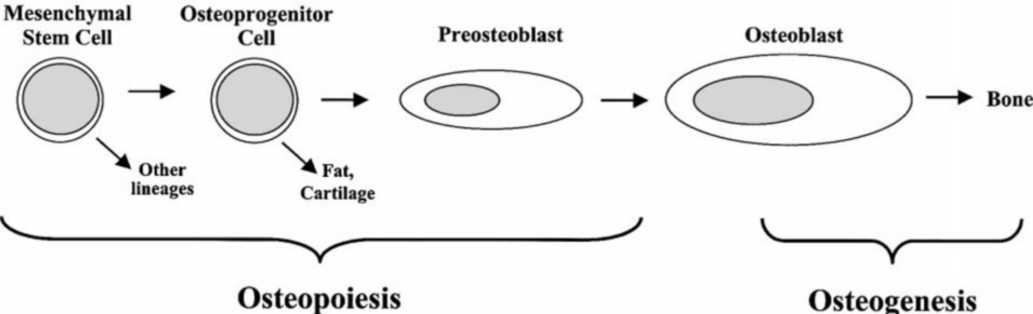

Figure 10. Osteogenic differentiation of MSC [modified (Long 2001)*]*

Differentiated MSCs can typically be characterized *in vitro* by deposition of calcium phosphate mineral and expression of osteogenic genes and proteins including ALP, BMP-2, BMP-4, BMP receptors I and II, PTH-receptor, type I collagen, bone sialoprotein, Runx-2, OC, osteonectin and OPN (Zuk *et al.* 2002, Strem *et al.* 2005, Zuk *et al.* 2001, Halvorsen *et al.* 2000, Halvorsen *et al.* 2001, Huang *et al.* 2002). In addition novel marker, heat shock protein (Hsp) 27 is suggested to precede other osteoblastic markes, such as ALP and collagen I (Hronik-Tupaj *et al.* 2011).

The first clinical case report of a microvascular custom made ectopic bone flap was reported by Mesimäki et al (Mesimäki *et al.* 2009).

4.3. Effect of External Stimulus on Osteogenic Differentiation of Adipose Stem Cells

In the literature, multiple osteogenic differentiation methods have been used, such as modification of culture media, use of GFs and osteogenic scaffold, gene transfer and physical stimuli. This chapter presents current differentiation methods concentrating on ES.

4.3.1. The Effect of Osteogenic Medium and Growth Factors

ASCs cultured in the presence of ascorbate-2-phosphate, dexamethasone and β-glycerophosphate induce osteogenic differentiation in approximately two weeks (Bunnell *et al.* 2008, Rada 2009). In one study where surface proteins and osteogenic markers were examined from osteoblasts and ASCs cultured in OM, both cell lines were detected to express CD10, CD44, CD59 and CD105 between days 7 and 15. It was also noticed that the growing rate of ASCs was significantly faster than that of osteoblasts. Another difference was more distinct expression of OC in osteoblasts. (Trentz *et al.* 2010)

In addition, foetal bovine serum (FBS) is commonly used component since it promotes MSC expansion. FBS provides various vital nutrients and GFs. Nevertheless, the current trend is to obtain serum-free media since FBS, and also human serums, contain a high risk of disease transmission and high lot-to-lot variability. (Bieback *et al.* 2009, Bieback *et al.* 2010) Also 1,25-dihydroxyvitamin D_3 has been used to trigger osteogenic differentiation (Rada 2009, Halvorsen *et al.* 2000, Halvorsen *et al.* 2001).

Several GFs have been studied with ASCs. GFs can be added either to the culture medium or integrated into the scaffold material. Also GF injections have been tested after implantation of the cell seeded scaffold (Shen *et al.* 2006).

BMPs belong to the TGF-β superfamily (Knippenberg *et al.* 2006). They induce differentiation of MSCs into osteochondrogenic and osteoblast precursor cells (Wan & Cao 2005) and can be divided into subgroups by their degree of sequence homology. BMP-2 and BMP-4 belong to the BMP-2/-4 subgroup whereas BMP-5, 6, 7 and 8 belong to the osteogenic protein-1 subgroup. Most of the members of the both groups induce formation of bone and cartilage *in vivo*. (Knippenberg *et al.* 2006) BMP-2 has been shown to induce osteogenic differentiation of BMSCs and ASCs *in vitro* and *in vivo (Dragoo et al. 2003, Rickard et al. 1994)*. Other BMPs, including BMP-4, 6, 7 and 14 are also reported to induce osteogenic differentiation of ASCs (Shen *et al.* 2006, Al-Salleeh *et al.* 2008, Kemmis *et al.* 2010).

FGF-2 has been used for osteogenic and chondrogenic differentiation. It is involved in angiogenesis and tissue regeneration, which has been reported to be based on the inactivation of IGF and TGF-β pathways. (Ito *et al.* 2008)

Purified VEGF has been used to induce vascularisation to bone tissue engineering constructs; however, it has very short half life, and thus it performs low efficacy in the tissues (Scherberich *et al.* 2010). Enhanced vascularisation can be achieved by culturing ASCs with endothelial cells as demonstrated by Scherberich *et al.* (Scherberich *et al.* 2007). GFs involved in osteogenic differentiation of MSCs are presented in Table 6.

Table 6. Role of GFs in bone tissue [Modified (Devescovi et al. 2008)*]*

GF	Cell source	Biological effect	Effect on bone
BMP	osteoblast, chondrocyte, endothelial cell	Osteoinduction, chondrogenesis	Migration of osteoprogenitors, induction of proliferation, differentiation and matrix synthesis
FGF	Magrophage, monocyte, bone marrow stromal cell (BMSC), chondrocyte, osteoblast, endothelial cell	Angiogenesis, proliferation of fibroblast and smooth muscle cells of vessels	Chondrocyte maturation (FGF-1), Osteoblast proliferation and differentiation, inhibition of apoptosis of immature osteoblasts, induction of apoptosis of mature osteocytes, bone resorption (FGF-2)
IGF	Osteoblast, chondrocyte, hepatocyte, endothelial cell	Regulation of growth hormone effects	Osteoblast proliferation and bone matrix synthesis, bone resorption
PDGF	Platelet, osteoblast, endothelial cell, monocyte, macrophage	Proliferation of connective tissue cells, monocyte/macrophage and smooth muscle cell chemotaxis, angiogenesis	Osteoprogenitor migration, proliferation and differentiation
TGF-β	Platelet, osteoblast, BMSC, chondrocyte, endothelial cell, fibroblast, macrophage	Immunosuppression, angiogenesis, stimulation of cell growth, differentiation and ECM synthesis	Undifferentiatied MSC proliferation, osteoblast precursor recruiting, osteoblast and chondrocyte differentiation(but inhibition of terminal differentiation), bone matrix production, recruitment of osteoclast precursors
VEGF	Osteoblast, platelet	Angiogenesis	Conversion of cartilage into bone, osteoblast proliferation and differentiation

To overcome the problems with short half-life of GFs, high costs, side effects and loss of activity during the preparation, gene transfer can be used. This can be done by engineering them either to secrete differentiation factors or synthesize transcription

factor to induce their differentiation. (Satija *et al.* 2007) For instance, Lee *et al.* delivered BMP-2/Runx-2 bicistronic vector into ASCs and reported significant increase in bone formation (Lee *et al.* 2010). However, gene transfer has various risks due to its significant manipulation of cells.

4.3.2. Effect of Mechanical Stimulation on Mesenchymal Stem Cells

The effect of mechanical loading has been extensively studied with MSCs where fluid flow has been shown to have a significant effect on the osteogenic differentiation of the cells. Other mechanical strains conducted with MSCs include flow perfusion (fluid flow through porous scaffold), scaffold stretching and hydrostatic pressure. Different types of mechanical loading can also have different osteogenic effects. For example, hydrostatic pressure affects mainly on formation of an ECM through regulation of gene and protein expression of collagen type I and contributes to further maturation, whereas fluid flow, flow perfusion and scaffold stretching have an impact on many stages of differentiation including increase in expression levels of early osteogenic markers, formation of an ECM and further maturation of MSCs and ECM. (Potier *et al.* 2010)

When considering cellular actions during mechanical strain, current research supports a four-stage cell-mediated theory of mechanotransduction:

1) Mechanocoupling: physiological loads are converted into local mechanical signals experienced by bone cells.
2) Biochemical coupling: the sense of load is transformed into biochemical response.
3) Signal transmission: biochemical response results downstream signaling within and between the cells.
4) Effector response of the bone cells: regeneration, modelling or resorption of the bone matrix. (Duncan & Turner 1995)

Response to the mechanical stimulation depends on the differentiation status. Interestingly, Pommerenke *et al.* reported differences in the calcium-dependent mechanostransduction between BMSCs and osteoblasts showing that the cell lines were displaying different kinetics in intracellular calcium concentration following the mechanical stimulation of integrins (Pommerenke *et al.* 2002, Pommerenke *et al.* 1996). This is also demonstrated with ES of MSCs presented in the next chapter.

