Tampere University

Mari Lallukka

BIOACTIVE GLASS/GELATIN HYBRID BIOMATERIALS FOR BONE TISSUE ENGINEERING

Faculty of Medicine and Health Technology
Master's thesis
February 2020

ABSTRACT

Mari Lallukka: Bioactive Glass/Gelatin Hybrid Biomaterials for Bone Tissue Engineering
Master's Thesis
Tampere University
Master's Degree Programme in Bioengineering
February 2020

Hybrid biomaterials combining bioactive glasses (BAG) and natural polymers such as gelatin, are potential alternatives for bone tissue engineering applications. The inorganic and organic phases in a hybrid interact at a molecular scale and are connected via covalent bonds due to coupling agents such as 3-glycidoxypropyl trimethoxysilane (GPTMS). Owing to the strong covalent bonding between phases it is possible to create materials with controllable degradation (both aqueous and enzymatic), and bioactivity. Furthermore, the rheological properties of the materials can be adjusted to increase the mechanical properties and/or allow shaping of the hybrid into a specific shape.

In this study, hybrids with either silicate glass S53P4, or Mg and Sr-doped borosilicate glass B12.5 –Mg 5- Sr 10 ("mix") combined to organic gelatin phase were synthesized with different weight ratios between phases: 30/70 (glass/gelatin), 15/85, 5/95 and 1/99. The aim of this study was to assess *in vitro* properties of these hybrid biomaterials. The bioactivity and degradation behaviour of the hybrids were investigated in Simulated Body Fluid (SBF) and in enzymatic collagenase solution. SBF dissolution experiment was carried out for two weeks, and enzymatic degradation experiment for six hours. At every time point, samples' mass loss and ion release behaviour were studied, and SBF samples' pH changes were measured.

For further characterization of the hybrids, rheological measurements were performed to understand the gelation behaviour of the hybrids, and thermogravimetric analysis (TGA) to assess the inorganic/organic phase ratio.

In addition, the biocompatibility of BAG/gelatin hybrid materials were evaluated by culturing human-derived mesenchymal stem cells (hBMSCs) in contact with hybrid samples and with hybrid dissolution product extracts. Furthermore, the ion concentrations in cell culturing medium upon culturing were also measured.

Based on the results, the hybrids were stable in aqueous solutions, and exhibited controlled ion release suggesting hydroxyapatite (HA) layer precipitation. The hybrids were also more resistant to enzymatic degradation than the gelatin alone. However, based on the Live/Dead results all hybrid compositions showed an inhibitory effect in hBMSC proliferation after 72 hours of culturing, possibly due to too high reactivity or release of unreacted compounds, such as the coupling agent GPTMS. Further cell studies and optimization of the hybrid biomaterial are needed to confirm the suitability of hybrids as a biocompatible bone tissue engineering scaffold material.

Keywords: hybrid biomaterial, bone tissue engineering, bioactive glass, *in vitro* bioactivity, bone marrow –derived mesenchymal stem cells, biocompatibility, rheology

TIIVISTELMÄ

Mari Lallukka: Bioaktiivinen lasi/gelatiini hybridibiomateriaalit luukudosteknologian sovelluksissa
Diplomityö
Tampereen yliopisto
Biotekniikan diplomi-insinöörin tutkinto-ohjelma
Helmikuu 2020

Bioaktiivista lasia ja luonnon polymeerejä, kuten gelatiinia yhdistävät hybridibiomateriaalit, ovat vaihtoehto luukudosteknologian sovelluksiin. Hybridimateriaalin epäorgaaninen ja orgaaninen osa vuorovaikuttavat molekyylitasolla, sillä ne sitoutuvat kovalenttisesti kemiallisten kiinnitysaineiden, kuten 3-glysidyloksipropyylitrimetoksisilaanin (GPTMS), ansiosta. Tämän vahvan sitoutumisen ansiosta hybridit ovat helpommin muotoiltavissa, niiden bioaktiivisuutta ja liukenemisnopeutta sekä mekaanisia ominaisuuksia voidaan säädellä tarpeen mukaan.

Tässä työssä tutkittiin useita erilaisia hybridimateriaaleja. Gelatiinin kanssa yhdistettiin bioaktiivista lasia eri painoprosenttisuhteilla (30/70 (lasi/gelatiini), 15/85, 5/95 ja 1/99). Laseina käytettiin joko S53P4 -silikaattilasia tai Mg- ja Sr- ioneja sisältävää B12.5 –Mg 5- Sr10 -borosilikaattilasia. Työn tavoitteena oli vertailla ja tutkia hybridien *in vitro* -ominaisuuksia. Hybridien bioaktiivisuutta ja liukenemista tutkittiin sekä fysiologisia nesteitä simuloivassa SBF-liuoksessa että kollagenaasi-entsyymiliuoksessa. SBF -dissoluutiosarjaa jatkettiin kaksi viikkoa, ja entsymaattista hajotusta seurattiin kuuden tunnin ajan. Tietyissä aikapisteissä tarkasteltiin hybridinäytteiden massan muutosta, pH-arvoa sekä ICP-OES -mittauksella ionien vapautumista.

Lisäksi hybridien geeliytymistä tutkittiin reologisilla mittauksilla, ja todellista suhdetta epäorgaanisen ja orgaanisen osan välillä analysoitiin termogravimetrisillä mittauksilla.

Työn toisena tavoitteena oli arvioida hybridien biosoveltuvuutta. Ihmisen luuytimen mesenkymaalisia kantasoluja viljeltiin kontaktissa hybridinäytteiden sekä niiden liukenemistuotteiden kanssa. Lisäksi vapautuvat ionikonsentraatiot analysoitiin ICP-OES -mittauksella.

Tulosten perusteella hybridit käyttäytyivät stabiilisti nesteympäristössä, ja liukenivat vapauttaen tasaisesti bioaktiivisuutta osoittavia ioneja. Hybridit vastustivat entsymaattista hajotusta hieman pelkkää orgaanista gelatiinia tehokkaammin. Kuitenkin Live/Dead -koetulosten perusteella hybridit rajoittivat solujen jakautumista 72 tunnin viljelyn jälkeen. Solujen heikko uusiutuminen johtuu todennäköisesti hybrideistä ylimäärin vapautuvasta reagoimattomasta GPTMS – kiinnitysaineesta. Jotta hybridien bioyhteensopivuus, ja näin ollen mahdollinen käyttö luukudosteknologiassa, voidaan varmistaa, tulee hybridien koostumusta optimoida ja jatkaa soluviljelytutkimuksia.

Avainsanat: hybridi- biomateriaali, luukudosteknologia, bioaktiivinen lasi, *in vitro* bioaktiivisuus, luuytimen kantasolut, bioyhteensopivuus, reologia

PREFACE

This Master of Science thesis study was performed in the group of Bioceramics, -glasses and -composites at Tampere University. Cell culturing part of the experiments were conducted in cooperation with the Adult Stem Cell Group at Tampere University.

First, I want to thank Associate Professor Jonathan Massera for giving me the opportunity to work with such an interesting project and for welcoming me into his research group. I am especially thankful for all the advice and guidance he gave me regarding my thesis work and the whole world of academic research. I also want to express my gratitude to Associate Professor Susanna Miettinen for making it possible to include cell culturing experiments in my thesis. A special thank you goes also to Arjen Gebraad and Amel Houaoui, for guiding me into the world of stem cells and always having time for my questions.

I also want to thank Jannika Paulamäki and Jennika Karvinen for guiding me through the rheological experiments, and especially Jannika for all her advice and support during my whole thesis process. I would also like to thank Henriikka Teittinen for assisting me with my experiments and always cheering me on even during difficult times.

Finally, I want to thank everyone I had the honor to meet while working at the laboratories both at Hervanta and Kauppi campus, you all made this thesis project unforgettable and the labs feel like home. And last but absolutely not least, I wish to express special gratitude to my family and friends for being there for me and showing endless support during this journey.

Suwon, South Korea, 11.02.2020

Mari Lallukka

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

45S5	Bioglass, bioactive glass with composition of 45 wt% SiO_2, 24.5 wt% CaO, 24.5 wt% Na_2O, and 6.0 wt% P_2O_5
58S	bioactive glass (58% SiO_2, 33% CaO and 9% P_2O_5, based on mol%)
APTES	(3-Aminopropyl)triethoxysilane
B	Boron
BAG	Bioactive glass
BMP	bone morphogenetic proteins
Ca	Calcium
CF	C-factor, molar ratio of GPTMS and gelatin
DPBS	Dulbecco's phosphate-buffered saline
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ESC	embryonic stem cell
FGF-2	basic fibroblast growth factor
G*	complex shear modulus
G’	storage modulus
G’’	loss modulus
GPTMS	3-glycidoxypropyl trimethoxysilane
hASC	human adipose stem cells
hBMSC	human bone marrow-derived mesenchymal stem cells
HCA	carbonated hydroxyapatite
HCl	hydrochloric acid
HLA-DR	Human Leukocyte Antigen – DR isotype
ICP-OES	inductively coupled plasma optical emission spectrometry
ICPTES	3-(Triethoxysilyl)propyl isocyanate
IGF	insulin-like growth factor
iPSCs	induced pluripotent stem cell
LVE	linear viscoelastic range
MC3T3-E1	pre-osteoblastic cell line from mouse
Mg	Magnesium
mix	abbreviation for B12,5 –Mg5 –Sr10 doped borosilicate bioactive glass
mol-%	Mol percent
MSC	mesenchymal stem cell
Na	Sodium
P	Phosphorus
PCL	polycaprolactone
PDGF	Platelet-derived growth factor
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PGA	polyglycolic acid
PHB	polyhydroxybutyrate
PLA	polylactic acid
PLGA	poly lactic-co-glycolic acid
PRP	Platelet rich plasma
P/S	Penicillin/Streptomycin
S53P4	abbreviation for
SAOS-2	human primary osteogenic sarcoma cell line
SBF	Simulated Body Fluid
Si	Silicon
Sr	Strontium

tan δ	loss factor
TCP	tricalcium phosphate
TEOS	tetraethyl orthosilicate
TEVS	triethoxyvinylsilane
TGA	Thermogravimetric analysis
TGF-β	Transforming growth factor beta
VP	N-vinyl pyrrolidone
wt-%	Weight percent
α-MEM	Alpha Modifications Minimum Essential Medium
γ	shear rate
γ-PGA	gamma-polyglutamic acid
η	viscosity
τ	shear stress

1. INTRODUCTION

Bone –related diseases and subsequent bone loss are a major burden in terms of patient quality of life and economic impact. There are multiple causes for bone injuries and defects, such as osteoporosis, traumatic injury, orthopaedic surgeries and tumour resection. Currently the most used treatment for bone defects is autogenous bone grafting, where bone is collected from patient's own body (*autograft*), or from other humans, typically cadavers (*allograft*). (Brydone, Meek et al. 2010)

Autografts and allografts suffer from various limitations, such as lack of supply, risk of disease transmission and high cost. To overcome these restrictions, synthetic bone substitute biomaterials, such as metallic, ceramic, polymeric or composite biomaterials are used. However, there are multiple criteria for bone substitute materials to fulfil. They must be biocompatible, possess suitable degradation and mechanical properties, and ideally have osteogenic properties. Bioactive biomaterials, such as bioactive glasses (BAGs), and other bioceramics, such as β- tricalcium phosphate (TCP), are an attractive option to meet these needs. But despite of their bioactivity and osteoconductive properties, when bioceramics are used alone, they tend to be too brittle and difficult to shape. These concerns of long-term reliability *in vivo* limit their applications as bone replacing biomaterials. (Jones 2013)

For this reason, focus has turned more on composite materials combining BAG particles or fibres within a polymer matrix. However, this approach is not optimal either, due to the mismatch in degradation between phases leading to unpredictable *in vivo* behaviour, and limited contact with bone forming cells due to polymer phase masking the bioactive components. Due to these challenges, there is a need for a material that can mimic the natural bone tissue more effectively while being stable *in vivo* (Valliant E.M., Jones J.R., 2011).

Bone tissue engineering is a popular method to overcome the limitations of autografts and allografts. Its key components include biocompatible three –dimensional temporary template (*scaffold*) to guide new bone formation, bone forming cells to lay down bone tissue matrix, and an appropriate environment to mature the tissue construct. Ideally when combining all these components best results in terms of native bone graft mimicking construct would be achieved. (Amini, Laurencin et al. 2012)

Natural bone tissue could be described as a nanocomposite material consisting of organic phase (collagen fibrils) and inorganic carbonated hydroxyapatite (HCA) crystals. Therefore, a hybrid material in which inorganic and organic phases are interacting in nanoscale could better mimic natural bone's structural and functional properties.(Jones 2013) Opposite to conventional composites, in inorganic/organic class II hybrid material the phases are homogeneously dispersed and covalently linked to each other at the molecular level. The covalent links are facilitated by reaction with coupling agents, such as 3-glycidoxypropyl trimethoxysilane (GPTMS). This way the material can achieve synergistic effect combining the properties of both organic and inorganic phases. (Kickelbick G., 2006) In the future, inorganic/organic hybrid biomaterials could find potential use as a bone scaffold for bone tissue engineering applications.

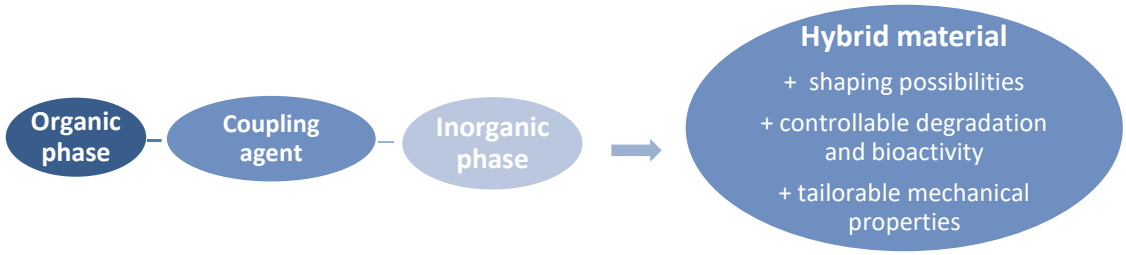


Figure 1. *Introduction of inorganic/organic hybrid biomaterial*

The objective of this study was to characterize novel bioactive glass/gelatin GPTMS-coupled hybrid biomaterials in terms of their bioactivity, degradation behaviour, rheological properties, and biocompatibility. This way valuable information about the materials suitability for bone tissue engineering applications could be assessed. For example, this type of scaffold material could be used as an injectable bone filler or putty-like material, which would be highly preferred by surgeons due to their ease of handling and tailorable properties (Fig 1).

Firstly, literature review including basics of bone biology and anatomy, current state of the art on hybrid biomaterials, rheology and stem cells/hybrid interactions for bone tissue engineering and regenerative medicine are covered. The materials and methods used to synthesize and characterize the newly developed materials are presented, followed by the results and discussion sections. Finally, conclusions are given, focusing on the limitations of the developed materials and suggestions for future work.

2. BONE TISSUE ENGINEERING

2.1 Structure of bone

Bone is a type of connective tissue consisting of bone cells and mineralized extracellular matrix. Its role in human body is to protect and support other organs and enable movement. It also acts as a calcium and phosphate storage. The inorganic part of bone, bone mineral, is found in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. The major structural organic building block of the bone matrix is type I collagen. Furthermore, other collagen types such as V, III, XI, and XIII are found in smaller amounts. In addition to collagen, other matrix proteins include proteoglycans, glycoproteins (*osteonectin, osteopontin, podoplanin, dentin matrix protein*), bone-specific vitamin K-dependent proteins (*osteocalcin, protein S*) and finally growth factors and cytokines (*BMPs, IGFs, TGF-β*). (Ross, Pawlina 2016) The components of bone matrix are summarized in Figure 2 below:

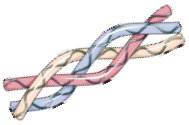
Bone	Matrix components 	Collagen (~90%)	type I types V (III, XI, XIII)
		Other proteins (~10%)	proteoglycans glycoproteins growth factors & cytokines bone-specific proteins
	Bone cells 	osteoprogenitors	
		osteoblasts	
		osteocytes	
		bone-lining cells	
		osteoclasts	

Figure 2. *Bone composition*

There are variety of cell types associated with bone. *Osteoprogenitor cells* are pre-osteoblast cells committed to bone cell differentiation. They are derived from mesenchymal stem cells, which are discussed more in detail later in this thesis. *Osteoblasts* are the matrix secreting cells, which are referred to as *osteocytes* once they get surrounded with secreted matrix. Some osteoblasts after bone deposition stay on the bone surface as bone-lining cells. In addition to cells creating new bone, cells resorbing bone also exist. These *osteoclasts* are present where bone is being remodelled or in the case of bone damage. (Ross, Pawlina 2016) The cells associated with bone are presented in Figure 2.

In general, bone tissue can be classified either as compact or spongy/cancellous bone. Denser compact bone is found outside of the bone while meshwork-like spongy bone forms the interior of the bone (Fig. 3). The porous meshwork is continuous and consists of bone marrow and blood vessels. Bone marrow is divided to either red or yellow marrow. Red marrow is the development site for blood cells and is most abundant in young individuals. For adults the fat cell-containing yellow marrow is more prominent.

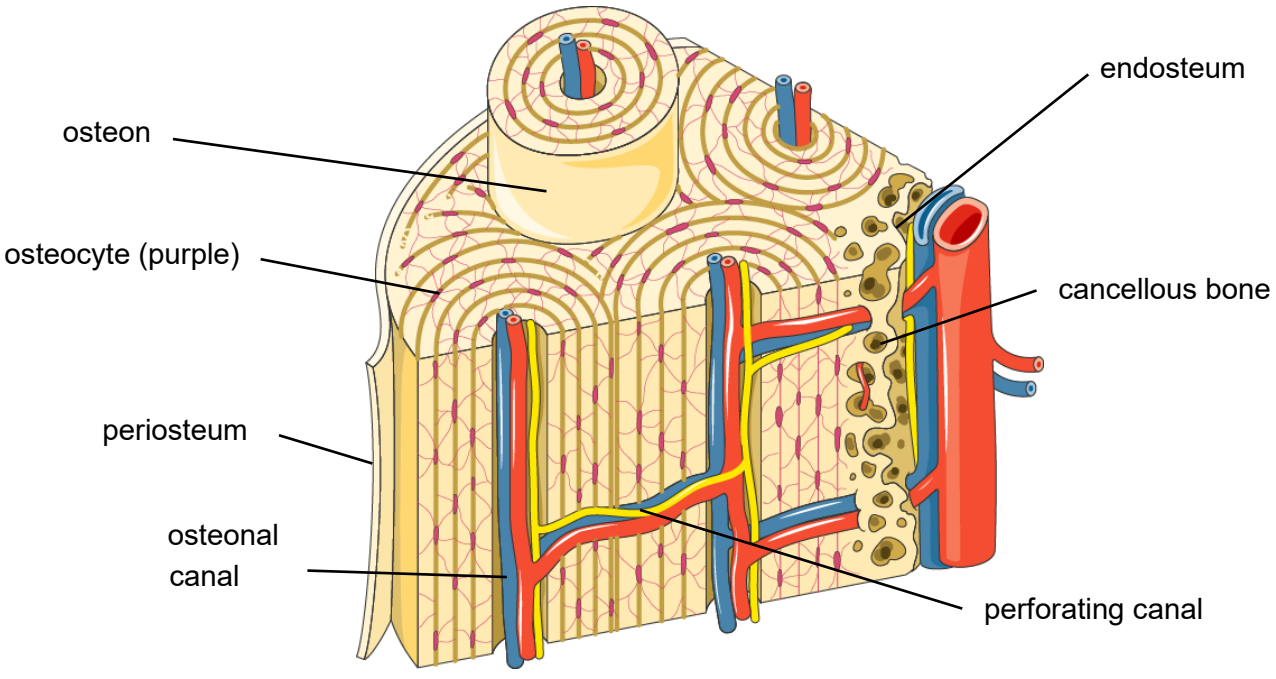


Figure 3. *Section of a mature bone*

As seen above, mature bone tissue is composed of cylindrical shaped structural units called osteons. In osteon there is bone matrix surrounding the central osteonal canal, which contains blood vessels and nerves. In addition to osteonal canals, there are perforating canals through periosteal and endosteal surfaces to reach osteonal canal and connect canals to each other. Periosteum is a fibrous membrane, which covers the outer surface of bones. Correspondingly, the lining facing the marrow cavity is referred to as endosteum. (Ross, Pawlina 2016)

2.2 Bone regeneration

Process of fracture healing consists of four main steps. These steps, including inflammation, proliferation, soft callus formation and finally hard callus formation are combined in Figure 4.

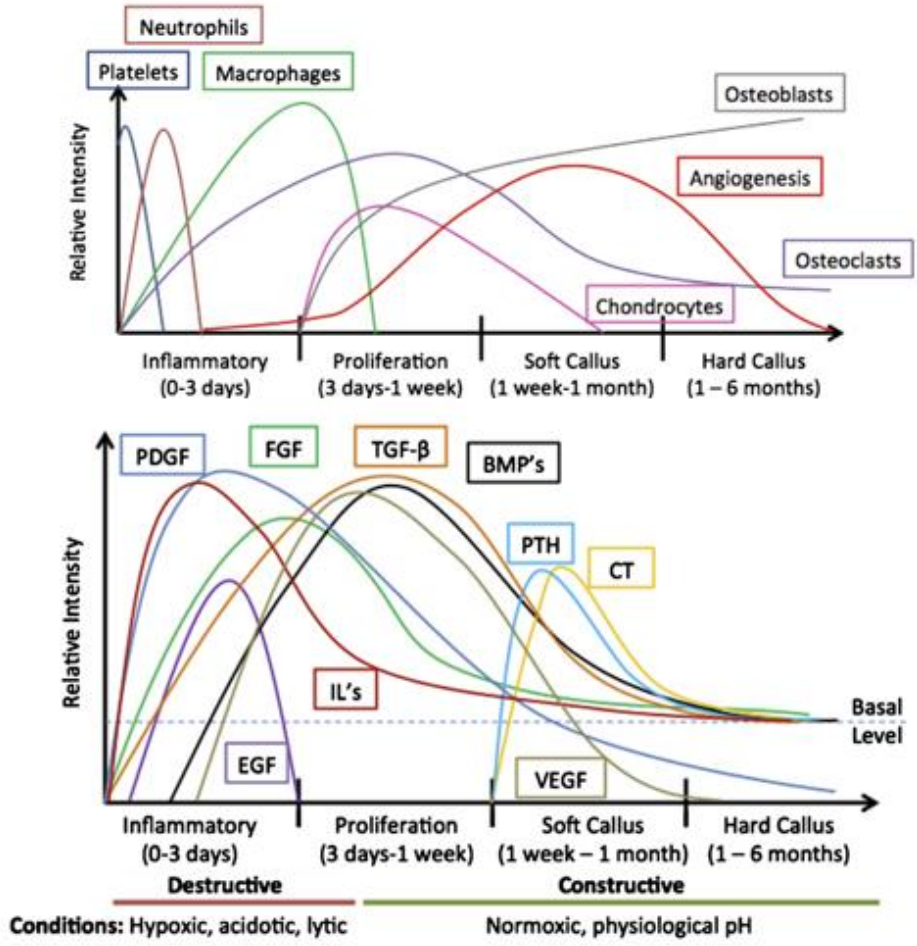


Figure 4. *Fracture healing model steps, adapted from (Arun, Alvarez, et al., 2014)*

First, hematoma or blood clot forms at the fracture site. This hematoma consists of platelets, leukocytes, macrophages, growth factors and cytokines, and it plays an important role in later bone regeneration. This step is often referred to as destructive phase, and it is characterized by inflammation, activity of pro-inflammatory cytokines, such platelet-derived growth factor (PDGF), or platelet-rich plasma (PRP), and local hypoxia. Hypoxia stimulates the formation of new vasculature (angiogenesis), which correspondingly leads to the second step of fracture healing process: recruitment of mesenchymal stem cells to the site. These cells can differentiate into chondrocytes and osteoblasts. Differentiation occurs under biological cues such as bone morphogenetic proteins (BMPs). Chondrocytes form cartilage matrix first, which is later substituted by bone formation by osteoblasts. For this reason, the third step includes the formation of soft cartilaginous scaffold, which is substituted in the last step by new deposited bone. Chondrocytes undergo programmed cell death (apoptosis), and finally over the course of months to years, osteoblasts and bone resorbing osteoclasts continue bone remodelling leading to indistinguishable new bone at the fracture site. (Arun, Alvarez et al., 2014)

2.3 Current methods to treat bone defects

The history of bone-grafting traces as back as the 17th century, when the first documented bone graft was performed by a Dutch surgeon Job van Meekeren. This bone graft was *a xenograft*, which refers to a tissue or organ derived from other species, in this case a piece of a dog skull. (Donati, Zolezzi et al. 2007) Currently, the standard treatment is *an autograft*, bone from the patient's own body, from donor site to defect site. The donor site is usually the top of the pelvis (iliac crest), or for spinal surgery it could also be bone spurs from the vertebrae. In addition to autografts, *allografts*, which are harvested from other humans (often cadavers), are used. Both options possess advantages but also multiple disadvantages. Autografts are preferred over allografts due to the increased graft integrity due to vascularization, however, they suffer from limited supply and discomfort to patients. Use of allografts eliminates donor site morbidity, but there is a risk for disease transmission, and increase risk for graft rejection. (Ghanbari, Vakili- ghartavol 2016)

Synthetic substitutes can overcome some limitations of autografts and allografts, since they are easier to sterilize, they are available in unlimited quantities and in different shapes and sizes. Synthetic bone graft materials can be made from natural or synthetic

polymers, ceramics, metals or composites. The most common ceramic materials for this use are often a mix of hydroxyapatite (HA) and tricalcium phosphate (TCP), manufactured in variety of forms including granules and porous blocks. (Jones 2012) Most research of polymers suitable for bone graft substitutes has focused on polylactic acid (PLA) , polyglycolic acid (PGA) and poly lactic-co-glycolic acid (PLGA) copolymers (Campana, Milano et al. 2014). Metallic implants made of, for example, titanium are strong and tough to resist crack propagation, and therefore they are suitable for load bearing applications. However, since metal is a bioinert material, fibrous encapsulation can occur, and healthy bone will never re-form on the site. Furthermore, the body is likely to eventually reject the implant, and higher risk for fatigue loading and infection exists. (Jones 2012)

Bioactive glass, which will be discussed more in detail in this work, is beneficial since it is bioactive, and, depending of its composition, can bond with both bone and soft tissue. However, concerns, such as brittleness and long-term mechanical reliability *in vivo*, are hindering their use as bone substitute materials. (Jones 2013) Furthermore, processing into three-dimensional scaffolds of the highly bioactive and commercially available silicate glasses remains a challenge due to their rapid crystallization during sintering (Massera, Fagerlund et al. 2012). For these reasons BAGs are often combined with for example biodegradable polymers to form composite materials. However, possible mismatch in the degradation rate between the glass and the polymer might lead to loosening of the glass phase, and to difficulties in predicting the overall degradation behaviour. (Jones 2013)

2.4 Bone tissue engineering

Current alternatives to bone grafts lack several important key properties. For instance, they cannot be loaded with osteogenic cells, they do not have sufficient mechanical properties, and they should include added compounds, such as vascular endothelial growth factor (VEGF) or bone morphogenetic proteins (BMPs) to promote vascularization and osteoinduction. Typically, such pro-angiogenic and osteogenic molecules are expensive and can have negative effects, such as ectopic bone formation (Oryan, Alidadi et al. 2014) Tissue engineering is making an attempt to solve these drawbacks.

Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function. (Langer, Vacanti 1993) In essence, functional living tissue can be fabricated using living cells, which are usually associated with a matrix or scaffold to guide new tissue development. *A scaffold* stands for a three-dimensional temporary support structure for tissue forming cells to synthesize new tissue in desired shape and dimensions. (Rahaman, Day et al. 2011) In the context of bone regeneration, mimicking the natural bone (*autograft*) is the best current option available. To achieve this, multiple widely accepted design criteria are made.

First of all, the scaffold needs to be three dimensional with interconnected pores, so that it is possible to seed cells and other relevant biological moieties inside, and that it can later support fluid flow, cell migration, and new tissue and blood vessel formation into the scaffold. In addition, the scaffold must be biocompatible, and it needs to promote osteogenic cell attachment and function. One other important aspect is the material degradation rate that, ultimately, should match the rate of new tissue formation without releasing any toxic by-products. Furthermore, the mechanical properties of the scaffold should mimic natural bone in order to survive physiological stresses at the implantation site in vivo. (Rahaman, Day et al. 2011)

Finally, other important aspects should also be noted, such as scalability (possibility for mass-production), easy sterilization, and the regulatory requirements needed to fulfil in order to get clinical use for the product. The user experience also matters, because the surgeons are known to prefer materials that could be cut to shape in theatre to fit the defect. (Gao, Rahaman et al. 2013, Jones 2013)

Some potential scaffold types for bone tissue engineering can be divided in three main categories: natural, synthetic and mineral-based. Firstly, natural scaffolds include biodegradable natural polymers and polysaccharides such as collagen, hyaluronic acid, alginate and chitosan. Challenge in using natural scaffolds is their lack of mechanical stability and difficulty to sterilize without disrupting their structure. The use of synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA), polydioxanone (PDS) and polycaprolactone (PCL) would improve the mechanical properties without compromising biodegradability. However, they lack osteoinductive properties, which could be achieved by using bioactive scaffold materials such as calcium phosphate ceramics and bioactive glass. (Montoro, Wan et al. 2014)

2.5 Stem cells in bone tissue engineering

The bone tissue engineering approach often involves the use of mesenchymal stem cells (MSCs) seeded into three-dimensional scaffold material and induced to generate new bone by osteoinductive cues. Especially bone-marrow derived mesenchymal stem cells (BMSCs) are used due to their high osteogenic potential. (Yousefi, James et al. 2016) Therefore, this chapter will focus on BMSCs after general introduction to stem cells.

Stem cell is defined as a cell that has the capacity to renew itself and to differentiate into other cell types of the body. Stem cells can be classified by dividing them into groups according to their differentiation potential. *Totipotent* stem cells are the earliest cells in mammal embryo development, and they can differentiate into all the possible cell types to form the whole embryo. *Pluripotent* stem cells, such as cells from the inner cell mass of the blastocyst, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) can differentiate into all the cells of all the three embryonic germ layers, but are not able to form a complete embryo. *Multipotent* stem cells, such as mesenchymal stem cells or other adult stem cells, can differentiate into multiple cell lineages. And finally, *unipotent* stem cells, such as spermatogonial stem cells, can only differentiate into one mature cell lineage. (Samadikuchaksaraei, Lecht et al. 2014)

Mesenchymal stem cells (MSCs) are multipotent stem cells capable of self-renewal, plasticity and ability to differentiate into multiple cell lineages. They are a heterogenous population of fibroblast-like cells from tissues of mesodermal origin. MSCs were first isolated and characterized from bone marrow in 1970s, but the term MSC came officially in the use only in early 1990s. Other typical sources to harvest MSCs include umbilical cord, placenta, blood, adipose tissue, and dental tissues. (Andrzejewska, Lukomska et al. 2019)

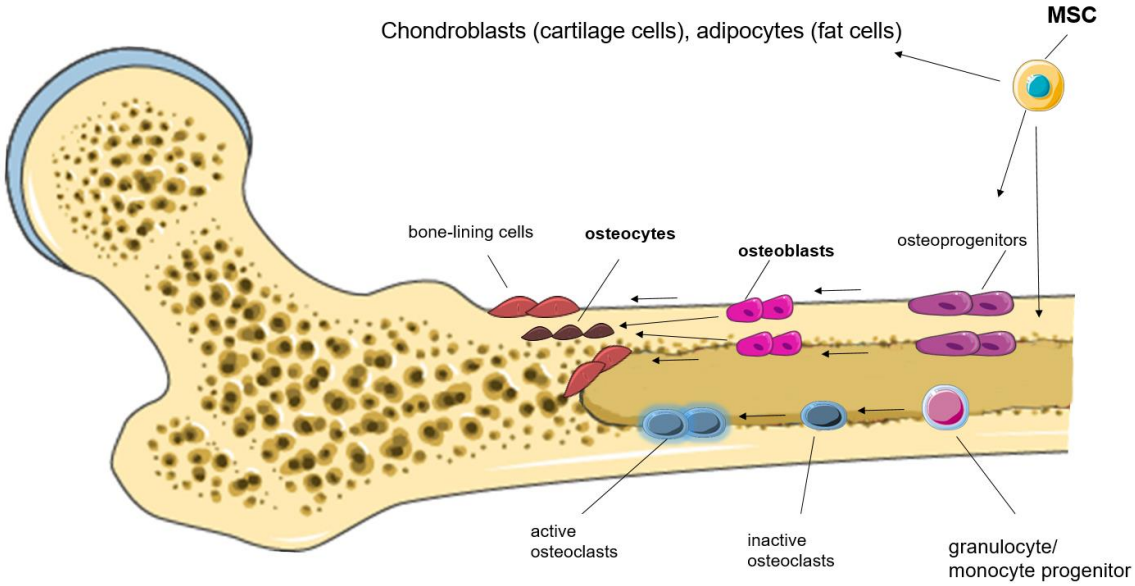


Figure 5. *MSC differentiation into multiple cell types*

Specific criteria to define MSCs exists. Firstly, MSCs need to be plastic-adherent in standard culture conditions. Secondly, ≥95% of the MSC population must express surface markers STRO-1, SB-10, Sh3 (CD73) and SH4 antigens as well as Thy-1 (CD90), TGF-β receptor type III endoglin (CD105), hyaluronic acid receptor CD44, integrin α1 subunit CD29, activated leukocyte-cell adhesion molecules (ALCAM, CD166), and possibly others. Thirdly, they should lack expression to the hematopoietic markers CD19/CD79a, CD34, CD45, CD11b/CD14 and HLA-DR. And finally, they must able to differentiate into osteoblasts (bone cells), chondroblasts (cartilage cells) and adipocytes (fat cells) under standard in vitro differentiating conditions (Fig. 5). (Dominici, Le Blanc et al. 2006)

Bone marrow derived stem cells (BMSCs) are considered to be promising in bone tissue engineering applications. They could be harvested directly from patient's own bone marrow, and they are thought to be responsible for natural bone repair process, where they differentiate into osteoblasts. As seen in Figure 6, they are fibroblast-like cells with spindle-like and stellate morphology.