Signal transmission between the cells is also important for transmitting mechanical signals. Fluid flow has been demonstrated to increase the generation of nitric oxide (NO) (Klein-Nulend *et al.* 1998, Mullender *et al.* 2004) which is essential for maintenance of bone mass according to animal experiments (Turner *et al.* 1996). It takes part into mechanotransduction signalling path ways in MSCs (Liu *et al.* 2010). Knippenberg *et al.* conducted pulsating fluid flow studies with ASCs and reported bone cell-like response, such as increased NO production (Knippenberg *et al.* 2005). Also

gap junctions and indirect communication via diffusible messengers may cooperate to transmit mechanical signals (Scott *et al.* 2008).

4.3.3. Effect of Electrical Stimulation on Mesenchymal Stem Cells

The thickness of the cell membrane is 10 nm and it exhibits 0.1 V potential difference. This corresponds to field strength of 10^6−10^7 V/m, which halts weaker electric field (EF) to inter the cell. Even though high-field strengths are needed for cell penetration, EF can interact with internal signalling path ways by coupling to sensor mechanisms at the cell membrane. Electromagnetic field can penetrate the cell membrane without any mediators. (Funk *et al.* 2009)

Cells may feel small EFs through multiple ways; namely, converse flexoelectricity, where EF applied to one part of the cell causes changes in membrane tension (Zhang *et al.* 2001), activation and clustering of surface receptors, or other coupling mechanisms that include different types of calcium channels and signal-transducing molecules, such as integrins. (Sun *et al.* 2007, Funk *et al.* 2009) Specific sensors for EF at the cell membrane are ion channels, voltage-sensitive phosphatase and lipid phosphatase (Funk *et al.* 2009). Especially, phospholipase C (PLC) is demonstrated to couple to ES in MSCs and osteoblasts (Sun *et al.* 2007). The effect of EF on cell membrane and intracellular reactions is presented in Figure 11.

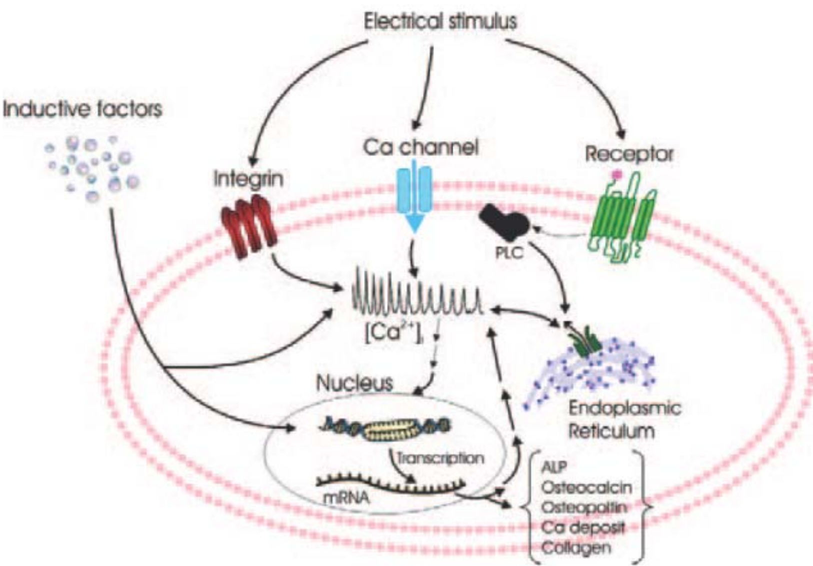

Figure 11. Potential electrocoupling mechanisms in the bone cell. (Sun et al. 2007)

External ES has been shown to induce various cellular and molecular responses including microfilament reorganization, cell surface receptor redistribution, changes in intercellular calcium dynamix, galvanotropic cell orientation and migration, enhanced differentiation, proliferation, angiogenesis and protein biosynthesis (Titushkin *et al.* 2011).

Even though ES has been acknowledged as an effective bone fracture treatment method, there is just limited clinical evidence from it (Kuzyk & Schemitsch 2009). The effect of external ES is stated to be based on the same phenomenon that mechanical strain generates electrical potentials in the bone (Yasuda 1953, Becker *et al.* 1964.). Compression generates electronegative potential and tension electropositive potentials. (Otter *et al.* 1988).

The parameters chosen for *in vitro* ES studies promoting osteogenic differentiation of MSCs are often mimicking some of the 3 kinds of ES treatment used for bone fractures: direct current (DC), capacitive coupling and inducting coupling. DC can be direct or pulsatile type which includes monophasic and biphasic (also classified as AC) waveform. Usually 5 to 100 μA is delivered. (Kuzyk & Schemitsch 2009, Black 1987). Capacitive coupling uses AC to create an EF. Frequencies of 20−200 Hz are applied resulting in 1−100 mV/cm electric field (Kuzyk & Schemitsch 2009). Inductive coupling embodies pulsed electromagnetic field (PEMF) which creates EF within the fracture site. Magnetic field is dependent on the current applied and the target tissue. (Kuzyk & Schemitsch 2009) Applied electromagnetic fields (EMFs) are varying from 0.1 to 20 G and create an EF of 1 to 100 mV/cm within the bone (Aaron & Steinberg 1991).

The results of ES studies for osteogenic differentiation of MSCs *in vitro* are almost consistent with the increase in cytosolic free calcium and cytoskeletal tension (Sun *et al*. 2007, Titushkin *et al.* 2009, McCullen *et al.* 2010, Hammerick *et al.* 2010). Also osteogenic differentiation appears to be common outcome for many studies (Sun et al. 2007, McCullen *et al.* 2010, Tandon *et al.* 2009, Hammerick *et al.* 2010, Hronic-Tupaj *et al.* 2011). However, due to the different research techniques and parameters used, the results can widely differ from each other. Research concerning ES for osteogenic applications is reviewed in Table 7 and explained more detailed below.

Table 7. ES studies conducted with MSCs, osteoblasts or osteoblastic cells for bone applications.

Cells	ES parameters	Me-dium	Results and discussion	Author
BMSCs	0.1 and 1 v/cm DC or 1 Hz sinusoidal AC	OM and BM	**Proliferation:** 3-fold cell growth under osteogenic induction and ES **Differentiation:** ES alters the calcium oscillation (reduction in calcium spikes) to resemble those of osteoblasts. ALP activity increased ES can couple phopholipase C → release of internal calcium → cell differentiation?	(Sun *et al.* 2007)
	10 V/cm		Invasive. Caclium oscillation abolished (probable membrane damage) .	
hMSCs	2 V/cm DC EF for 60 min.	OM	**Differentiation:** decreased cytoskeleton elasticity of MSCs (actin cytoskeleton reorganization) and increased membrane cytoskeletal interaction of MSCs → typical for mature osteoblasts	(Titushkin & Cho 2009)
hASC	1, 10, 100 or 1000 V/cm, sinusoidal 1 Hz for 5 min periods	OM	**Differentiation:** Increase in intracellular calcium in 1, 10 and 100 V/cm EF. 1000 V/cm induced cell death immediately.	(McCullen *et al.* 2010)
	1, 3 or 5 V/cm, sinusoidal 1 Hz, 4h/day for 14 days	OM	**Proliferation:** 5 V/cm had best proliferation at day 7, control group was superior at day 14. **Differentiation:** 1 V/cm was superior and there was significant increase in mineralization between day 7 and 14. → Enhanced calcium deposition.	
hASC	6 V/cm DC, 2−4 h	BM	Orientation perpendicular to the direction of the EF. Disassembly of gap junctions. **Differentiation:** upregulation of VEGF, FGF and thrombomodulin.	(Tandon *et al.* 2009)
mouse ASC	Pulsed ES, 6 V/cm, 50 Hz, 6h/day	BM	**Differentiation:** increase in cytoskeletal tension, ALP, OPN, RunX-2, Col I, cytosolic free calcium **Proliferation:** no significant change in proliferation.	(Hammerick *et al.* 2010)
hMSC	20 mV/cm, 60 kHz, 40 min/day, 28 days	OM	**Differentiation:** Increase in ALP mRNA and Collagen I mRNA beginning from day 15. Increase in heat shock protein 27 beginning from day 10.	(Hronik-Tupaj *et al.* 2011)