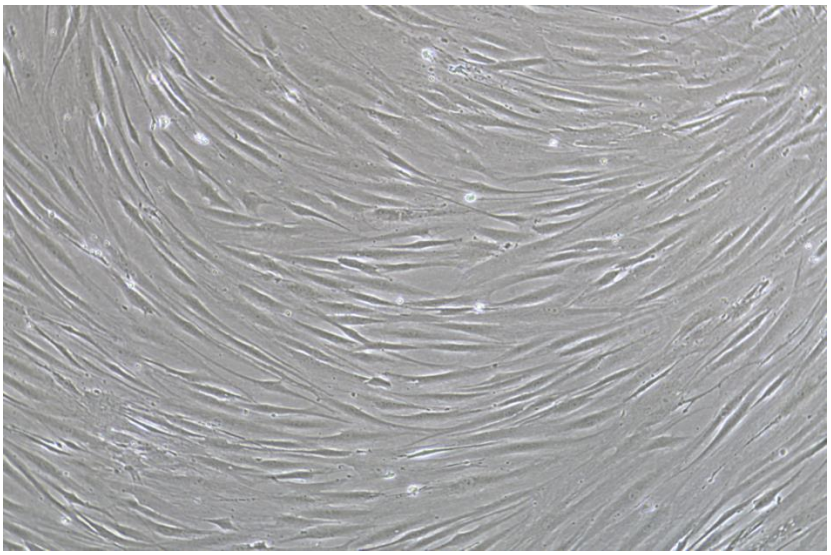


Figure 6. *Morphology of BMSCs in culturing flask, x10 objective: spindle, stellate, irregular*

MSCs have been shown to have hypoimmunogenic properties: they are able to modulate immune cell phenotypes and to immunosuppress the local environment. However, their use is limited by the low frequency within bone marrow stroma. In addition, MSCs in general have high variation between different donors. For instance, the age, gender, extraction site, and physical condition can play important role in the quality of harvested MSCs. (Andrzejewska, Lukomska et al. 2019) Other challenges include high cost of serum and growth factor supplements needed in their in vitro expansion (Montoro, Wan et al. 2014).

BMSCs, and other MSCs such as adipose stem cells (ASCs) have been widely studied with many silicate bioactive glasses, and found to support their proliferation and differentiation functions in *in vitro* cell culture (Bosetti, Cannas 2005, Radin, Reilly et al. 2005, Rahaman, Day et al. 2011). In contrast, borate and borosilicate bioactive glasses have shown lower ability to support cell proliferation due to their faster degradation rate and pH increasing effect of releasing boron (Rahaman, Day et al. 2011, Ojansivu, Mishra et al. 2018). However, despite the reduced cell proliferation, the dissolution products of the glass have been found to stimulate osteogenic commitment and upregulate endothelial markers (Ojansivu, Mishra et al. 2018).

The use of hBMSCs with hybrid materials will be discussed in more detail in the following chapter.

3. HYBRID BIOMATERIAL

Conventional bioactive glass-containing composites are usually prepared by incorporating bioactive glass particles in biodegradable polymer matrix. Most used polymers include polyesters such as polylactic acid (PLA), polyglycolic acid (PGA), and poly(lactic-co-glycolic) acid (PLGA). Polymers, such as PLA or polyhydroxybutyrate (PHB), have also been applied as coatings to highly porous glass-ceramic foam scaffolds to improve resistance to fractures, but there are doubts over the effectiveness in terms of cellular response. (Jones 2013) However, these conventional composites suffer from multiple limitations. One concern is the lack of bioactive surface, such as in the case of polymer coatings on BAGs. Also the polymer matrix can “mask” bioactive glass particles, which leads to osteoprogenitor cells not being able to encounter glass but only polymer (Fig. 7). Other issues include mismatch in the degradation rate of the composites components, which causes instability of the scaffold. For example, the commonly used polyesters degrade by self-catalytic hydrolysis, which leads to rapid loss of mechanical properties. (Valliant, Jones 2011)

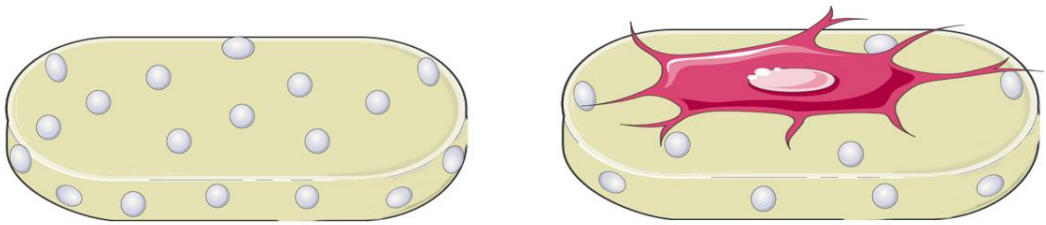


Figure 7. *Issue with conventional composites: bioactive glass particles (white) embedded in polymer matrix. Cells can contact only few particles limiting bioactivity (Jones 2012)*

In order to overcome these issues, a new type of biomaterial named *hybrid material* has been developed. Hybrid materials act as one phase due to their interpenetrating organic-inorganic networks interacting at the nanoscale (Novak 1993). With hybrids it is possible to combine properties of different phases effectively: for example, the high bioactivity of BAG, toughness of polymers, and controlled congruent degradation by balancing the ratio of the phases. Hybrids differ from nanocomposites because their components

cannot be distinguished above nanoscale. The so-called true hybrid behaves as one material phase, while it exploits the benefits of each individual phase. (Mahony, Yue et al. 2014)

Hybrid materials can be divided into two classes depending on the interactions between inorganic and organic components (Fig. 8). *Class I hybrids* contain only weak bonding, such as hydrogen bonds, or van der Waals forces. They are usually prepared by incorporating a soluble polymer into inorganic sol, and entrapping polymer into silica network during network condensation. The challenges of these hybrids include the lack of chemical bonds between the inorganic and the organic phases, which can lead to rapid dissolution behaviour (Valliant, Jones 2011, Poologasundarampillai, Maçon 2016). *Class II hybrids* differ from class I hybrids because they have covalent bonding between the components. In order to form covalent bonds, *a coupling agent* (a molecule) is needed. In earlier studies the use of silica-containing polymers, such as polydimethylsiloxane (PDMS) was investigated, but due to biostability of those polymers, they are not suitable for tissue engineering applications (Novak 1993, Valliant, Jones 2011).

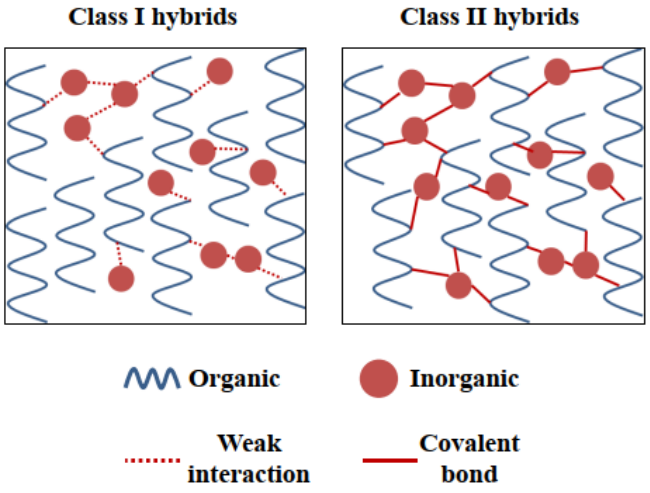


Figure 8. *Class I and Class II hybrid classification, adapted from (Mondal 2018)*

Inorganic-organic solids are usually prepared by exploiting the sol-gel process. The used polymer is added early to the process so that the silica network can form around it. The conventional sol-gel process is modified in terms of thermal processing, since most hybrid systems need aging and drying below 100 °C. In addition, control of the pH -value is very crucial in this process: it affects the functionalization of the polymer, and the

gelation of the silica network. For instance, too acidic pH might cause degradation of certain polymers during the synthesis. (Jones 2013)

There are multiple challenges involved in hybrid material development. First, hybrid synthesis is complex and challenging due to the heavy chemistry involved. Also since the inorganic phase of hybrids have been mainly from tetraethyl orthosilicate (TEOS) in previous studies, the main challenge has been the difficult incorporation of calcium ions into the silicate network. It is essential to incorporate calcium if the material is meant to have the osteoinductive properties of bioactive glasses. (Jones 2013) In sol-gel synthesis adding of metal salts with calcium require high temperature treatment above 400 °C. Alternatively, the use of calcium alkoxide precursors in room temperature might lead to impossible handling of the hybrid sol due to premature gelation. Therefore, mostly pure silica-polymer hybrids are done with heterogeneous calcium deposits outside the silicate network. The issue with these deposits is their rapid dissolution: they get washed away in aqueous environment, causing burst release of ions and rapid pH increase. (Lao, Dieudonné et al. 2016)

3.1 Inorganic component: Bioactive glass

Bioactive glass was discovered by Professor Larry Hench at the University of Florida in 1969. He discovered a degradable glass with composition 46.1 SiO_2- 24.4 Na_2O- 26.9 CaO - 2.6 P_2O_5 (mol%), which was later named as 45S5 Bioglass. Bioglass was found to form strong bond to bone, which started a whole new field of bioactive ceramics. (Hench, Splinter et al. 1971)

Bioactive material can be defined as a material that stimulates a beneficial response from the body, particularly bonding to host tissue. (Hench 2006) Bioactive glass is especially beneficial in bone applications, because it doesn't only bond with bone rapidly, but also stimulates bone growth away from the bone-implant surface (*osteoinduction*) by stimulating genes associated with osteoblast differentiation. While glass bonds to bone, carbonated hydroxyapatite layer (HCA) starts to precipitate at the surface. HCA is very similar to natural bone mineral, and it is believed to interact with collagen fibrils to integrate with bone. The reason for these osteoinductive properties of bioactive glass lies in the dissolution products, such as soluble silica and calcium ions that stimulate osteogenic cells to produce bone matrix. (Hench 2006)

It is important to understand the atomic structure of glass in order to study its properties. In general, bioactive glasses consist of glass formers, network modifiers and

intermediates. Silicate glasses consist of silica tetrahedra connected by -Si-O-Si- bridging oxygen bonds, Si being the network forming atom. Network modifiers include Na and Ca, since they disrupt the Si network by forming non-bridging oxygen bonds (Fig. 9). It has been shown that P is isolated from silica network, and no P-O-Si bonds form unless the glass contains more than 50 mol% of P. This explains why P is rapidly lost upon immersion in aqueous solution. In the case of borate- or phosphate glasses the network former is either boron trioxide (B_2O_3) or phosphorus pentoxide (P_2O_5), respectively. (Stanić 2017)

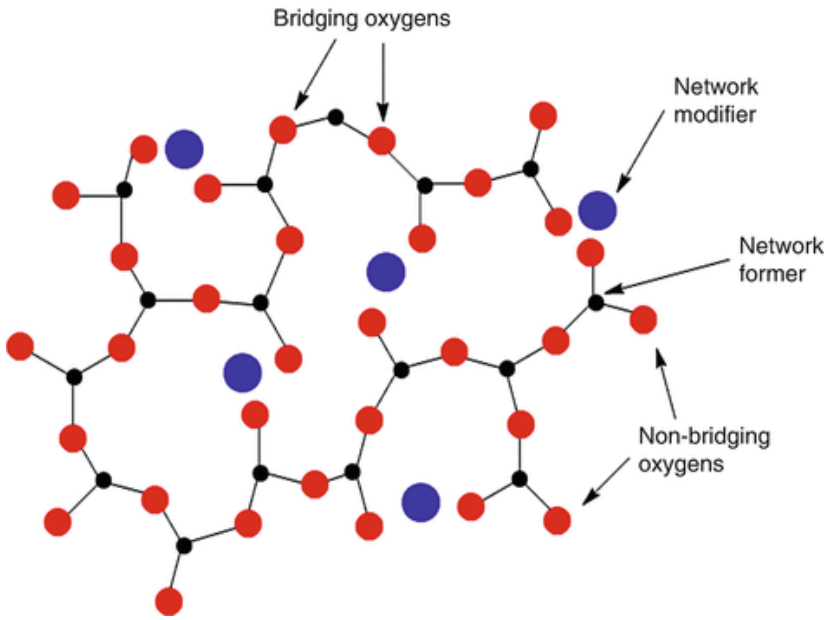


Figure 9. *Bioactive glass network, adapted from (Stanić 2017)*

It has been shown that the accumulation of dissolution products from the glass leads to changes in the glass chemical composition and change in the surrounding pH, which leads to HCA nucleation. (Hench 1998, Jones 2012) The whole multistep process can be described as follow:

Firstly, rapid ion exchange occurs on the glass surface: H^+ ions from the solution are exchanged with network modifier cations Ca^{2+} or Na^+ of the glass. This ion exchange creates silanol bonds Si-OH on the glass surface:

$$\text{Si-O}^-\text{Na}^+ + \text{H}^+ + \text{OH}^- \rightarrow \text{Si-OH}^+ + \text{Na}^+(\text{aq}) + \text{OH}^-$$

The pH value increases due to both the consumption of H+ ions, and the release of alkali-metal and alkaline-earth ions. Because of that, the cation depleted silica-rich region starts to form near the glass surface. Phosphate, if present in the glass composition, is lost from composition during this phase, because it is isolated from the silica network. High local pH leads to OH^- ions attacking the silica network, breaking Si-O-Si bonds. Soluble silica is lost as silicic acid $Si(OH)_4$ to the solution, and more silanols are left on the glass surface:

$$\text{Si-O-Si} + H_2O \rightarrow \text{Si -OH} + \text{OH -Si}$$

After these steps occurs the condensation of Si-OH groups near the glass surface and the repolymerization of silica rich cation-depleted layer. Next, Ca^{2+} and $PO4^{3-}$ groups migrate through the silica rich layer and from the solution. They form an amorphous CaO-P_2O_5 layer on top of the silica gel layer (Fig. 10). Finally, OH^- and carbonate $(CO_3)^{2-}$ from solution are incorporated in the amorphous CaO-P_2O_5 film which crystallizes into HCA layer.

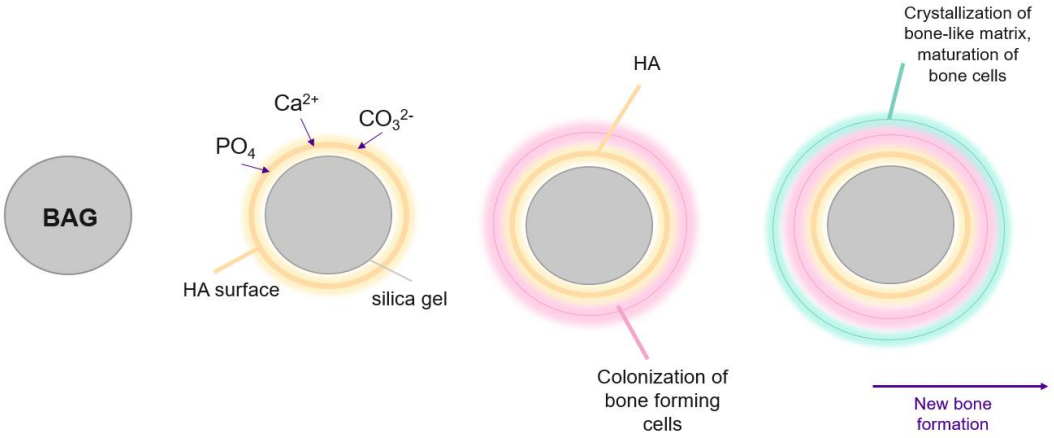


Figure 10. *Mechanism of HCA layer formation on the surface of BAGs*

After the formation of crystalline HCA layer, following steps are hypothesized to occur:

First, the biological moieties get absorbed into the HCA layer, following by the action of macrophages, attachment and differentiation of stem cells, and finally the generation and crystallization of matrix. (Jones 2012)

Bioactive glasses can be divided into many subgroups. In addition to conventional silicate glasses such as 45S5 or S53P4, for example phosphate- and borate- based glasses have been developed. Furthermore, doping of conventional bioactive glasses, as well as borate/phosphate variant, with metal ions such as Mg, Sr, and Ag, or trace elements such as Cu, Zn and Sr can lead to changes in crystal structure, thermal stability, morphology, solubility and chemical and biological properties. In the scope of this thesis work, especially important properties include the favourable biological responses such as enhanced angiogenesis, osteogenesis and antibacterial activity. Especially in the case of bone tissue engineering applications, understanding the role of inorganic ions in bone metabolism is crucial. (Hoppe, Güldal et al. 2011, O'Neill, Awale et al. 2018)

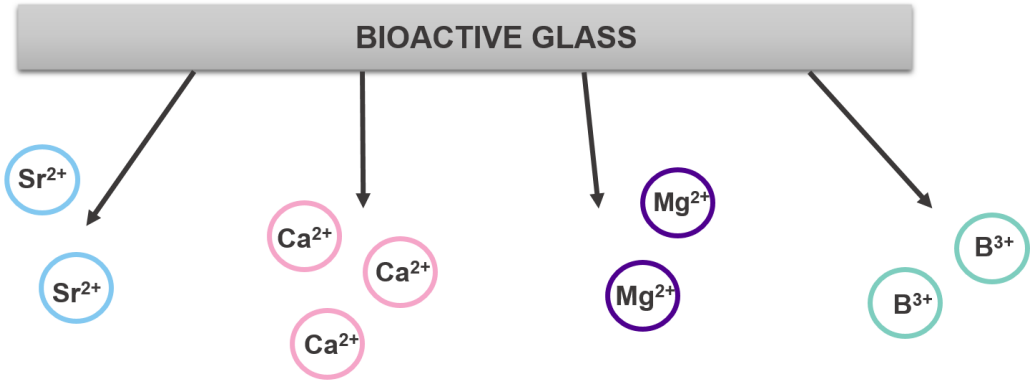


Figure 11. *Ions with osteogenic properties (Mouriño, Vidotto et al. 2019)*

Magnesium is one of the main trace elements in human body and plays an important role in the bone development. Mg-ions doped in a glass network are found to stimulate new bone formation, and increase bone cell adhesion and stability (Zreiqat, Howlett et al. 2002, Yamasaki, Yoshida et al. 2002).

Strontium is structurally, physically and chemically very similar to calcium, which is why it has been widely studied in the context of bone regeneration. For instance, Sr -ions are found to be promising in treatment of osteoporosis by inhibiting bone-resorbing osteoclast activity (Meunier, Slosman et al. 2002), and beneficial to bone formation in vivo (Marie, Ammann et al. 2001, O'Donnell, Candarlioglu et al. 2010, Lao, Jallot et al. 2008, Gentleman, Fredholm et al. 2010).

Boron, even though toxic in high concentration, is an essential trace dietary element. It has been found to stimulate bone formation, and RNA synthesis in fibroblast cells (Dzondo-Gadet, Mayap-Nzietchueng et al. 2002).

The modification of the conventional silicate glass network also affects its thermal and degradation properties. In the context of BAG dissolution, the rate of HCA layer formation indicating bioactivity highly depends on the glass composition. The lower the silica content, for example in the case of adding modifying cations, the less connected silica network, and this leads to more rapid dissolution of the glass (Hench 1998).

Addition of boron has been found to form a phase separated glass with regions rich in SiO_2, and B_2O_3.Because the borate phase dissolves faster due to higher solubility, borosilicate glasses have increased dissolution rate in aqueous environment compared to silicate glasses.(Massera, Claireaux et al. 2011, Tainio, Salazar et al. 2020) Partial substitution of Ca with Mg has been found to increase durability and contribute toward stronger glass network. (Massera, Hupa et al. 2012)

Two main ways to fabricate bioactive glass include the conventional melt-quenching route and the chemistry-based sol-gel route. In melt-quenching the oxides are melted together at high temperatures in a platinum crucible, and then quenched in a graphite mould or in water. The sol-gel reaction takes place in room temperature, where the glass precursors undergo polymer-type reactions to form a gel. The gel consists of a wet inorganic network of covalently bonded silica, which is then dried and heated to form a glass. The conventional 45S5 and other commercial glasses are prepared by melt-quenching, and ternary composition glasses such as 58S are fabricated by sol-gel method. (Jones 2013)

The main difference between melt-quenched and sol-gel glasses is that melt-derived glasses are dense while sol-gel glasses tend to have inherent nanoporosity (Sepulveda, Jones et al. 2001). This porosity increases surface area and thus the reactivity. TEOS (tetra-alkyl orthosilicate) is typically used as a sol-gel precursor. Catalytic conditions of sol gel process can be acidic, basic, or neutral, and it impacts greatly the structure of the inorganic network formed. (Novak 1993) The main disadvantage of sol-gel method is the possible shrinking and cracking during gel drying caused by drying stresses that are generally attributed to large capillary forces generated in very small pores of the gel (Novak 1993).

3.2 Organic component: Polymers

The possibilities of preparing an inorganic organic hybrid are almost endless: for example, wide range of both natural and synthetic organic polymers as well as inorganic metal-oxygen fragments and cationic species can be incorporated in hybrid materials (Jones 2013).

The choice of polymer can be based on a variety of factors. First, in order to exploit hybrids for tissue engineering applications, controllable degradation is important. Therefore, the used polymer should be biodegradable. However, it is also essential to consider the method of polymer degradation, for example whether it occurs by hydrolysis or by enzymatic degradation. Chain scission occurs by reaction between water and polymer, where the water splits the polymer chains. Autocatalysis, due to acidic oligomers that catalyse further degradation due to local pH increase, leads to rapid and unpredictable dissolution. This can be a challenge with conventional polyesters like PGA, PLA and their copolymers. In *in vivo* conditions enzymatic degradation happens resulting in reduced chain length of polymers, mainly on the surface because water penetration is slower than the rate of degradation. Therefore, even though the size of the scaffold becomes smaller, the bulk structure is maintained. These types of degrading scaffolds provide longer mechanical stability for the tissue to regenerate.(Dhandayuthapani, Yoshida et al. 2012)

The next important criterion, in the case of hybrids, is whether it is possible to form covalent bonds between the inorganic phase and the polymer. This criterion is often fulfilled using bifunctional coupling agents, which are discussed more in detail in the next chapter. Finally, when choosing the suitable polymer often natural tissue is mimicked. In bone tissue engineering applications collagen would be a preference because bones are mainly consisting of it. However, collagen is not very soluble and therefore for example gelatin, or chitosan are other potential alternatives.(Shirosaki, Osaka et al. 2012)

Collagen as the most abundant component of the organic part of natural bone would be the ideal polymer choice for bone tissue engineering scaffolds. In addition, due to the strong triple helix structure of its amino acids, appropriate mechanical properties can be achieved. It is often derived from bovine skin and tendons, porcine skin and rat tail.(Parenteau-Bareil, Gauvin et al. 2010) Due to its xenogenous nature, there are risks associated with the use of collagen, such as possible transmission of diseases. In addition, challenges arise from its insolubility. Collagen also varies from batch to batch: for example, its degree of cross-linking might vary. Other issues include religious and cultural considerations due to the use of porcine derived collagen, and regulatory concerns. (Valliant, Jones 2011).

Gelatin is one attractive option to act as organic phase in hybrid since as a denatured form of collagen, it is a major constituent of natural extracellular matrix of all tissues. Gelatin is also more a practical choice cost wise than collagen, and it is easily available. Gelatin retains the functional groups of collagen along its chains but it is soluble in water, which is another benefit. Disadvantages include that the amino acid chains are not

necessarily uniform when they come from natural source. Therefore, it is difficult to accurately define how many covalent bonds will form between gelatin and silica, as it is not known how many functional groups each gelatin molecule will have. (Jones 2013)

Other polymers studied for the use in inorganic organic hybrids include for example chitosan and polycaprolactone (PCL). Moreover, multiple synthetic polymers such as polyethylene glycol (PEG) are also experimented in hybrid research. (Jones 2013)

3.3 Coupling agents

The coupling agents are usually bifunctional short chain polymers containing alkoxysilane groups on one end and another functional group on the other end. The type of functional group depends on the polymer. These groups might also be as side chains instead of end of the chains. (Valliant, Jones 2011).

Most common coupling agents include organosilanes such as 3-glycidoxypropyl trimethoxysilane (GPTMS), 3-aminopropyltriethoxysilane (APTES), and 3-isocyanatopropyltriethoxysiloane (ICPTES) (Mahony, Yue et al. 2014) (Fig. 12).

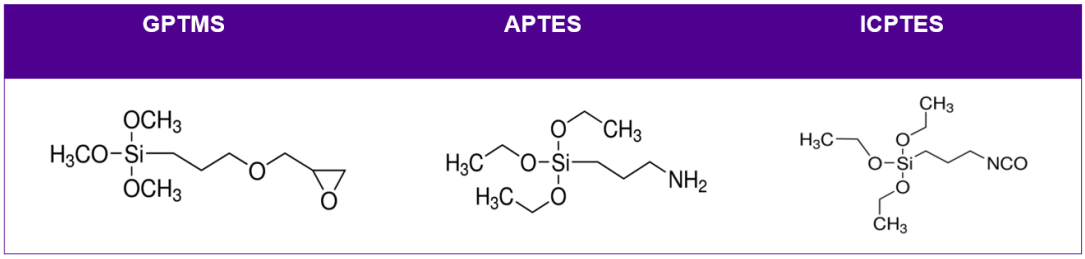


Figure 12. *Chemical structure of most common coupling agents*

Out of these options GPTMS is the most widely used in terms of hybrid materials due to its inexpensive price, and the ease of polymer functionalization. With GPTMS the polymer functionalization can be carried out in single step reaction. GPTMS has an epoxy ring on one end, and three methoxysilane groups on the other end of the molecule. The epoxy ring is very susceptible to nucleophilic attack, and therefore polymer containing nucleophilic groups such as –OH or –COOH can be functionalized with GPTMS. (Ren, Tsuru et al. 2001) The hypothesized reaction between GPTMS and natural polymer such as gelatin are shown below (Fig. 13):

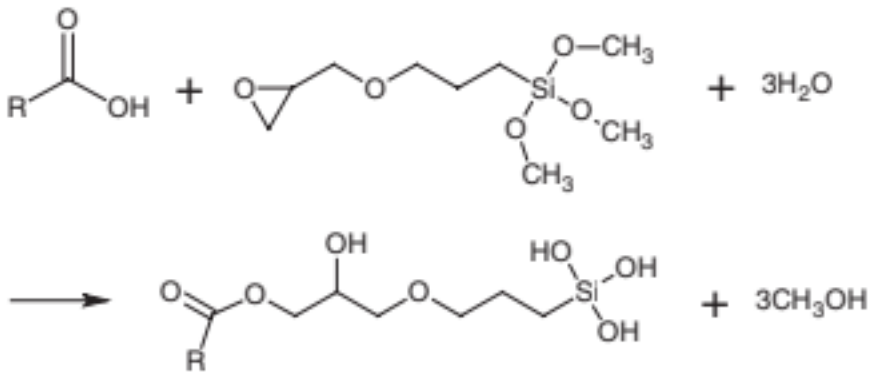


Figure 13. *Hypothesized reactions between GPTMS and gelatin, adapted from (Ren, Tsuru et al. 2001)*

In addition, it is hypothesized that the silanol groups of the GPTMS-functionalized gelatin would react to those of bioactive glass (Fig. 14).

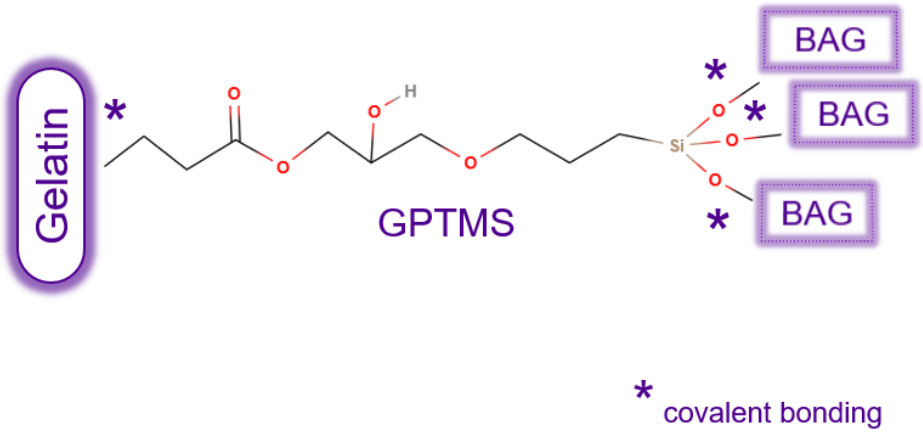


Figure 14. *Theoretical structure of BAG/gelatin class II hybrid biomaterial*

Like mentioned earlier, other options for coupling agents exist. For example, in the case of ICPTES, the isocyanate functional groups are highly toxic. Therefore, during the hybrid synthesis it is challenging to ensure the removal of all unreacted ICPTES. In addition, ICPTES is found disadvantageous due to its preferential reactivity towards H_2O. For this reason, anhydrous solvents must be used, to which for example often used gelatin is insoluble. (Connell, Gabrielli et al. 2017).

While coupling agents are often used, other ways to crosslink natural polymers exist. These crosslinkers include for example glutaraldehyde, 1-ethyl-3-(3-

dimethylaminopropyl)carbodiimide hydrochloride (EDC), and genipin. From those, glutaraldehyde is the most common and efficient one. Its aldehyde groups at both ends bond covalently to amine groups of polymer chain. It is easily available, inexpensive, but concerns exist due to its cytotoxicity upon degradation. (Bigi, Cojazzi et al. 2001) EDC is also cytotoxic if it remains unreacted in the solution. It is soluble in water and reacts with carboxyl groups on the polymer. Genipin is a non-cytotoxic alternative to glutaraldehyde and EDC but it is found much less effective (Bigi, Cojazzi et al. 2002). Due to the reasons explained above, these crosslinkers have not been studied in detail in the context of hybrid biomaterials.

Due to the competitive nature of polymerization reactions of inorganic and organic network of the hybrid, pH value of the solution is very essential for the functionalization to occur. Gabrielli et al. reported that slightly acidic conditions are needed to obtain a functionalization through nucleophilic attack because the catalysis is too slow at a neutral pH. Nevertheless, if acidity is increased too much, it can result in hydrolysis of the epoxy ring to the corresponding diol being prevalent reaction, which limits the nucleophile attack. Precipitation process is most prevalent in basic conditions, which leads to epoxy rings remaining closed. The optimization of these reactions would need careful considerations of the reaction times and pH values present in hybrid synthesis (Gabrielli, Russo et al. 2013)

3.4 Current state of hybrid biomaterial research

The synthesis of the first organic-inorganic hybrids traces all the way back to early 1980s, more commonly referred to as “organically modified silicates” or “ormosiles” (Jones 2012) In these hybrids starting materials were often polydimethylsiloxane (PDMS) or also currently widely used tetraethyl orthosilicate (TEOS). More modern approach to hybrids is to focus on covalently coupled class II hybrids (Table 1).

Table 1. *Selected class II hybrids for bone tissue engineering applications*

Organic phase	Inorganic Phase	Coupling agents	Cells	Scaffold fabrication	Reference
Gelatin	SiO_2-$Ca(NO_3)_2$	GPTMS	MSCs	freeze-drying	(Mahony, Tsigkou et al. 2010)
Gelatin	SiO_2 - CaO	GPTMS	L929 mouse fibroblasts	microsphere leaching	(Lao, Dieudonné et al. 2016)
γPGA*	SiO_2	GPTMS	osteosarcoma	foaming	(Poologasundarampillai, Ionescu et al. 2010, Poologasundarampillai, Yu et al. 2012)
chitosan	SiO_2	GPTMS	-	freeze-drying	(Shirosaki, Tsuru et al. 2009)
PEG	SiO_2	ICPTES	SAOS2	rapid prototyping	(Hendrikx, Kascholke et al. 2016)
PCL*, Poly(VP-co-TEVS)	borophosphosilicate glass	GPTMS	MC3T3-E1	compression molding, salt leaching	(Mondal 2018)

* poly-γ-glutamic acid (γPGA), polycaprolactone (PCL), N-vinylpyrrolidone (VP), triethoxyvinylsilane (TEVS)

Unquestionably the most common hybrid combination so far includes silica-gelatin hybrids prepared by sol-gel -route. (Ren, Tsuru et al. 2001, Mahony, Tsigkou et al. 2010, Mahony, Yue et al. 2014, Lao, Dieudonné et al. 2016). As seen from the Table 1, hybrids have been mainly synthesized by using TEOS as the inorganic sol gel precursor instead of melt-quenched BAG particles. However, many different organic polymers are found to be suitable to couple with a coupling agent, such as gelatin, chitosan and polycaprolactone (PCL).