Cells	ES parameters	Me-dium	Results and discussion	Author
BMSC	24h/day, Pulsed BEC, 100 Hz, 250 µs pulse duration with 1.5 µA/cm^2 or 25 µs with 15 µA/cm^2	OM and BM	**Proliferation (in BM):** increased most in lower level of amplitude and longer duration **Differentiation (in OM and BM):** increased VEGF production. Osteogenic differentiation increased 3 days after the end of the stimulation. 250 µs with 1.5 µA/cm showed the best proliferation also over the control.	(Kim *et al.* 2009)
Osteo-blasts	BEC, 4.2 A/ m^2 20 Hz, pulse duration 0.4 ms. 1 h/day, 3 weeks		**Proliferation:** increased **Improvement in osteogenic functions:** ALP, Col I and calcium deposition improved	(Ercan & Webster 2010)
osteo-blastic cells	20mV/cm, 60 kHz, 300 µA/cm^2, 30 min-24 h		**Proliferation:** increased **Differentiation:** increases the level of TGF-b1 mRNA	(Zhuang *et al.* 1997)

Proliferation of MSCs under ES was reported to be increased in 2 studies, which both used OM (McCullen *et al.* 2010, Sun *et al.* 2007). Other proliferation studies with MSCs, presented in Table 7, used BM. Kim *et al.* (2009) showed increased proliferation under pulsed BEC, whereas Hammerick *et al*. (2010) did not detect any differences with pulsed AC. In addition, osteoblasts have been studied with continuous BEC resulting also in increased proliferation (Ercan & Webster 2010).

All the studies reported differentiation under ES via various different methods. Immediate changes in respond to ES were detected through calcium imaging, where the detected increase in intracellular calcium is explained by activation of calcium channels and release of calcium from intracellular calcium stores. (Hammerick *et al.* 2010, McCullen *et al.* 2010, Sun *et al.* 2007). The strength of the ES defines the mechanisms behind the changes in intracellular calcium concentration; for instance, the strength of ES determines the kinetics of PLC redistribution in the cell membrane which further regulates the release of intracellular calcium (Khatib *et al.* 2004).

Intracellular oscillation of calcium concentration is suggested to be involved in mediating differentiation by Sun *et al.* (2007). Calcium oscillation was reported to decrease to a level similar to that found in the terminally differentiated osteoblasts. However, the amplitude of the MSCs remained the same. DC at 0.1 or 1 V/cm and oscillatory mode at 1 V/cm had similar effect on calcium spikes in BM. Effect of ES was also studied in OM, which appeared to increase proliferation by 3-fold compared to OM alone. Also significantly higher ALP activity was found with the effect of ES. (Sun *et al.* 2007)

Changes in mechanical properties of cytoskeleton have been reported by Hammerick *et al.* (2010) and Titushkin *et al.* (2009). Hammerick reported increase in elastic modulus under pulsed 6 V/cm 50 Hz AC referring to increase in stress fiber formation. Instead, Titushkin stated reduction in elastic modulus when stimulating cells

with DC of 2 V/cm. Nevertheless, both authors concluded that the skeletal changes preceded to osteogenic differentiation.

Interestingly, VEGF is reported to increase under continuous BEC with MSCs and osteoblasts, which is not the usual case with other waveforms (Kim *et al.* 2009, Ercan & Webster 2010). Pulsatile type BEC also promotes non-accumulation of charged proteins and maintenance of constant current and pH in the media, which makes it preferable for biological conditions. (Kim *et al.* 2006, Huang *et al.* 1999, Bodamyali *et al.* 1999)

EXPERIMENTAL PART

5. RESEARCH METHODS AND MATERIAL

5.1. Polypyrrole Synthesis

2D polypyrrole samples were coated electrochemically on special 24-well plates, where the bottom of the well plate had been removed and replaced with conductive golden coated polyethylene terephtalate film. The set-up of electrochemical polymerization is presented in Figure 12. The golden films were attached to the well bottom by using medical grade silicon elastomer (Dow Corning Corporation, Midland, USA).

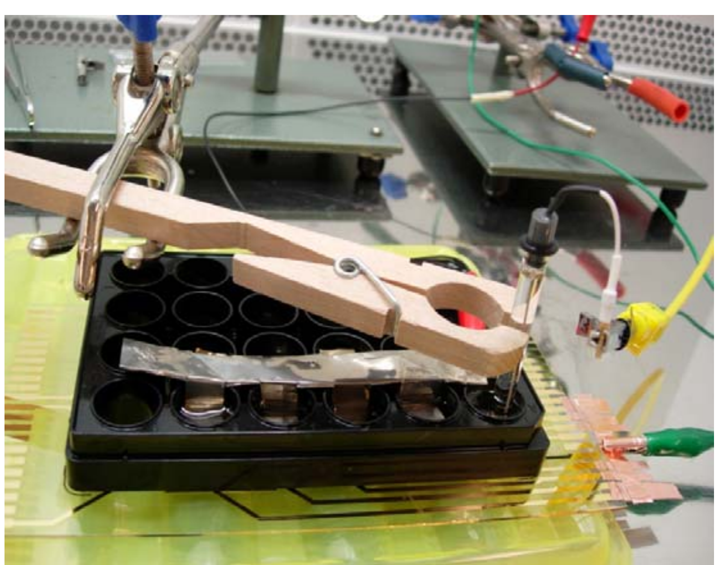

Figure 12. Electrochemical polymerization of 2D PPy samples.

Polymerization solution was made 1 day before polymerization. 0.07 ml of Py (Sigma Aldrich, St. Louis, USA) and 1 mg of HA from Streptococcus equi (Sigma Aldrich) or CS A from bovine trachea (Sigma Aldrich) was added to 1 ml of water and stirred overnight. Polymerization was performed in the stimulation well plates. Working electrode was attached to the golden sheet and counter electrode to a platinum net which was framed to be in touch with the working solution when placed on top of the stimulation plate. Potential was kept constant with value of 1.0 V during the polymerization of all the layers. Stimulation plates and plate covers were sterilized by gamma irradiation (BBF Sterilisationsservice GmbH, Kernen, Germany).

5.2. Cell Culture Methods

5.2.1. Adipose Stem Cell Isolation and Cell Culture

The adipose tissue was obtained from a tissue harvest from surgical procedure of 3 different female donors of age 46–65 years. The ASC isolation was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District. The isolation procedure based on the method of Haimi *et al.* was started with washing the sample with Dulbecco's Phophate buffered saline (DPBS; Lonza, Basel, Switzerland) (Haimi *et al.* 2009). The tissue was processed into small pieces with scissors and digested with collagenase type I (1.5 mg(ml; Invitrogen, Califormia, USA) at 37 °C in a gyratory water bath for 45–60 min with intermittent shaking every 15 minutes. After centrifugation, the uppermost layers of fat and connective tissue were discarded and a cell pellet on the bottom filtered with filter of 100 µm (Falcon®, Becton Dickinson Labware, New Jersey, USA) to remove cellular debris. After a second centrifugation, the cell pellet was washed with distilled water to remove residual non-adherent red blood cells. The third centrifugation was made together with the BM consisting of Dulbecco's Modified Eagle Medium/Ham's Nutrient mixture F-12 (DMEM/F-12 1:1 Invitogen), 10% fetal bovine serum (FBS; Invitrogen), 1% L-glutamine (GlutaMAX I; Invitrogen) and 1% antibiotics/antimycotic (100 U/ml penicillin, 0.1 mg/ml streptomycin; Invitrogen). The remaining pellet consisting of ASC was resuspended with the BM and transferred into T-75 polystyrene flasks (Nunc, Roskilde, Denmark) for expansion. The experiments were carried out at passages 4.

Before cell seeding, CS-PPy and HA-PPy wells were rinsed with DPBS and pre-treated with BM for 48 h at 37 ºC. Cell density of 30,000 cells in 1.0 ml of medium were seeded on each well: CS-PPy and HA-PPy coated plates with or without ES were studied standard polystyrene culturing plates acting as control samples. Cells were let to attach for 24 h before first ES.

On the first stimulation day, BM of half of the samples was replaced by osteogenic medium (OM) that contained 10 mM of β-glycerophosphate, 250 µM of L-ascorbic acid-2-phosphate and 5 nM of dexametasone in addition to the BM. Medium was changed twice a week for all the samples. Cells were examined at day 7 and 14 in all the experiments.