However, due to the challenging nature of hybrid synthesis chemistry, and several issues in hybrid composition optimization, such as the choice of the coupling agent, there is still a long way to release the full potential of hybrid biomaterials.

4. RHEOLOGY

Rheology is the deformation and flow behaviour of materials. Literally the meaning of rheology is "*flow science*": the term *"rheology"* originates from the Greek word "*rheos",* meaning *"river*", *"flowing"* or "*streaming*". (Mezger 2012) It is dependent on the material's inner structure, the outside forces stressing the material, and finally the ambient conditions, such as surrounding temperature.

In the context of hybrid materials rheology is interesting due to the viscoelastic nature of the material. By studying rheological properties of hybrids, important information about its gelation as a function of time or temperature could be assessed. These properties are essential for example when designing bioinks for extrusion-based bioprinting, which is a popular scaffold preparation method. (Ozbolat, Hospodiuk 2016)

Rheological behaviour of a material can be classified as shown in Table 2 below: (Mezger 2012)

Table 2. *Classification of rheological behaviour*

LIQUIDS		**SOLIDS**	
ideally viscous	**viscoelastic**	**viscoelastic**	ideally elastic
flow behaviour	**flow behaviour**	**deformation behaviour**	deformation behaviour
flow/viscosity curves	creep tests, relaxation tests, oscillatory tests		

4.1 Oscillatory measurements and detection of gel point

Oscillation measurements, where the plate system oscillates instead of rotation, are used because, in this way, more viscous samples are not destroyed too easily and can be studied. In addition, with oscillation, it is possible to measure materials within their linear viscoelastic range (LVE), which indicates the range in which the test can be carried out without destroying the structure of the sample. (Murata 2012)

In the two-plate model the upper plate is moving, while the lower plate remains stationary. The sample is sandwiched between these plates to study its deformation behaviour (Fig. 15).

Figure 15. *Two-plate system with 20 mm diameter plate geometry*

Shear modulus G can be express as:

$$G = \frac{\tau}{\gamma} \qquad (1)$$

where τ is the shear stress and γ is the shear deformation / shear strain. Shear modulus G describes the material's strength or stiffness, and it is influenced by time and temperature. *Shear stress* can be defined as:

$$\tau = \frac{F}{A} \qquad (2)$$

where F is the shear force applied to stressed material, and A is the area of the upper plate. Unit of shear stress is [N/m^2] or [Pa]. *Shear deformation* can be defined as:

$$\gamma = \frac{s}{h} \qquad (3)$$

where s is the deflection path from rest to maximum deflection, and h is the distance between plates.

Oscillation frequency can be specified either as angular frequency ω in [rad/s] or as the frequency f in [Hz]. These two can be conversed to each other as follow:

$$\omega = 2\pi \cdot f \qquad (4)$$

Viscoelastic material can be described by 1) their storage modulus and 2) their loss modulus.

Complex shear modulus G^* [Pa] is used since values that are determined in harmonic periodic fashion in sinusoidal processes like oscillation are written in complex form:

$$G^* = \frac{\tau_A}{\gamma_A} \qquad (5)$$

Storage modulus G' (G prime) stands for the stored deformation energy by the sample during deformation process, such as shearing. Materials which store deformation energy ultimately stay in unchanged shape after a load cycle. Therefore, G' measures the elastic behaviour of the sample.

Loss modulus G'' (G double prime) measures the lost deformation energy during deformation. In other words, the structure of the material changes, and energy is spent during the process. Materials that behave that way include samples that flow either partially or completely. With flow there is relative motion between the units of the structure, which causes frictional forces between the components. Ultimately, frictional heat is created. A part of this heat energy heats up the sample, and another part may be lost in the form of heat to the surrounding environment. Irreversible deformation behaviour occurs, and therefore, G'' measures the viscous behaviour of the sample. (Mezger 2012, Murata 2012)

The relationship between G*, G' and G'' using phase-shift angle δ can be seen in Figure 16:

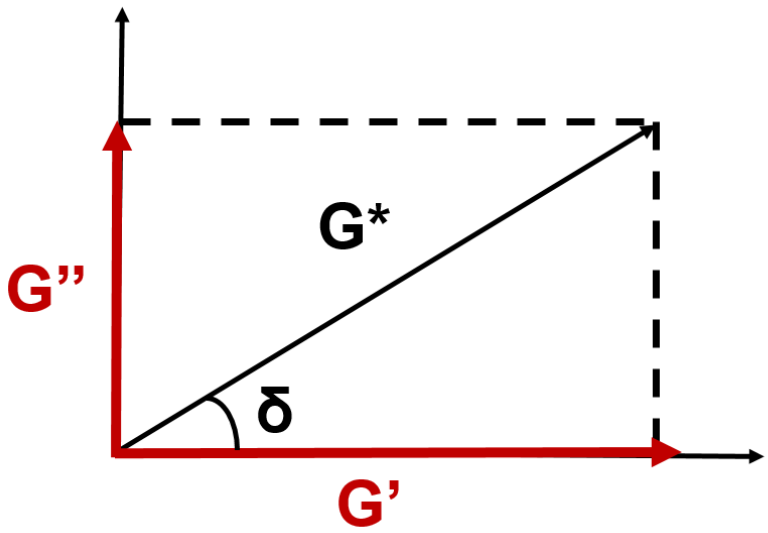


Figure 16. *The relationship between G*, G' and G'' using phase-shift angle δ*

From this figure we get:

$$\tan\delta = \frac{G''}{G'} \qquad (6)$$

This is referred to as the loss factor, which is a measure of the lost and stored deformation energy. This way the ratio of the viscous and elastic portion of the viscoelastic deformation behaviour can be defined. For instance, ideal elastic behaviour happens when $\tan\delta = 0$, and G' dominates G''. Correspondingly, ideally viscous behaviour is expressed as $\tan\delta \to \infty$, where G'' completely takes over G'. (Mezger 2012)

In the case of gel formation, hardening and curing processes, sol/gel transition point (gel point) is reached when $\tan\delta = 1$, and the ratio of G' and G'' is the same. (Fig. 17)

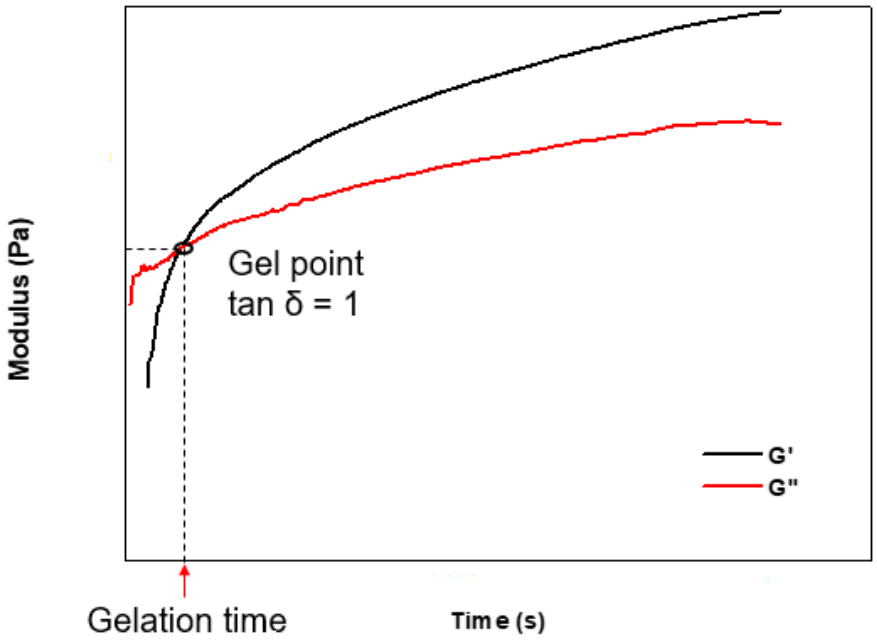


Figure 17. *Sol/gel transition point (gel point)*

In general, the relationship between G' and G'' is summarized below:

- G' < G'', viscoelastic liquids
- G' = G'', sol-gel transition point, gel point
- G' > G'', viscoelastic solids

4.2 Temperature-dependent flow behaviour

The effect of temperature on the flow and deformation behaviour of measured sample can be assessed with rheological temperature ramp measurements. In these measurements, viscosity η is determined as a function of the temperature. For example, it is possible to investigate the softening/melting temperature, or solidification temperature of the sample by this type of rheological measurement. Viscosity can be defined for ideally viscous liquids at a constant temperature as:

$$\eta = \frac{\tau}{\gamma} \qquad (7)$$

where τ is the shear stress, and γ is the corresponding shear rate. Unit of viscosity is [Pas] (Pascal seconds, 1 Ns/m^2).

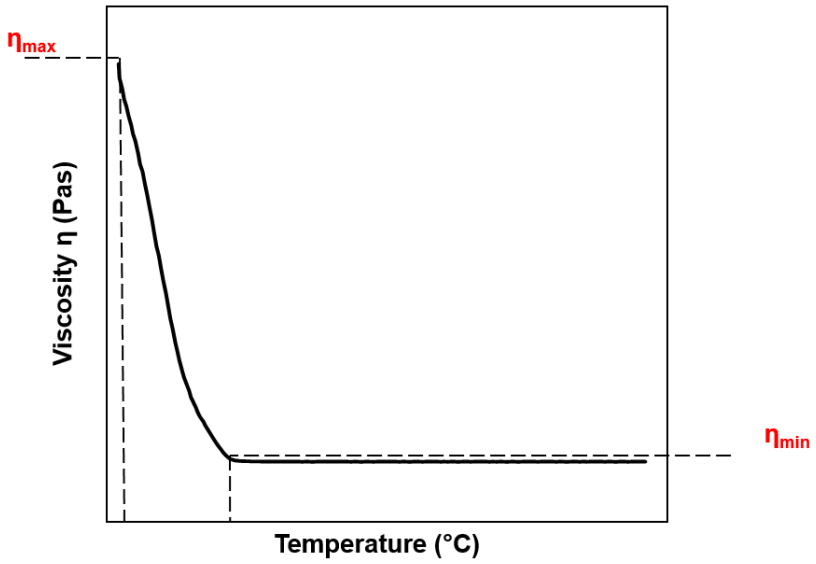


Figure 18. *Viscosity as a function of temperature*

As seen in Figure 18, the temperature is commonly represented on a linear scale on the x-axis of η(T) –diagram, having viscosity as y-axis either on linear or logarithmic scale, depending on the range of viscosity values measured. η_{min} shows the viscosity minimum and it is called softening or melting temperature. η_{min} shows the viscosity maximum usually giving information about the crystallization or freezing point of the sample. (Mezger 2012)

5. MATERIALS AND METHODS

The objective of the study was to synthesize two different bioactive glass/gelatin hybrid materials and characterize their *in vitro* dissolution properties and biocompatibility using human bone marrow derived mesenchymal stem cells (hBMSCs).

The experimental part of the work was conducted as a cooperation between the Bioceramics, -glasses and –composites group, and the Adult Stem Cell group at Tampere University.

5.1 Materials

Two bioactive glass compositions were used in hybrid synthesis: commercially available S53P4 (BonAlive®) as a control and a borosilicate glass, based on the S53P4, where 12.5% of the SiO_2 was replaced with B_2O_3,and CaO was partly substituted with Sr and Mg (labelled “mix”/BMgSr) (Tainio 2016). The glasses were melted, in air, at 1400 and 1200 °C, respectively. They were they annealed at 520 and 500 °C respectively to remove residual stress and further crushed to obtained powder, using a ball mill. The particle size used was < 38 µm. Both BAGs were made at the laboratory of tissue engineering and biomaterials, and the full protocol can be found elsewhere (Tainio, Salazar et al. 2020). The compositions (in mol%) of both glass compositions are shown in Table 3 below:

Table 3. *Used bioactive glass compositions in mol-%*

	SiO_2	B_2O_3	CaO	Na_2O	P_2O5	MgO	SrO
mix	47.12	**6.73**	6.77	22.66	1.72	**5.00**	**10.00**
S53P4	53.85	-	21.77	22.66	1.72	-	-

In addition to bioactive glass, gelatin from porcine skin (Type A 300, Sigma) was used along with 3-Glycidyloxypropyl trimethoxysilane (GPTMS) (Sigma), and 0.01 M hydrochloric acid (HCl) diluted from 1M Titripur Reag. Ph Eur, Reag. USP (Sigma).

Hybrids were prepared by first dissolving gelatin into 0.01 M HCl at 50 mg/ml concentration, at 37 °C. GPTMS (v = 3.68 ml) and bioactive glass (m = 535,7 mg) were added simultaneously to match the C-factor 1000, and the final solution was left to rotate in a UVP Hybridizer Hybridization oven (Analytik Jena US LLC, California, USA) until gelation. The protocol is summarized in the Figure 19 below:

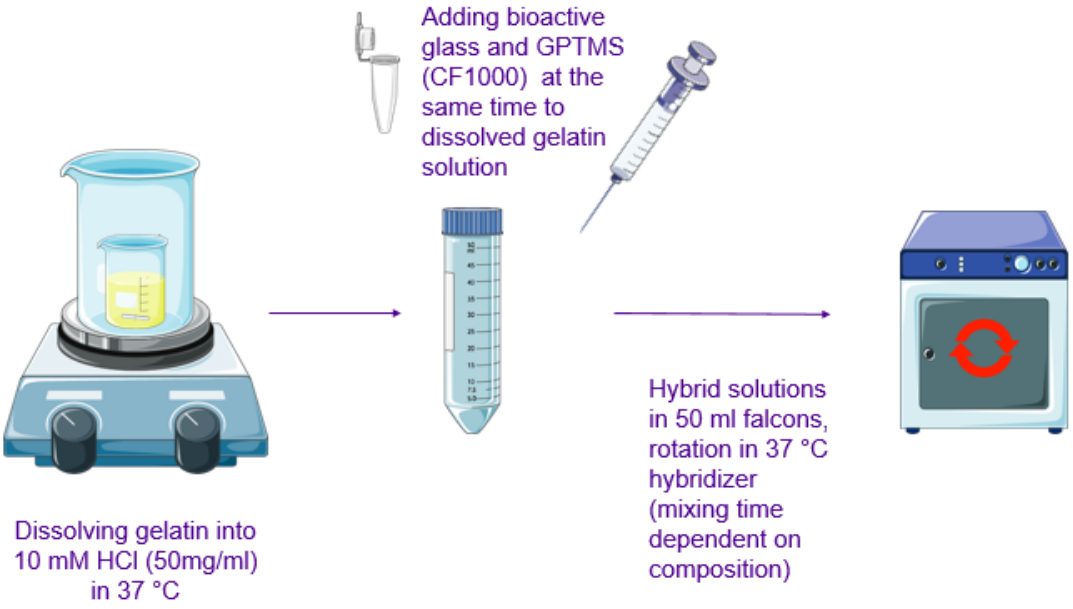


Figure 19. *Synthesis protocol for hybrids*

In this study the C-factor (CF), which is referred to as the molar ratio between GPTMS and gelatin ($\frac{GPTMS\ (mol)}{gelatin\ (mol)}$), was maintained constant at 1000.

Various weight ratios of gelatin and BAG were tested. The weight ratio was found to influence the gelation time. Times before gelation in the hybridizer are reported in the Table 4. Gelation time was assumed to be from the time of mixing all the reagents to complete gelation of hybrid into solid gel. For the composition 30/70 rheological properties were measured. In this case the hybrid gels were not let to completely gelate, but instead after visible increase of viscosity (transforming from liquid to honey-like consistency) rotation was stopped.

Table 4. *Gelation times for hybrids*

		Full gelation time	Rheological measurements
S53P4/gelatin	**30/70** **15/85** **5/95** **1/99**	~4h ~6h ~12h ~24h	~3h 30 min - - -
mix/gelatin	**30/70** **15/85** **5/95** **1/99**	~1h 30 min ~4h ~6h ~11h	~1h 15 min - - -

5.2 Methods

5.2.1 In vitro dissolution

Dissolution test for hybrid materials were performed in simulated body fluid (SBF), which has similar ion concentration to blood plasma (Table 5). SBF test is used to assess the ability of the material to induce precipitation of hydroxyapatite layer (bioactivity). In addition, hybrid dissolution was also tested in collagenase (enzymatic degradation) solution. SBF buffer solution was prepared following the protocol presented by Kokubo et al (Kokubo, Kushitani et al. 1990). Specific details about SBF preparation is presented in the Appendix.

Table 5. *Ion concentrations of blood plasma and SBF buffer solution (mmol/l) (Varila, Fagerlund et al. 2012)*

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5

Hybrids were synthesized for SBF dissolution test according to the protocol described earlier. The following day after synthesis gels were cut into small pieces ranging from 450 mg to 510 mg. Sample pieces were placed into 30 ml falcon tubes. The solution to mass ratio was kept constant at 1mL/35mg. The time points for SBF test included 24, 48, 72, 168, and 336 hours. All the measurements were done in triplicates except SBF controls without hybrid samples. SBF dissolution samples were kept between time points in Multitron AJ 188g (Infors, Bottmingen, Switzerland) shaking incubator at 37 °C and 100 RPM.