5.2.2. Cell Attachment and Viability

Cell attachment and viability were evaluated qualitatively using live/dead-staining probes (Molecular Probes, Eugene, OR, USA). The staining method was based on the probes resulting in different fluorescence reactions of dead and viable cells. CellTracker™ Green (5-chloromethylfuorescein diacetate, CMFDA), Molecular probes) is a membrane permeable dye that freely diffuses into living cells and reacts with proteins and peptides, resulting in green fluorescence. Ethidium homodimer-1 (EthD-1; Molecular Probes) enters cells with damaged membranes and binds to nucleic acids, thereby producing a bright red fluorescence in dead cells.

First, the medium was discarded from the wells, which were subsequently washed with DPBS. Cells were incubated with DPBS-based dye-solution containing 0.5 μM of CMFDA and EthD-1 for 45 min at room temperature protected from the light. After discarding the working solution, PPy samples were cut off from well plates and examined with fluorescence microscope (Olympus IX51, Olympus Finland PLC, Vantaa, Finland).

5.2.3. Cell Proliferation

Cell proliferation was studied by CyQuant® Cell Proliferation Assay Kit (CyQuant; Molecular Probes, Invitrogen) which measures quantitatively the total amount of DNA in the sample. The experiment was performed according to the manufacturer's protocol. Briefly, on the day of the analysis, the medium was discarded from the wells and the wells washed with DPBS. The cells were suspended with 0.1% Triton-X 100 buffer (Sigma-Aldrich) to lyse them and frozen at -70 ºC overnight or for a longer period of time. After thawing, the Triton-cell lysates were collected into eppendorf tubes, centrifuged, and pipetted as 3 parallel samples in 96-microplate (Nunc). CyQuant® GR Dye was added into each well suspending the cell lysate thoroughly. The fluorescence was measured with microplate reader (Victor 1420 Multilabel Counter, Wallac, Turku, Finland) at 405 nm.

5.2.4. Osteogenic Differentiation

ALP activity was determined by using ALP Kit (Sigma-Aldrich) and mineralization by using Alizarin Red S (Sigma-Aldrich) according to the manufacturer's protocol. ALP catalyses the hydrolysis of p-nitrophenyl phosphate in alkaline solution liberating p-nitrophenol which appears as a yellow color. The rate of p-nitrophenol formation is proportional to the catalytic concentration of ALP present in the sample.

The same samples were used for ALP as for CyQuant analysis. Each sample suspended with Triton-X 100, was transferred into 3 parallel wells of MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA). The

samples were incubated with 50% alkaline buffer solution (2-amino-2-methyl-1-propanol, 1,5 mol/l, pH 10.3; Sigma-Aldrich) and 50% of stock substrate solution (p-nitrophenyl phosphate; Sigma-Aldrich) for exactly 15 min at 37 ºC. To stop the reaction sodium hydroxide (1.0 mol/l, Sigma; Aldrich) was added. The samples were transferred into 96-well plate (Nunc). The intensity of the colour was measured at 405 nm using a microplate reader (Victor 1420).

5.2.5. Mineralization

Alizarin Red staining is used for detecting calcium compounds deposited in the ECM by the cells, which is characteristic for osteoblasts. Alizarin Red and Ca^{2+} ions precipitate to form red deposits in aqueous solutions.

The cultures were rinsed with DBPS followed by fixation in ice cold 70% ethanol (Altia Corporation, Helsinki, FIN) for 60 min at room temperature. Alcohol was removed and the samples were rinsed once with distilled water before adding the 2 % Alizarin Red S adjusted to pH 4.2 with ammonium hydroxide solution. Samples were incubated in Alizarin Red for 5 minutes and after Alizarin Red removal, washed three times with distilled water. Subsequently, samples were incubated in cetylpyridium chloride with gentle shaking for three hours in room temperature protected from the light. After incubation 3 parallel samples of supernatant were pipetted on a 96-well plate and absorbance measured at 544 nM using a microplate reader.

5.3. Electrical Stimulation

The counter electrodes were attached on the well plate cover as strings that had a projection into each well (Figure 13).

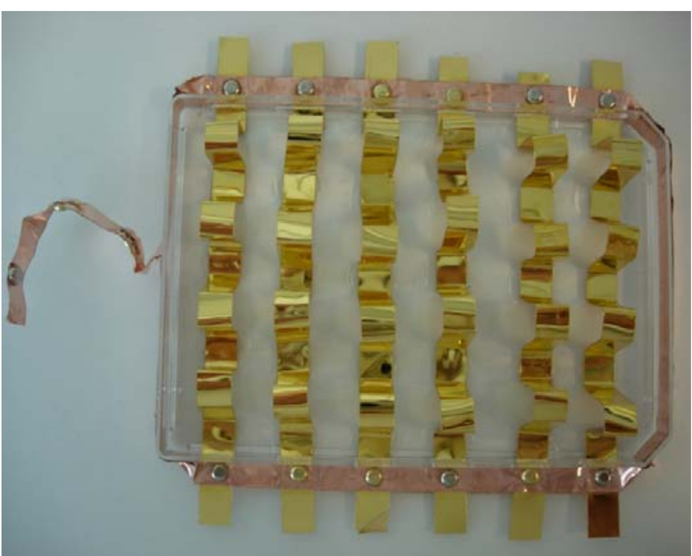

Figure 13. Counter electotrodes attached to well plate cover.

Each projection was slightly embedded into the medium in order to transfer electricity between the bottom and the cover electrode.

For electrical stimulation, the stimulations plates were divided into three groups according to the frequency used: a group with no stimulation, a group with a frequency of 1 Hz and a group with frequency of 100 Hz. All of these 3 groups included 4 subgroups: HA-PPy in OM and BM and CS-PPy in OM and BM. Thus in total, there were 12 different groups.

ES was performed in cell culturing incubator (37 º C, 5 % CO_2). ES set-up is presented in Figure 14.

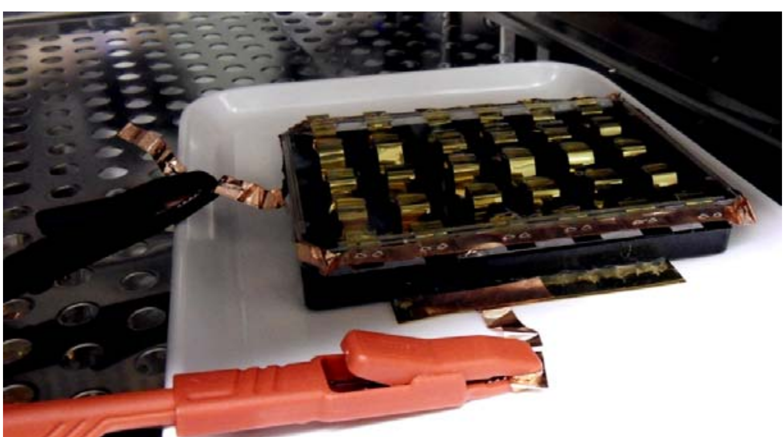

Figure 14. ES was performed inside cell culturing incubator.

Samples were stimulated for 4 h a day during 14 days with BEC at amplitude of ±0.2 V. Frequency, which changed according to the stimulation group, was generated by function generator (Tektronix AFG 3021B, Beaverton, USA). The duration of the pulse was 2.5 s in positive amplitude and 2.5 s in the negative amplitude. From function generator, signal proceeded to timer and further on to resistor where signal distributed to 2 directions: to oscilloscope (Tektronix TDS 2024B) for signal detection and to the stimulation plates. The strength of the electric field was approximately 2 V/cm.

5.3.1. Statistical Analysis

Statistitical analyses of DNA and ALP results were performed with SPSS version 18. The data from quantitative experiments of all three repeats was reported as mean ± standard deviation. The results were considered significant when $p<0.05$.
Statistical examinations were tested using one-way analysis of variance (ANOVA) with bonferroni post hoc correction for multiple corrections. The effects of different culturing periods (7 vs. 14 d), surface materials (HA vs. CS) and stimulation set-up (no ES vs. ES of 1 Hz vs. ES of 100 Hz) were evaluated from combined data of 3 experiments.

6. RESULTS

6.1. Cell Viability

Live/dead staining showed no differences between stimulation groups at any of the time points when the samples were examined with fluorescence microscope (Figure 16 and 18). Instead, there was a notable difference between HA-PPy and CS-PPy. Surprisingly on HA-PPy samples, the cells had formed clusters in both media and all 3 stimulation groups at both time points (Figure 15 and 17). Clusters were partly detached from the surface. Within all of the samples in both media, mainly green fluorescence was detected, which reflected good biocompatibility of all CS-PPy samples and non-harmful effect of the ES.