At each time point pH values of each SBF solution were measured at 37.00 ± 0.02 °C with Mettler Toledo SevenMulti MP 225 pH-meter (Mettler-Toledo International Inc., Greifensee, Switzerland) From the extracted solution the ion concentrations were analysed with Inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent Technologies, California, USA). Samples for ICP-OES were prepared by diluting the samples 10 times to 1M nitric acid (HNO_3). The analysed wavelengths are listed in the table below:

Table 6. *Wavelengths for ICP-OES*

	B	Ca	Mg	Na	P	Si	Sr
Wavelength (nm)	249.772	396.847	285.213	589.592	213.618	288.158	407.771

In addition, mass loss of the immersed hybrid pieces was calculated:

$$mass\ loss = \frac{m_0 - m_1}{m_0} \cdot 100\ \% \qquad (8)$$

Before weighing the sample pieces were washed with 96% ethanol to remove excess SBF and dried gently with paper.

The collagenase degradation test was conducted for 0.5h, 1h, 2h, 3h, 4h, 5h, and 6h. Hybrid gels were synthesized similarly as for the SBF dissolution test. The following day after synthesis gels where cut in small pieces (300-380 mg) in order to fit into 48 well plates. All measurements were done in triplicates, and between time points samples were left in 48 well plate in physiological temperature 37 °C inside incubator.

0.1% collagenase solution was prepared by mixing collagenase enzyme (from *Clostridium histolyticum*, type IA, crude, Sigma), TRIS-HCl (50 mM) and $CaCl_2$ (0,36 mM) into distilled water. After dissolving all the reagents, the pH value was adjusted to 7.4 at 37 °C by adding drops of 1M NaOH. Collagenase solution was added to wells with samples to match ratio of 1000 mg of sample per 3 ml. At every time point ICP-OES samples were prepared similarly as for the SBF samples. In addition, mass loss was calculated by using the equation (8) defined earlier.

5.2.2 Thermogravimetric analysis (TGA)

In order to analyse the glass and gelatin distribution of the hybrid materials thermogravimetric analysis (TGA) was conducted. STA 449 F1 Jupiter Simultaneous Thermal Analyzer (Netzsch Group, Selb, Germany) was used in measurements with Al_2O_3 crucibles. For both hybrids, small pieces of overnight stabilized gels were cut and left to dry in room temperature for 48 hours. In addition to time point 0 h samples, TGA analysis was also conducted to samples being immersed in SBF for 24, 48, 72, and 168 hours. Also, gels consisting of only gelatin and GPTMS were prepared and dried 24 hours, and then analysed. All measurements were done in triplicates. The TGA analysis

was conducted from room temperature to 600°C at 5K/min, in air. The organic content burns during heating and the inorganic glass part is left as residue.

5.2.3 Rheological measurements

In order to study the viscoelasticity of the hybrids rheological measurements were conducted. Rheological measurements were performed using Discovery Series Hybrid Rheometer 2 (TA Instruments, Delaware, USA) using 20 mm diameter parallel plate geometry.

Two different procedures were used: time sweep and temperature ramp. Timesweep measurements were performed at 37 °C for 1.5 h, with 20 mm diameter parallel plate geometry, and sample size of 350 µl. The mixing time inside hybridizer was 1 hour 15 minutes for mix hybrids, and 3 hours 30 minutes for S53P4 hybrids. These mixing times were chosen based on the visible viscosity increase of the hybrids, which is needed in order to be able to pipette samples and sandwich them between parallel plates. These mixing times were kept constant for each measurement. The moduli G' and G'' were detected at frequency of 1.0 Hz and 0.1% strain.

Temperature ramps were performed with temperatures ranging from 37 °C to 20 (18) °C, with 300s soak time at 37 °C, and ramp rate 0.5 °C /min. 20 mm diameter parallel plate geometry was used, with hybrid sample size of 350 µl. The samples for temperature ramps were prepared without adding coupling agent GPTMS, because it would cause gelation time-dependently, and therefore the real impact of the temperature on gelation would not be able to be studied. For this reason, 25 ml of 0.01 M HCl and 535,7 mg of BAG were mixed and rotated in hybridizer in 24 °C until visible increase of viscosity (1,5 hours for mix hybrid and 2 hours for S53P4 hybrid).

For all the measurements the upper plate of the parallel plate system was lowered until it was in contact with the sample surface and axial force on the instrument was 0.01 N. All measurements were conducted in triplicates.

5.2.4 Hybrid cytotoxicity testing

In the cell culturing part of this thesis work, the cells used were human bone marrow derived stem cells (hBMSCs), passages ranging from four to six. Used medium included 5% human serum (HS) and 1% of penicillin – streptomycin (P/S) antibiotics. The medium was sterile filtered using Stericup (Thermo Scientific Nalgene Rapid-Flow) before using in contact with cells to minimize the risk of contamination. Growth factor FGF-2 was added to culturing flasks (Biolite 75 cm^2 flasks and 25 cm^2 flasks, vented, Thermo Scientific) 5µl per 75cm^2 flask when expanding the cells. Adding FGF-2 has shown to enhance osteogenic potential, extend cells' lifespan and proliferative capacity. (Bianchi, Banfi et al. 2003, Martin, Muraglia et al. 1997) All the Live/Dead experiments were conducted in Nunclon delta surface (Thermo Scientific) wellplates.

All cell culturing and testing was performed in CellTech Laboratory and BMT laboratory at the Institute of Biosciences and Medical Technology (BioMediTech, Arvo building).

Cryopreserved human bone marrow derived mesenchymal stem cells (passages 4-6) were thawed according to instructions provided by Adult Stem Cell Group. Melted cell suspension was added into 5 ml of cold medium and centrifuged for 5 min 1000 rpm (Eppendorf centrifuge 5810 R, Eppendorf Ag, Germany). Cell pellet was then resuspended in 4 ml of warm medium and divided to two 75 cm^2 cell culturing flasks. (Flasks 2 ml + 8 ml medium inside). In addition, 5 µl of FGF-2 growth factor (10µg/ml) were added to both flasks. Flasks were left in the incubator at 37°C, and medium were changed every three days.

The general rule when culturing BMSCs is to seed 3000 cells per cm^2, which means approximately 225 000 cells per 75 cm^2 flask. When increase in proliferation rate is wanted, medium is changed only partially, and new FGF-2 is added only half the amount of usual (2.5 µl). It is also possible to slow down the proliferation by changing the whole medium.

When cells reach ~80% confluency, they were trypsinized (TrypLE Select 1X, ThermoFischer) according to given protocol, and counted using Bürker cell counting chamber. Seeding density was 25 000 cells per 1 cm^2 (per well in 48-well plate), so after counting cells were suspended to medium to match 25 000 cells/ml.

hBMSCs were seeded to 11mm diameter and ~3mm thickness cylinder-shaped samples (Fig 20).

Figure 20. *Hybrid sample for cell cultures, diameter 11 mm, height 3mm*

The hybrids for the cell samples were synthesized the way explained earlier, but all the reagents were sterile-filtered, and synthesis was conducted inside a laminar hood. Hybrids were rotated in hybridizer oven until preferred viscosity was reached, then they were casted into cut syringe moulds, and covered with autoclaved aluminium foil caps (Fig. 21). This way it was possible to ensure uniform size of the samples, and the samples were found to easily detach from syringes without breaking. For example, when casted directly to well plates, it was impossible to get samples out without breaking them.

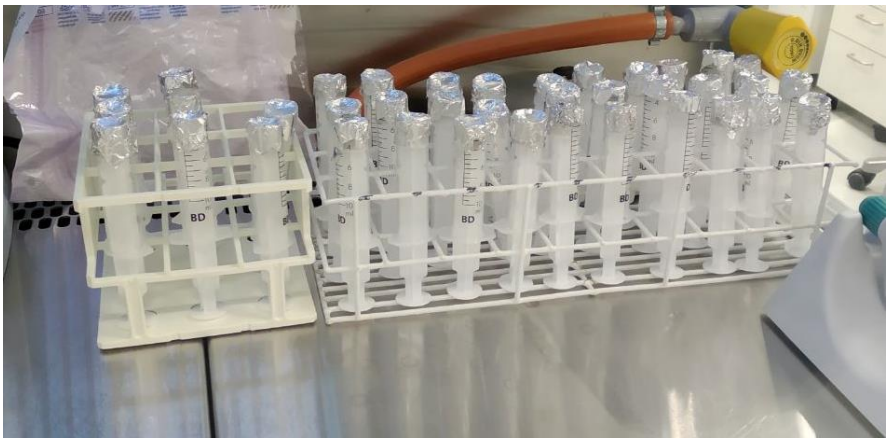


Figure 21. *Syringe moulds with autoclaved aluminium foil caps*

The moulds were let in room temperature inside laminar hood, and they were cut with 11mm diameter hollow puncher the next day.

Figure 22. *11mm diameter cylinder-shaped samples fit perfectly in 48 well plates*

Pre-incubation of the samples was performed in cell culturing medium for minimum 72 hours, in 37°C incubator. This way the initial burst release of ions can occur without cells, and it would wash away unreacted molecules.

Live/dead viability/cytotoxicity assay was used to detect cell proliferation and viability at time points 24h, 72h and 168h according to the instructions provided by the Adult Stem Cell Group. The Live/Dead working solution was prepared in concentration earlier optimized for well plates and bioactive glass scaffolds, summarized in Table 7 below:

Table 7. *The protocol to prepare Live/dead working solution*

Dye	Working concentration	Pipette	
		Dye	DPBS
EthD-1	0,25 µM	1,25 µl	10 ml
Calcein AM	0,50 µM	1,25 µl	

-20 °C stored dyes are warmed to room temperature and pipetted into Dulbecco's phosphate-buffered saline (DPBS). Wells to be analysed are first washed once with warm DPBS, and then working solution is added for 30-45 minutes. Wellplates are covered with an aluminium foil for protection against light. After incubation working solution is removed and DPBS added for visualization. Dead control is prepared by adding 1 % Triton-X to cells and incubating for 10 minutes in room temperature.

Microscopy of the stained samples was done using Olympus IX51 Inverted Fluorescence microscope, and by using the software DP Controller (2.2.1.227) and DP Manager

(2.2.1.195). (IX51, Olympus, Tokyo, Japan, equipped with a fluorescence unit and a camera DP30BW, Olympus). When visualizing the blue filter is for living cells stained by Calcein AM, and the green filter is for necrotic cells stained by EthD-1. Images were treated using ImageJ software. The overall cell culturing method is summarized below in Figure 23:

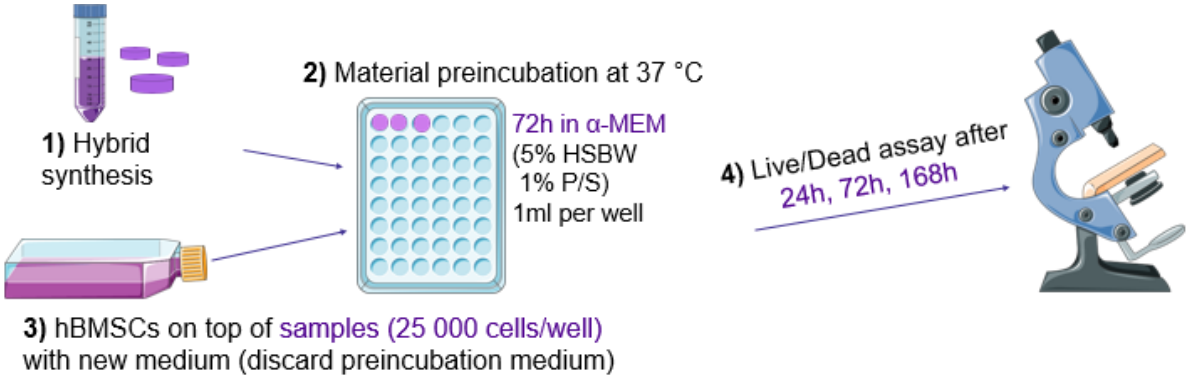


Figure 23. *Protocol for cell culturing, direct method*

Cells were cultured also using other method. In order to assess the effect of degradation byproducts from hybrids to cells, cells were cultured also in contact with preincubation medium without actual hybrid sample pieces. From pre-incubation immersion medium is collected from different time points and already seeded cells are cultured with this immersion medium. This is shown in the Figure 24:

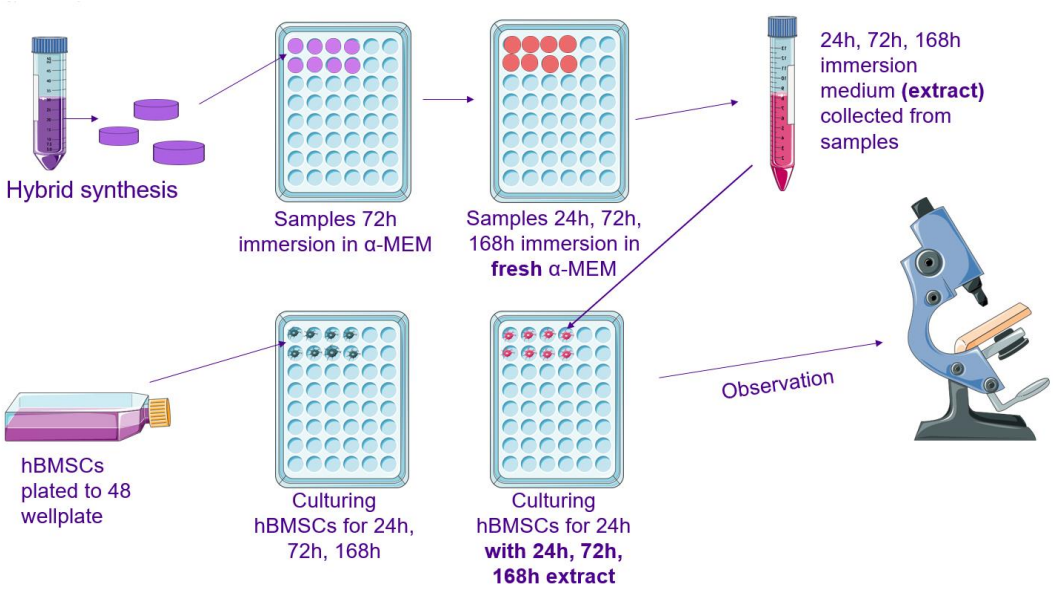


Figure 24. *Protocol for cell culturing, indirect method*

From both methods ICP- samples were collected in 15 ml falcon tubes and diluted to distilled water in ratio of 1:10. ICP samples were stored in freezer until ICP-OES analysis. The wavelengths presented in Table 6 earlier were also used for these ICP samples.

6. RESULTS

6.1 pH measurements

pH – values of hybrids during their synthesis were measured in order to assess pH value changes during the gelation process. Table 8 presents approximate pH values during hybrid synthesis process for both hybrids. All pH-values were measured at 37.00 ± 0.02 °C, except the measurements with pH-paper. Due to different gelation times and changes in viscosity of hybrid solutions pH values were measured at different times.

Table 8. *pH-values of 30/70 hybrids during synthesis*

		S53P4 30/70	mix 30/70
Directly after mixing	pH-paper pH meter	8 7.64 ± 0.02	9 8.54 ± 0.02
0.5h-1h in rotation, no visible change in viscosity	pH paper pH meter	8 8.29 ± 0.02	10 9.07 ± 0.02
Increasing viscosity	pH-paper pH meter	9 8.56 ± 0.02	10 9.43 ± 0.02
After overnight stabilization (fully gelated)	pH paper pH meter	9 -	10 -

For both hybrids, the pH value was observed to increase during synthesis. For mix (borosilicate) hybrid pH values were slightly higher. However, due to increasing viscosity of hybrids in synthesis there is no information whether the pH keeps rising during and after gel point.

6.2 Thermogravimetric (TGA) analysis

TGA analysis was conducted to assess the hybrid resistance to thermal decomposition and to evidence successful grafting of GPTMS to gelatin and BAG. Thermal decomposition of BAG/gelatin hybrid consists of two steps: first the decomposition of organic gelatin in lower temperatures, and finally of BAG in very high temperatures.

Organic-inorganic crosslinking would result in higher resistance to thermal decomposition. (Lao, Dieudonné et al. 2016) In our case, only decomposition of gelatin was assessed, and therefore temperature was raised only until 600 °C. This way we can assess whether our hybrids in reality display the theoretical 30/70 weight ratio between bioactive glass and gelatin.