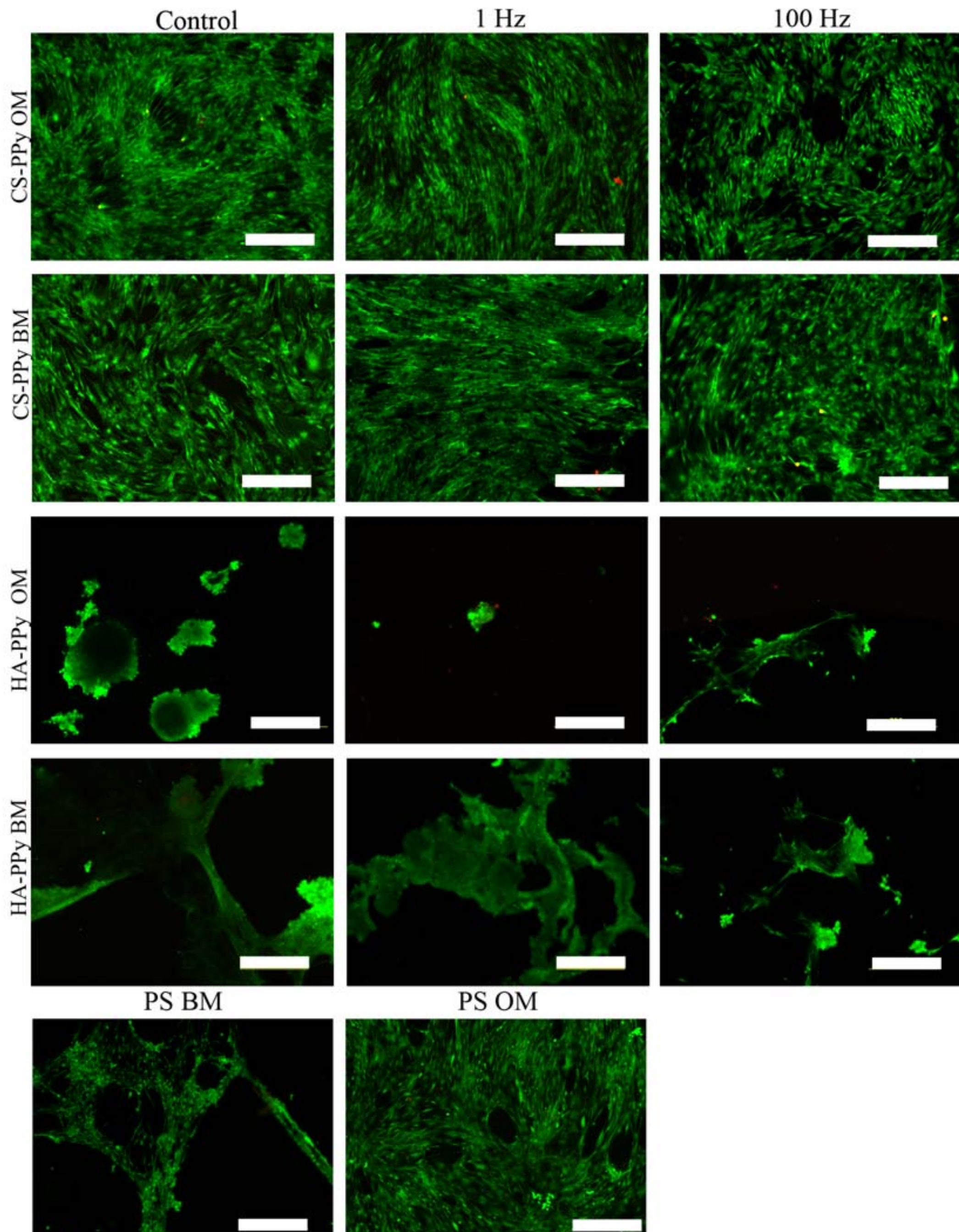

Figure 15. Live/dead staining at 7d time point. Scale bar 500 µm.

Interestingly, ASCs grew better and more homogenously on CS-PPy samples than on standard cell culture plastic. The difference between cell growths was the most obvious in BM (Figure 16).

CS-PPy samples showed morphological differences between the culture media. Some cells on CS-PPy in the BM contained perforations at both time points (Figure 17). The amount of perforations varied widely between CS-PPy in BM samples, and was not as obvious on CS-PPy samples in OM.

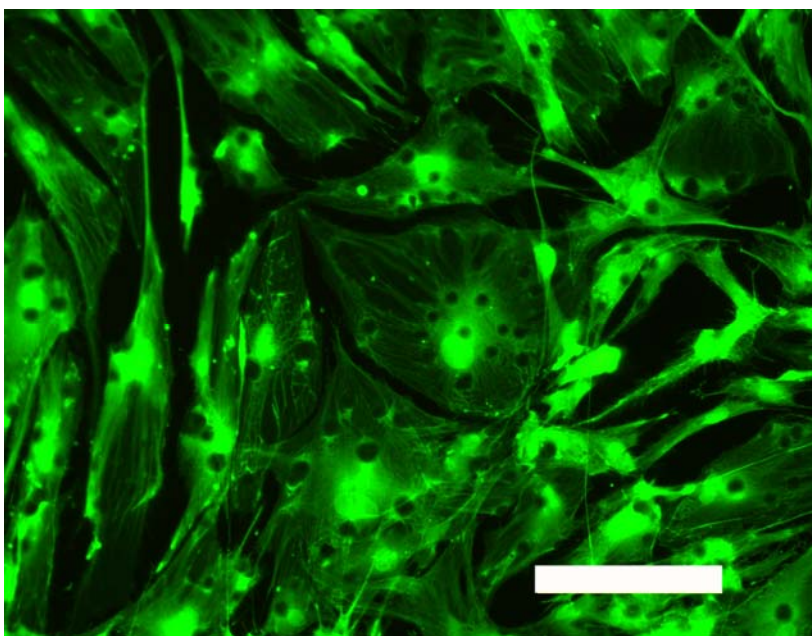

Figure 16. *Perforations were seen on CS-PPy in BM in all stimulation groups. Scale bar 200 µm.*

At day 14, cell number increased to day 7 as shown in Figure 17. Also, the differences between media became clearer in ASCs on CS-PPy showing higher cell number and attachment in OM compared to BM. ASCs on PS showed similar trend in growth of cell number as CS-PPy samples.

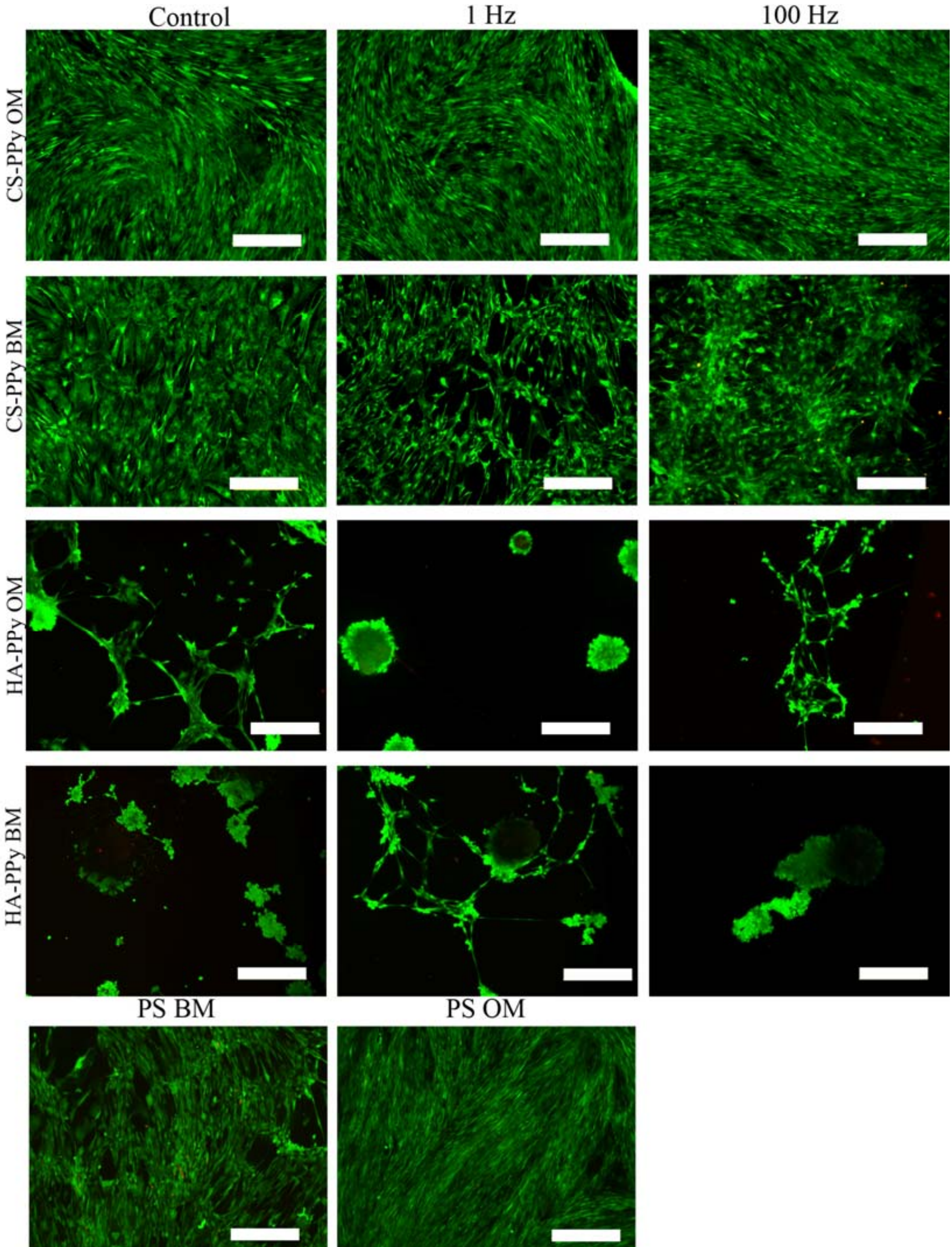

Figure 17. *Live/dead staining at 14d timepoint. Scale bar 500 µm.*

The difference in cell number between PS and CS-PPy was not as obvious as day 7, which could be due to different appearance of the cell shape.

6.2. Proliferation

The cell number was notably higher on all the samples cultured on OM. The DNA content of ASCs is presented for both media in Figure 18. In addition, the cell number

did not increase in BM as it did in OM. ASCs on HA-PPy and CS-PPy controls had significantly higher DNA content in OM compared to BM at day 14, and for HA-PPy also at day 7 (not shown in figure).

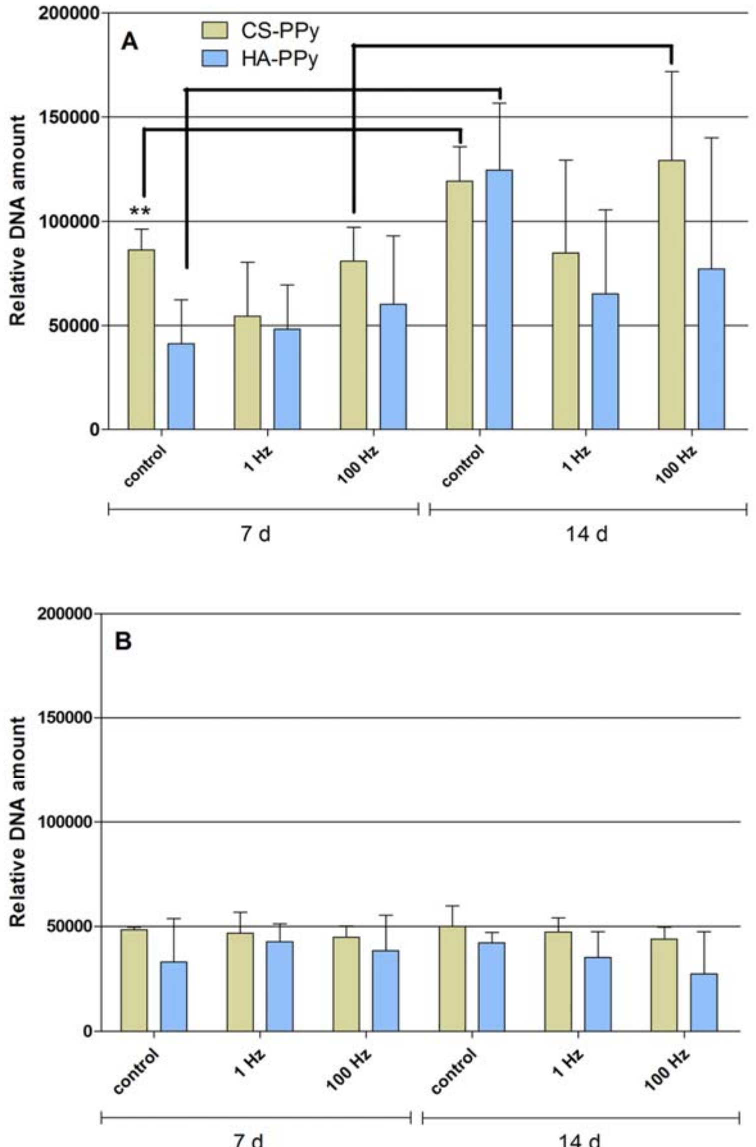

Figure 18. *DNA content of ASCs in OM (A) and BM (B). Bars connected with lines present significant difference between the time points (p<0.05).**)present significant increase in cell number between the materials.*

CS-PPy surfaces supported cell growth better than HA-PPy surfaces, which showed strongest trend in OM. The difference was significant at day 7 in the controls. There was also a slight trend in stimulation parameters in OM where 1 Hz stimulation was the weakest to support ASC proliferation.

Interestingly ASCs in BM did not show any trend within the stimulation parameters. ASCs in CS-PPy in OM had significantly higher DNA content with 100 Hz stimulation than those in BM (not shown in figure).

At day 7, all the non-cellular scaffolds of 1 and 100 Hz gave slightly higher background compared with controls (data not shown). However at day 14, all the non-cellular scaffolds expressed same absorbance as the controls at day 7 and 14.

6.3. Osteogenic Differentiation

ALP activity was highest in ASCs cultured in OM at day 14, whereas any ALP activity could not be detected reliably in BM cultured ASCs (Figure 19). ALP activity increased with time, but not significantly on HA-PPy samples due to a huge standard deviation. ALP activity varied strongly within the patients resulting in a large standard deviation. There was a mild trend that ASCs cultured on HA-PPy had higher ALP activity compared to ASC on CS-PPy. However, the difference was not significant at any of the time points with or without stimulation. In addition there were no differences between the stimulation groups.

Alizarin Red staining was done to measure the mineralization of the matrix; however, this method did not work properly with the materials and gave a strong background staining and inconsistent absorbance values as shown by Figure 20.

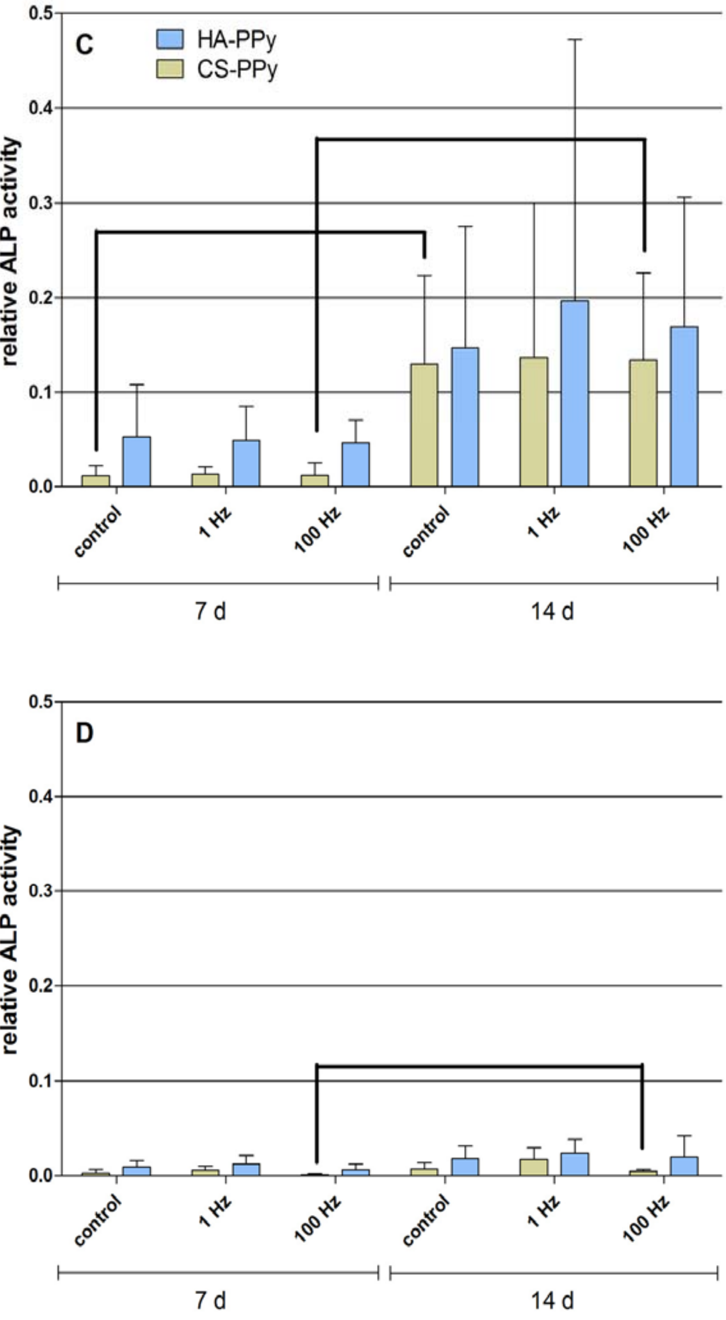

Figure 19. *ALP activity of ASCs cultured in OM (C) and BM (D). Bars connected with lines present significant difference between the time points ($p<0.05$).*