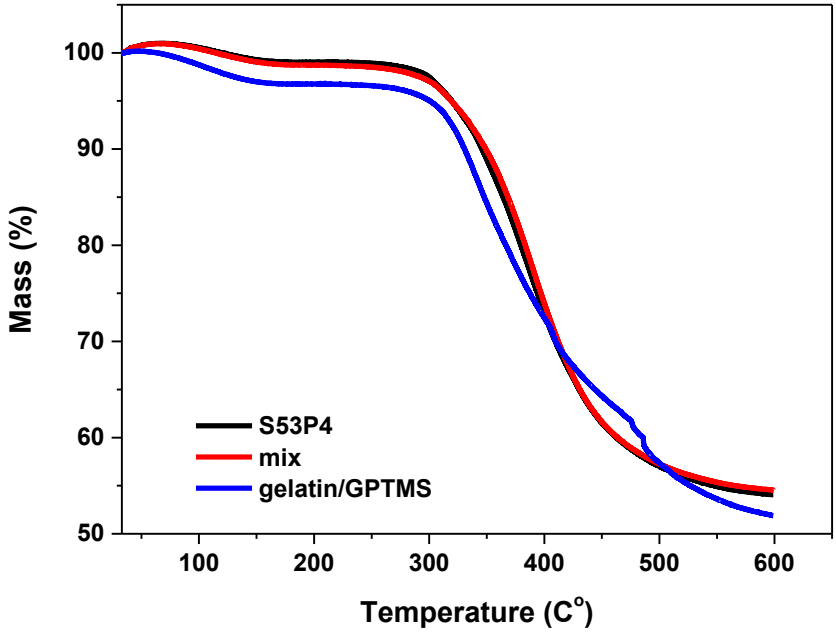


Figure 25. *Residual mass of S53P4 & borosilicate (mix) hybrids compared to gelatin/GPTMS*

Residual masses of both hybrids and gel made of only gelatin and GPTMS are shown in Figure 25. Higher residual mass for hybrids indicate higher resistance towards dissolution and slightly improved stability. However, differences are relatively small.

For triplicates combined values are displayed in Table 9 below using standard deviation to calculate error:

Table 9. *Residual masses of S53P4 and mix hybrids*

	Residual mass (%) at 600 °C
Gelatin & GPTMS	51.89
S53P4	54.73 ± 1.24
mix	53.44 ± 1.22

By using the measured residual masses, it is possible to calculate back and estimate the weight ratio between BAG, gelatin and GPTMS.

$$\textit{residual mass at 600 °C, gelatin \& GPTMS}(1 - \boldsymbol{x}) + \boldsymbol{x} = \textit{residual mass at 600 °C (hybrid)}$$

$\boldsymbol{x}$ = *percentage of BAG remaining*

Using this formula percentages of S53P4 and mix can be calculated:

- S53P4 ~6%
- B12,5-Mg5-Sr10 ~2%

The calculated values represent the hybrid composition quite accurately. Because the theoretical composition of 30/70 wt-% between BAG and gelatin didn't take account the mass of added GPTMS, the real amount of BAG is much less than theoretical 30 %. The density of GPTMS being 1.07 g/ml at 25 °C, it can be estimated that the more realistic weight ratio would be ~69% of GPTMS, ~22% of gelatin, and ~9% of BAG.

6.3 In vitro dissolution

From the SBF dissolution firstly the pH changes of different hybrid compositions were studied (Fig 26). Error stated by the pH- meter manufacturer (± 0.02) was used, unless the error measured from triplicates was bigger. In that case, the error was stated as the standard deviations of the triplicates.

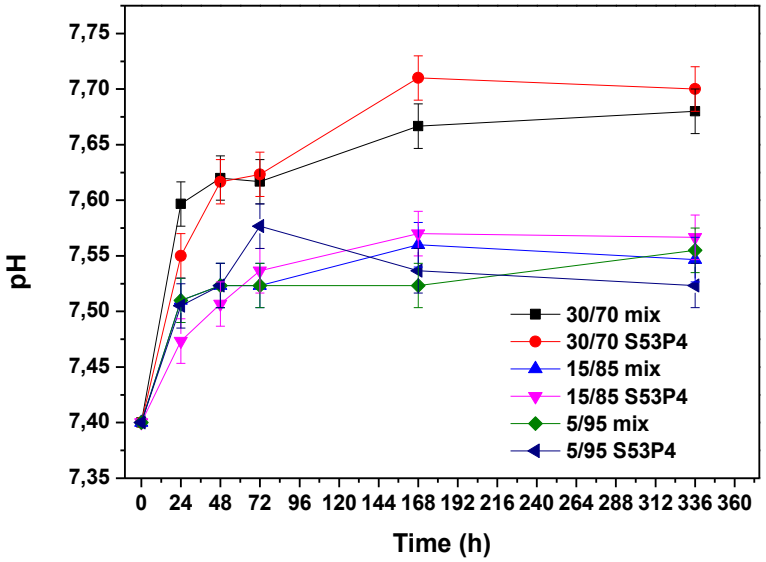


Figure 26. *pH value changes in SBF immersion for 2 weeks*

As seen in Figure 26, in general, higher glass content leads to higher pH increase. Clearly for 30/70 hybrids pH increased the most, while other compositions containing less BAG showed more subtle pH increase. pH can be seen to increase most rapidly during the first 72 hours in SBF immersion, and to stabilize at 2 weeks of immersion.

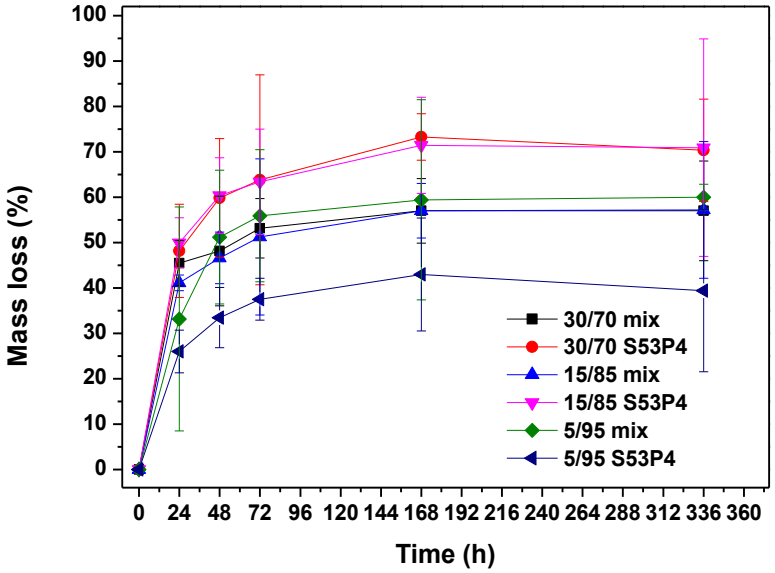


Figure 27. *Mass loss (%) upon dissolution in SBF for up to 2 weeks*

As seen in Figure 27, all hybrids showed mass loss upon immersion in SBF. Regardless of the glass/gelatin ratio the mass loss was rapid for up to 24h, then slowed down up to 72h and finally was fairly stable until 2 weeks. The mass loss was more pronounced in materials with higher glass content and more subtle for the materials containing less glass particles. The mass loss was also found to be significantly higher for hybrids containing the silicate glass compared to the one (with similar glass content) containing the borosilicate bioactive glass.

In addition to pH and mass loss, Si, B, P, Ca, Mg and Sr ion release patterns from hybrids were determined with ICP-OES measurement (Fig. 28-30).

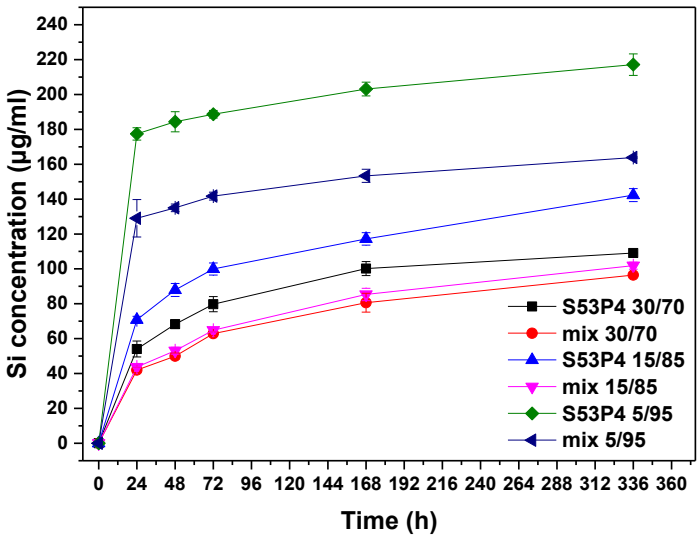


Figure 28. *Si concentration in SBF immersion as a function of immersion time*

All hybrid compositions show increasing amount of Si in solution as a function of immersion time. The quantity of released Si was higher in the case of silicate bioactive glasses than for the borosilicate counterpart. Furthermore, it is important to note that the Si release was highest for the 5/95 hybrids, and lowest for 30/70 hybrids. This indicates that the higher the glass content the lower the Si release in solution.

Figure 28 and 29 presents the Ca and P concentration, respectively, in the solution as a function of immersion time.

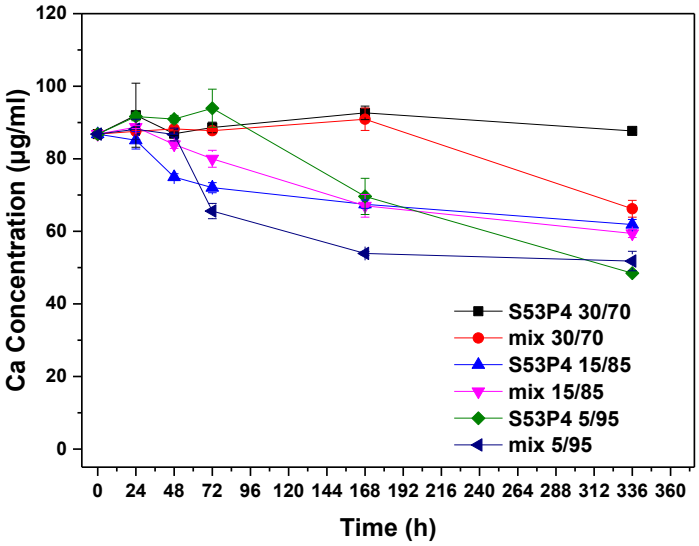


Figure 29. *Ca concentration in SBF immersion as a function of immersion time*

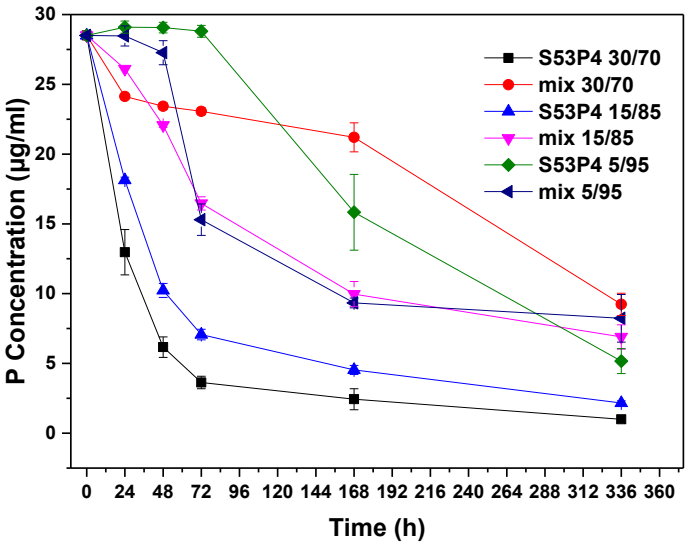


Figure 30. *P concentration in SBF immersion as a function of immersion time*

Before discussing the Ca release, it is important to remind that the mix glass contains Sr and Mg which were replaced for Ca. Therefore, in the mix glass the Ca content is significantly lower than for the S53P4 glass.

With increasing immersion time, the Ca content remained fairly constant in the case of the hybrid containing S53P4 in 30/70 ratio. The hybrid containing similar glass content

but of the borosilicate glass the Ca content decrease at 2 weeks of immersion. All other samples exhibit a decrease in the Ca content starting between 24 and 72 h of immersion depending on the materials compositions.

The P concentration, however, showed a significant decrease in concentration, for all materials with increasing immersion time, already at 24h of immersion, except for the 5/95 samples that exhibit a decrease in P at longer time. In general, the higher the glass content the more rapid the drop in P. Also, the silicate glass leads to faster decrease in P. For all hybrid compositions both Ca and P concentrations decreased in 2 weeks of SBF immersion, indicating HCA layer formation on BAG surface.

In addition, thermogravimetric analysis for post-SBF immersion hybrids was conducted. This would give information about the effect of SBF immersion on the stability of the hybrids. Figure 31 presents the TGA curves from hybrids made using mix glass, after synthesis (t=0h) and after immersion in SBF (t=24, 48, 72, 168h).

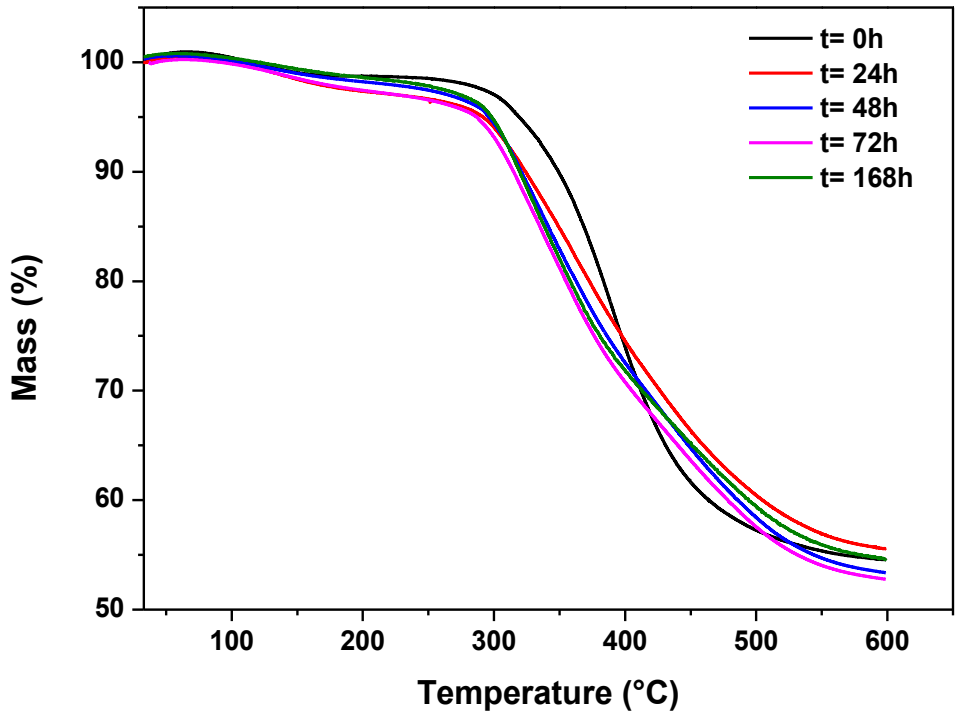


Figure 31. *Residual mass of borosilicate hybrids (mix) before (t=0h) and after SBF immersion (24h)*

First of all, once can notice that the shape of the curve, post immersion resembles more the TGA curve of the gelatin/GPTMS without bioactive glass presented in Figure 25.

For borosilicate hybrids it can be seen that hybrid before SBF immersion is slightly more stable than same hybrids after immersion. This is detected by observing the shape of the curve compared to later time points, mass loss upon heating is detected to be slightly slower. No big differences between the SBF immersion hybrids is detected, their behavior upon heating is very similar, with similar amount of residual mass after heating to maximum temperature.