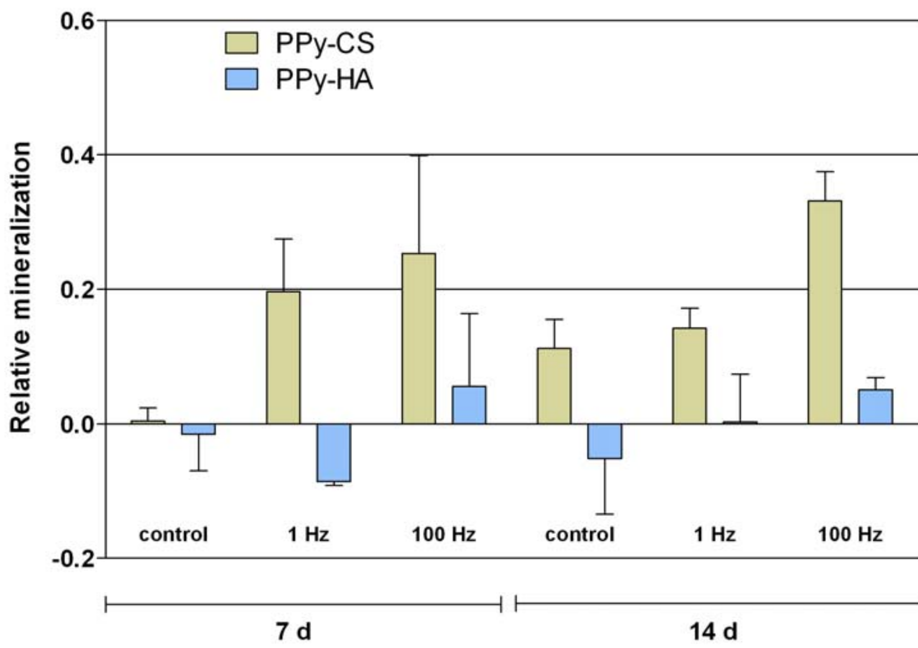

***Figure 20**. Mineralization of ASCs cultured in OM. The data represents ASC mineralization from 1 patient.*

Alizarin Red method was discarded from the results since the protocol was not compatible with the material. All the 3 repeats in both media showed very inconsistent and partly negative absorbance values.

7. DISCUSSION OF THE RESULTS

The purpose of this study was to evaluate the effect of dopant material, the effect of ES with two different frequencies and the effect of medium on viability, proliferation and osteogenic differentiation of ASCs. Selection of the best parameters requires balancing between proliferation and osteogenic differentiation, since both must occur in the regeneration of the bone.

7.1. The Effect of Material

2D surfaces, in general, do not correspond to the requirements of bone tissue scaffold. Therefore, further investigation of the coating in this study need to be conducted with 3D scaffolds (Porter *et al.* 2009). Moreover, electrochemical polymerization requires conductive surfaces for polymer deposition, which leads to the problem when coating non-conductive scaffolds, such as commonly used PLLA. Therefore with non-conductive 3D structures, chemical polymerization is required. In this method, the conductivity of the scaffold is created by additional oxidizing agent, which alludes to the fact that the 2D surface material in this study is not directly applicable to those of 3D models. Nevertheless, this study brings information about HA-CS comparison under ES.

Cell viability and attachment

ASCs grew homogenously in CS-PPy samples cell expansion being even better than on standard PS wells. In contrast, ASCs clustered on HA-PPy samples, which may be due to the poor attachment of the cells. HA used in this study was produced by bacteria, *Streptococcus equi*. The manufacturer guarantees that protein impurities are lower than 1 % so there can be protein residues which may affect on the ASC response to the HA-PPy.

HA is widely used in chondrogenic applications due to its presence in cartilage. It has been reported to induce aggregation of ASCs, which is one of the earliest signs of chondrogenesis. (Wu *et al.* 2010) This could allude to suitability of HA-PPy for chondrogenic applications, thus, whether the clusteration in this study was triggered by the aggregative effect of HA-PPy, needs to be studied in the future. Also, other HA sources, such as human-based HA, should be also investigated.

Another explanation for cell clustering could arise from topography of the material since nanoscale surface roughness is reported to affect on osteoblast adhesion as well as proliferation and differentiation (Gittens *et al.* 2011, Palin *et al.* 2005, Webster *et al.* 2000, Dalby *et al.* 2006). HA-PPy coating has been reported to have

notably rougher surface structure and lower modulus compared with CS-PPy when same weight percentage of dopant is used in the polymer synthesis (Gelmi *et al.* 2010). Surface roughness depends on the nature of the doping species, dopant concentrations and current density during the synthesis (Serra Moreno *et al.* 2008). Moreover, film thickness can be adjusted by current density during the polymerization, which has significant effect on surface properties (Gilmore *et al.* 2009). For instance, increase in film thickness in various PPy doped films or increase in dopant concentration has been reported to result in rougher surface structure, which can have a strong influence on cell adhesion. (Serra Moreno *et al.* 2008, Gilmore *et al.* 2009). In addition, film thickness was reported to affect especially on surface roughness of HA-PPy and lesser extent on CS-PPy (Gilmore *et al.* 2009). In one study, osteoblasts that were seeded on smooth surface of heparin-PPy or HA-PPy films, showed better cell adhesion and proliferation than those films that exhibited irregular surfaces (Serra Moreno *et al.* 2008) Irregular surface roughness could therefore offer one explanation why cells clustered and detached from HA-PPy surfaces in this study. However, the surface roughness was not measured, and hence, the final conclusions cannot be performed based on surface roughness. This would be important to investigate in the future studies.

ASCs cultured on both coatings showed good viability especially in OM. Despite the perforations in CS-PPy samples, the cell viability followed similar pattern with PS. Most of the perforations were observed in ASCs on CS-PPy in BM encompassing control and both stimulation groups. Also few ASCs on CS-PPy in OM had perforations in a lesser amount. The reason, why these areas were especially detected on CS-PPy in BM, remained unclear.

Proliferation and differentiation

In this study, HA-PPy showed stronger effect on cell differentiation, whereas CS-PPy supported significantly more ASC proliferation. According to the live/dead samples, cell clustering and cell detachment may have lowered the cell number on HA-PPy samples. Also, high standard deviations in DNA detection and ALP could be partly explained by detachment of the cells in some samples. Moreover, results from ALP activity in OM illustrated considerable high patient variation, and the rate of differentiation varied widely among the patients, which could have increased standard deviation as well. One explanation to the standard deviation may also be the quality of the materials, which varied along the 3 patients. This was observed in live/dead staining and macroscopically. Macroscopical examination revealed topographical and colour differences between the studied 2 PPy coatings.

BM did not support proliferation of ASCs on neither of the samples as the amount of DNA did not increase within time. In contrast, OM supported both proliferation and differentiation of ASCs on both HA-PPy and CS-PPy. However, since ALP activity is also dependent on cell number, increase in cell number increases also the total ALP activity that is detected. Only ASCs in OM exhibited differences between stimulation groups. Medium had the strongest effect on differentiation and proliferation

of ASCs, but also the effect of material was considerable. ALP activity increased significantly in ASCs cultured on CS-PPy still having lower ALP activity compared to HA-PPy at day 14.

Both, CS and HA have been reported to support osteogenic differentiation of MSCs *in vitro* (Kawano *et al.* 2011, Rentsch *et al.* 2009). One mechanism behind it has been recently reported to be their ability to regulate BMP-2 activity (Kawano 2011).

Interestingly, mechanical actuation of PPy films occurs during ES due to diffusion of electrolytes in and out of the polymer to retain the charge balance on the polymer backbone. Therefore, CS-PPy and HA-PPy expand during the reduction of the polymer. (Gelmi *et al.* 2010) In a study of Gelmi *et al.* (2010), strain caused by BEC was higher in CS-PPy films than HA-PPy due to CS-PPy's lower modulus and thickness. Actuation causes shear stress to the cells due to fluid flow, which can enhance for example osteogenic differentiation (Klein-Nulend *et al.* 1998, Mullender *et al.* 2004, Knippenberg *et al.* 2005). Nevertheless, it remains to be determined whether or not actuation played a significant role in this study and which one of the materials exhibited higher strain.

Material conductivity is essential factor because it defines the electric current mediated to the cells. Conductivity is dependent on several factors, such as film thickness, area of electric flux and amount of dopant. Slight variation in the quality of the samples causes also changes in conductivity. Nevertheless, conductivity should remain relatively stable during our experimental period. Poor conductivity of the samples of medium can also lead to temperature elevation, which can have an effect on cellular reactions. Interestingly, there was slight but clear increase in background noise in DNA detection at day 7 within all the stimulated samples, which could allude to ion exchange between medium and PPy under ES. However, background levels decreased back to normal baseline at day 14.

Alizarin Red staining was used to assess mineralization of the extracellular matrix in this study. However, it was not suitable with this material due to high absorption levels of the material and very inconsistent absorbance values. Therefore protocol needs further optimization. Perhaps more exhaustive rinsing stage of the samples could improve the usability of the method.

7.2. The Effect of Electrical Stimulation

ES with the studied parameters did not seem to have any notable effect on viability, proliferation or differentiation of ASCs.

Cell viability and attachment

Cell viability and attachment did not to vary under ES according to the live/dead staining. ASCs on HA-PPy detached and formed clusters similarly regardless of the stimulation groups.

Proliferation and differentiation

Any kind of trends in DNA detection were not seen in BM, which is supported by the result of Hammerick *et al.* (2010) who did not recognize any differences in proliferation under pulsed AC in BM. By contrast, Kim *et al.* (2009) and Ercan *et al.* (2010) reported proliferation under BEC. Importantly, Kim *et al.* used pulsatile BEC waveform which is very similar compared with our study. In the study of Kim *et al.*, two different parameter configurations were compared with the same pulse rate of 100 pulses/s (100 Hz): 250 µs with lower amplitude and 25 µs with higher amplitude. It occurred that longer pulse duration with lower amplitude had stronger proliferative effect. Nonetheless, the duration of the pulse of 250 µs is considerably shorter and the amplitude (1.5 $\mu A/cm^2$) lower than in our study when 100 Hz frequency is used. Another notable difference was the stimulation period. Kim *et al.* used static stimulation and proliferation was measured already at days 1, 3 and 5. So the question remains whether proliferation had increased in longer experimental periods, such as 7 d or 14 d. Our study did not have time point of 1d which could have been useful for determining early respond to ES.

According to the literature, ES may enhance the proliferative effect of OM (McCullen *et al.* 2010, Kim *et al.* 2009, Sun *et al.* 2007). This, however, was not confirmed by our study since any clear differences in DNA content between control and stimulation groups were not detected. McCullen *et al.* (2010) reported the best proliferation at the strongest (5 V/cm) electric field using 1 Hz frequency. Our daily stimulation period and total duration of our study were similar to McCullen's but the biggest difference was the sinusoidal waveform and static stimulation, which is why the electric fields cannot be directly compared. McCullen *et al.* observed increase in intracellular free calcium when electric field strength was increased. However, different frequencies and electric field magnitudes result in different mechanisms for increased cytosolic calcium, and thus, the threshold for proliferation or differentiation can vary according to stimulation parameters.

When comparing frequencies in OM, 1 Hz was weaker than 100 Hz to promote proliferation. Even though there were no significant differences between control or stimulation groups, the results suggests that cells did experience ES configurations differently in OM than in BM. Natural bone resonance is around 300 Hz, which is why lower frequencies are usually chosen in the experiments (Fujita *et al.* 1983). Both, 1 Hz and 100 Hz frequencies have been reported to promote proliferation. However to our knowledge, BEC has been tested only with frequencies higher than 20 Hz. In case of ASCs on HA-PPy, DNA content was highest at 100 Hz at day 7, whereas at day 14, ES seemed to inhibit proliferation of ASCs on HA-PPy in osteogenic conditions. This could be due to poor adhesion of cells which may have detached during ES and possibly actuation.

Similarly, osteogenic differentiation under ES did not occur in BM and could not be determined reliably in OM due to huge standard deviation in ALP activity. This

is contradictive to most studies (Hammerick *et al.* 2010, Kim *et al.* 2009, Sun *et al.* 2007, Tandon *et al.* 2009). For instance, Sun *et al.* (2007) reported that 1V/cm ES of the MSCs in BM caused a decrease in the Ca^{2+} oscillations, which again, resembles those of terminally differentiated osteoblasts. They further conclude that ES can be applied to undifferentiated MSCs to alter the oscillation pattern, which according to Sun *et al.* is likely to be involved in mediating differentiation. In addition, Kim *et al.* reported increase in ALP activity under BEC in OM compared to control until at day 10. ALP activity however was lower than control at the other time points. Kim *et al.* concluded that that their BEC parameters were mitogenic rather than osteoinductive since any osteogenic differentiation markers were not increased. (Kim *et al.* 2009) The same conclusion from our BEC parameters cannot be drawn.

The reason, why enhancement in proliferation and differentiation were not detected under ES in this study, is not clear. The strength of the electric field is within the same levels as in other studies, but the parameters in this study are unique, thus the results cannot be directly compared with those of others. There were several factors that could have affected the precision and reliability of the study. For instance, corrosion of the stimulation system and ion precipitation from medium might have occurred. In addition, medium plays an important role as current mediator in this study, which is enabled by different electrolytes. The conductance of the medium can vary due to different electrolyte concentrations within the lots or electrolyte exchange between PPy-coated film and medium. Also, one essential variable in our study was the current density. This was due to practical reasons, since some stimulation plates consisted of 4 sample wells, whereas some of 8 sample wells. Therefore, as the voltage was kept constant, the current density was doubled with those of having only 4 sample wells. Inclusion of PPy could also have synergistic effects with ES. Most of the other studies use inert conductive surfaces or simply exploit medium as conductor. Therefore, various phenomena can occur when bioactive conductive materials are used for ES. It is unclear whether the possible synergy affected increasingly on standard deviation or impaired the effect of ES on proliferation and differentiation.

Kim *et al.* compared MSC mineralization under ES in BM and OM resulting in significant difference between non-stimulated and stimulated group in OM but not in BM, which suggests that effect of ES on differentiation is stronger in OM. However, to the best of our knowledge, this was the first study that systematically compares BM and OM under ES. Furthermore, literature lacks comparison of frequencies promoting osteogenic differentiation of MSCs. (Kim *et al.* 2009)

In order to fully determine whether the cells respond to ES, calcium imaging should be included in the stimulation experiments. If the similar calcium oscillation, as Sun *et al.* reports, is not seen with our parameters, then other parameters should be considered. In general, interpretation of literature was challenging due to different way to present parameters or lack of essential parameters.

8. CONCLUSION

The choice of medium exhibited the strongest effect on ASC proliferation and differentiation, but most importantly, the choice of dopant had an effect on cell response: CS-PPy supported proliferation significantly, whereas HA-PPy showed slightly but systematically stronger effect on differentiation. ASCs formed clusters on HA-PPy surfaces, which could be due to poor attachment of the cells. However, proliferation and differentiation were not notably compromised due to clusters and, hence there is a possibility that HA-PPy could be suitable for chondrogenic applications. ES did not have an effect on proliferation or differentiation but triggered a trend in CS-PPy samples in OM, which suggests that ASC respond to 1 Hz and 100 Hz differently. In case of proliferation, 1 Hz was not suitable for promoting cell growth, whereas 100 Hz had similar effects than the control conditions. Therefore, further optimization of ES parameters with PPy is required to enhance ASC proliferation and differentiation. CS-PPy coatings seem to offer suitable conductive surface for ASCs and therefore coating of scaffolds with PPy could provide good adhesion for the cells and enable ES through scaffold materials.

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