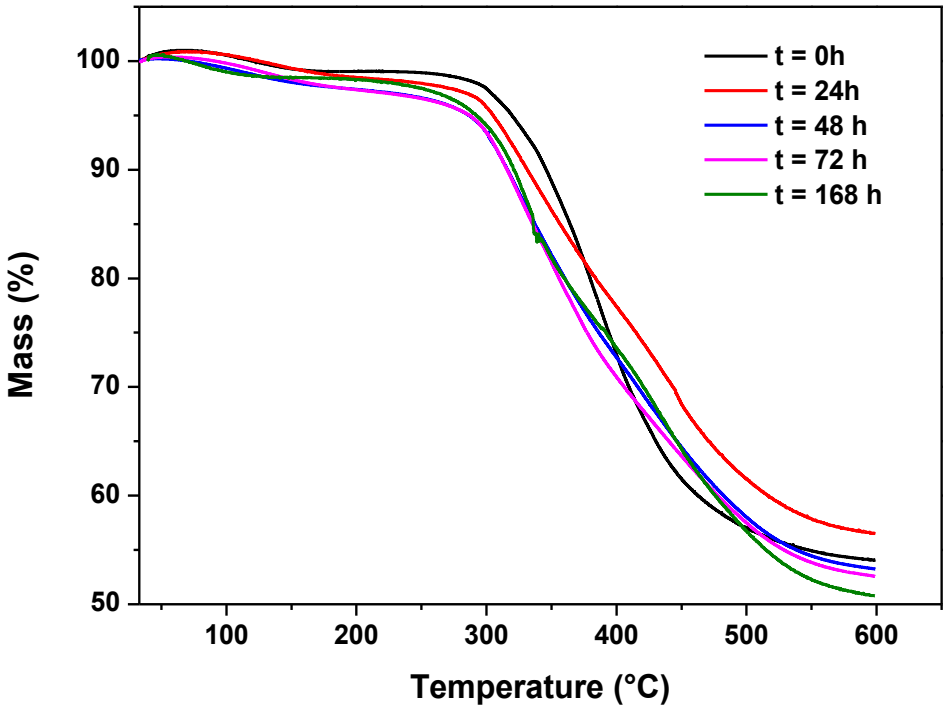


Figure 32. *Residual mass of S53P4 hybrids (mix) before (t=0h) and after SBF immersion (24h)*

As seen in Figure 32, S53P4 hybrids seem slightly more reactive in terms of the bioactive glass: the residual mass decreases upon SBF immersion time more than when compared to mix hybrids. S53P4 hybrids are probably also slightly more resistant against heating, because there is no as big difference between the behaviour of hybrid before and after SBF immersion.

For triplicates combined values are displayed in Table 10 below using standard deviation to calculate error:

Table 10. *Combined residual masses after SBF immersion*

	SBF immersion time (h)	Residual mass (%) at 600 °C
S53P4	24	59.96 ± 0.62
	48	50.70 ± 3.61
	72	51.11 ± 2.06
	168	50.26 ± 0.72
mix	24	55.42 ± 0.19
	48	53.77 ± 0.58
	72	53.64 ± 1.23
	168	54.25 ± 0.53

The residual masses are slightly larger for mix hybrids, but overall no big differences between two hybrids in terms of the residual mass as a function of immersion time. Overall, the residual mass decreases upon SBF immersion.

In vitro enzymatic studies are aimed at assessing the potential increased in stability, upon enzymatic attack, of the hybrids. In turn, this can give insight on the level of cross-linking between the organic and inorganic phase. Two different hybrid materials with same weight ratio of gelatin and glass (30/70) but different BAG, were studied to assess how hybrid composition alters the enzymatic degradation behaviour. This was analysed by measuring the mass loss of collagenase solution immersed hybrid samples, and by analysing their ion release by ICP-OES. Errors are stated as standard deviation, and all experiment is performed with three parallel samples.

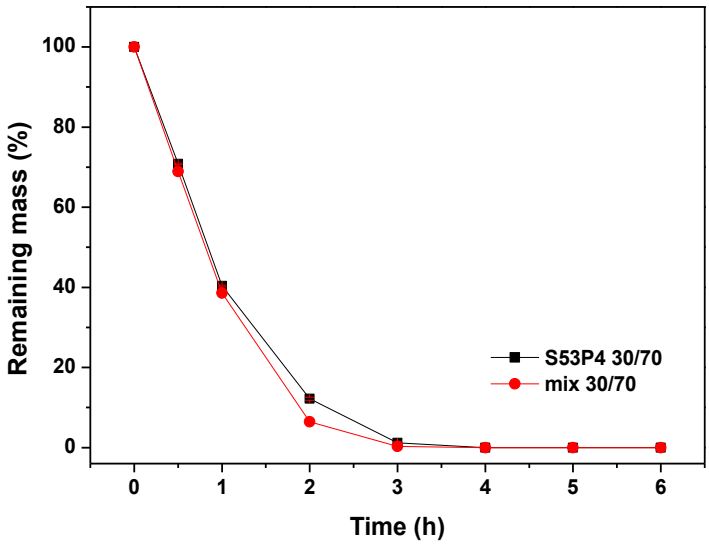


Figure 33. *Enzymatic degradation of hybrid materials by collagenase, mass loss in %*

Both hybrids degraded rapidly in collagenase solution immersion, as expected due to the high gelatin content of the hybrids. After one-hour immersion in collagenase solution mass loss of sample pieces were found to be over 50%. After three-hour immersion all samples were completely dissolved in collagenase solution. However, compared to plain gelatin, adding of GPTMS and BAG resulted in more stable gels.

From ICP-OES results (Fig. 34) it can be seen that the Si release increases until three hours for both hybrids, and after that all samples completely dissolved in solution. Clearly Si-O-Si bonds are undergoing destruction. In S53P4 composition there is slightly more Si, which explains higher Si release. Otherwise both hybrids have same C-factor, and both completely dissolved, so only reason for difference is the difference in glass composition.

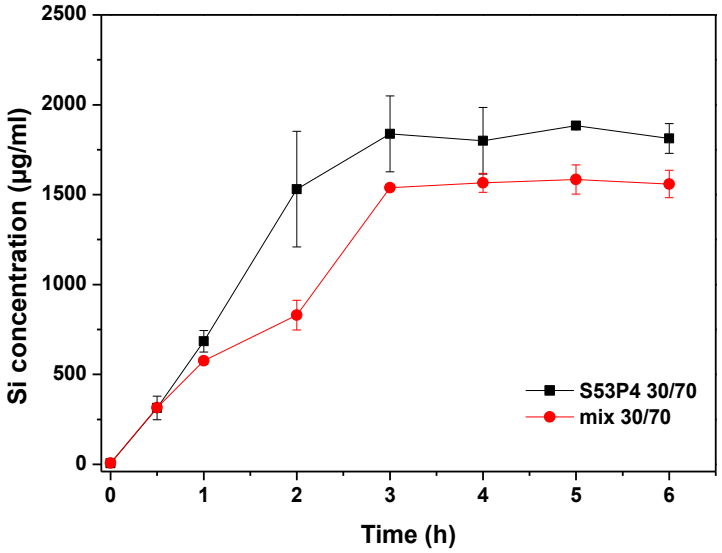


Figure 34. *Si release during enzymatic degradation*

Results for all ions individually are presented in Appendix.

6.4 Rheological properties

Rheological properties of hybrids were assessed in order to evaluate the gel point and other viscoelastic properties of the hybrids. Both hybrids in BAG/gelatin ratio 30/70 were

characterized through oscillatory time sweeps at 37 °C and through temperature ramps. Hybrids were mixed inside hybridizer until few minutes before expected gelation. Expected gelation time was possible to estimate from the visible increase in viscosity of the gels. Mixing time and the initiation time for rheological measurements was kept same for every measurement.

The G' and G'' moduli as a function of time for the 30/70 mix hybrid can be seen in Figure 35 below:

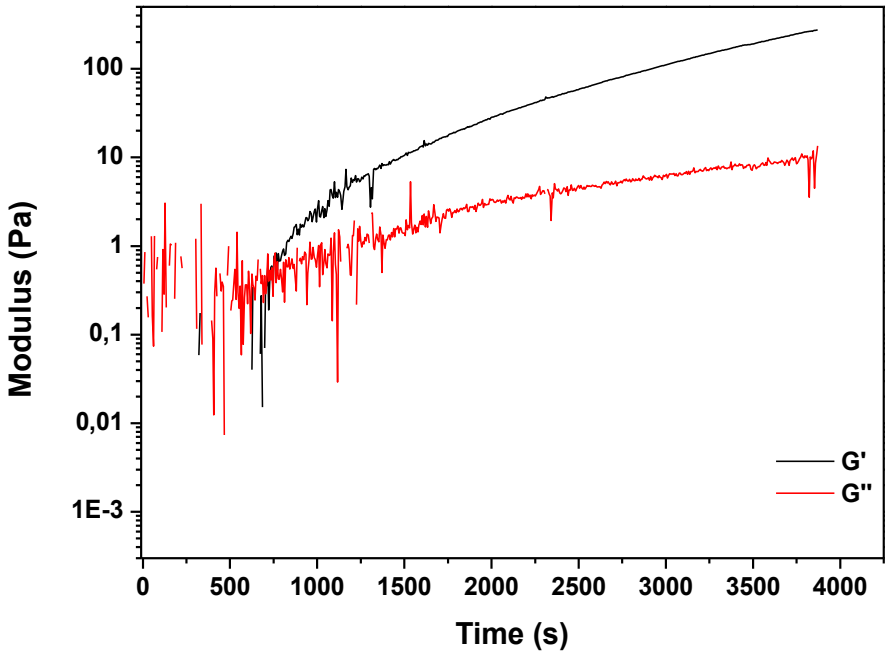


Figure 35. *G' and G'' as a function of time for 30/70 mix hybrid*

For borosilicate hybrid (mix) gelation point, where the moduli intersect, is detected approximately around 800 seconds. When the initial mixing time of 1h 15min is considered the overall gelation time for mix hybrid is estimated around 1h 30 min. Similar result is shown in Table 4. After gelation point both moduli can be seen to continue increasing.

The G' and G'' moduli as a function of time for the 30/70 S53P4 hybrid can be seen in Figure 36 below:

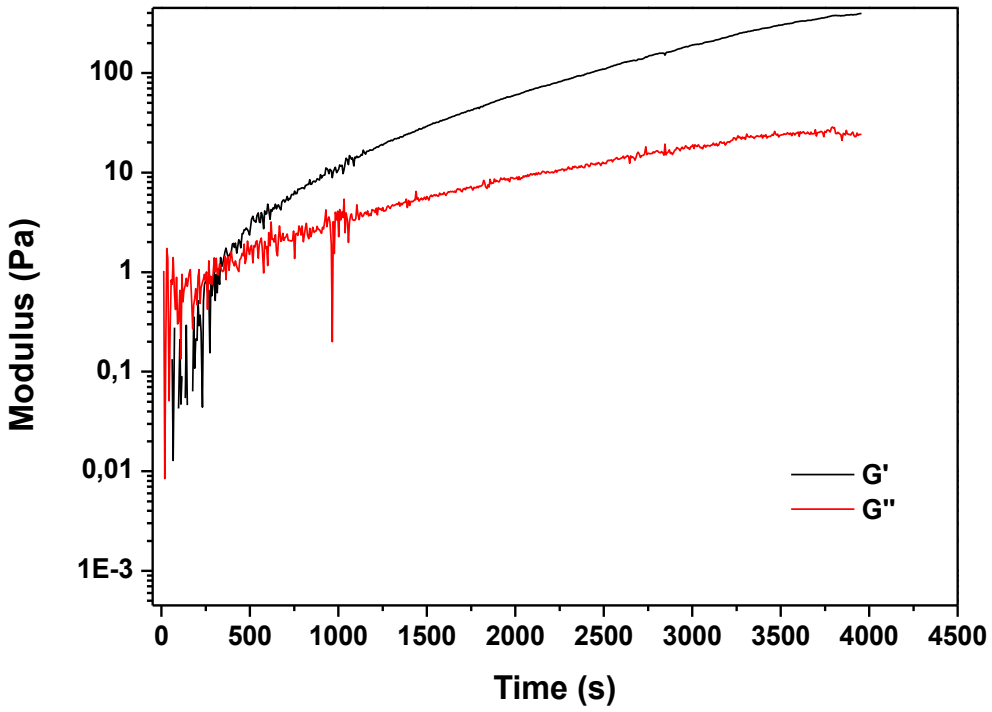


Figure 36. *G' and G'' as a function of time for 30/70 S53P4 hybrid*

For S53P4 hybrid similar behaviour in terms of gelation is detected. However, the time window between viscosity that is possible to use as time sweep sample, and full gelation point is slightly shorter than for borosilicate hybrid, less than 500 seconds. When the mixing time of 3h 30 min is considered, overall gelation time of approximately 3h 40 min is obtained. This slightly less than earlier measured 4h shown in Table 4. In addition, similarly than for mix hybrid, for S53P4 hybrid the both moduli keep increasing after the detected gelation point.

In addition to gelation point detection in means of time sweep measurements, temperature ramps for both hybrids without adding time dependent GPTMS are performed (Fig. 37). Gel containing only gelatin is used as a control.

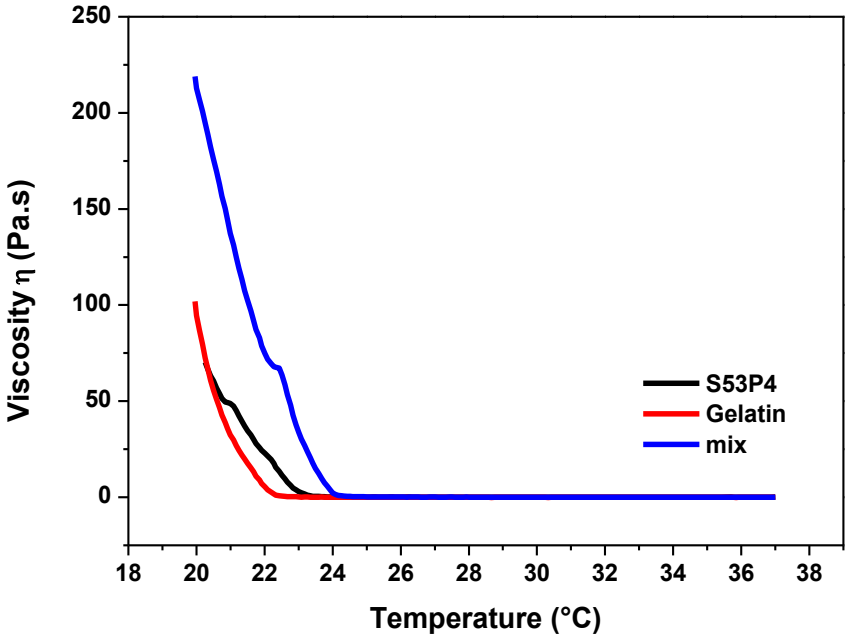


Figure 37. *Viscosity as a function of temperature for hybrids, gelatin as a control*

As seen in Figure 37, for all the materials the viscosity increases as a function of decreasing temperature. This indicates the gels becoming more solid in lower temperatures. S53P4 hybrid follows similar pattern in viscosity increase as a function of decreasing temperature than control gelatin. For borosilicate hybrid the viscosity increase is steeper and occurs earlier in slightly higher temperature (~24 °C).

6.5 Hybrid cytotoxicity

The biocompatibility of hybrid materials was assessed by culturing human bone marrow-derived stem cells (hBMSCs) in contact with hybrid materials. In addition, hBMSCs were cultured with hybrid extract, without direct contact with the samples.

Images from Live/Dead assay for 15/85, 5/95, and 1/99 wt-% compositions (BAG/gelatin) for both hybrids are seen below:

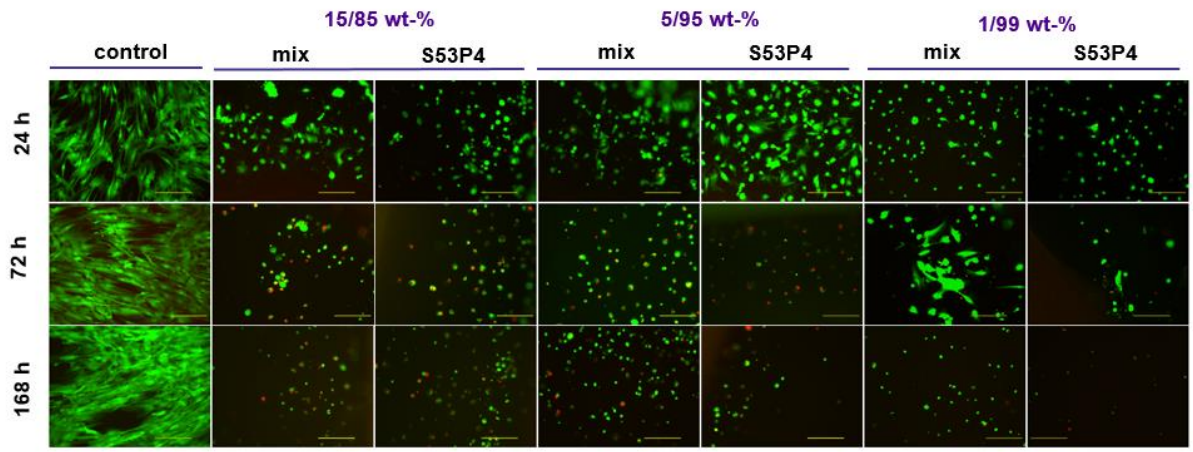


Figure 38. *hBMSC viability in contact with hybrid materials. Scale bar 200 µm.*

As seen in Figure 38, after 24h of culturing some elongated and attached cells can be seen for each composition, except for 1/99 wt% hybrids. However, after 72 hours, cell proliferation and attachment deteriorate rapidly, resulting in very low viability of hBMSCs. No big difference between the borosilicate or S53P4 glass is observed in terms of cell viability.

Similar results were received from imaging cells in contact with hybrid extracts, without contact with the material. Naturally when hBMSCs were already spread and attached to well plates, more viable cells are detected in images.

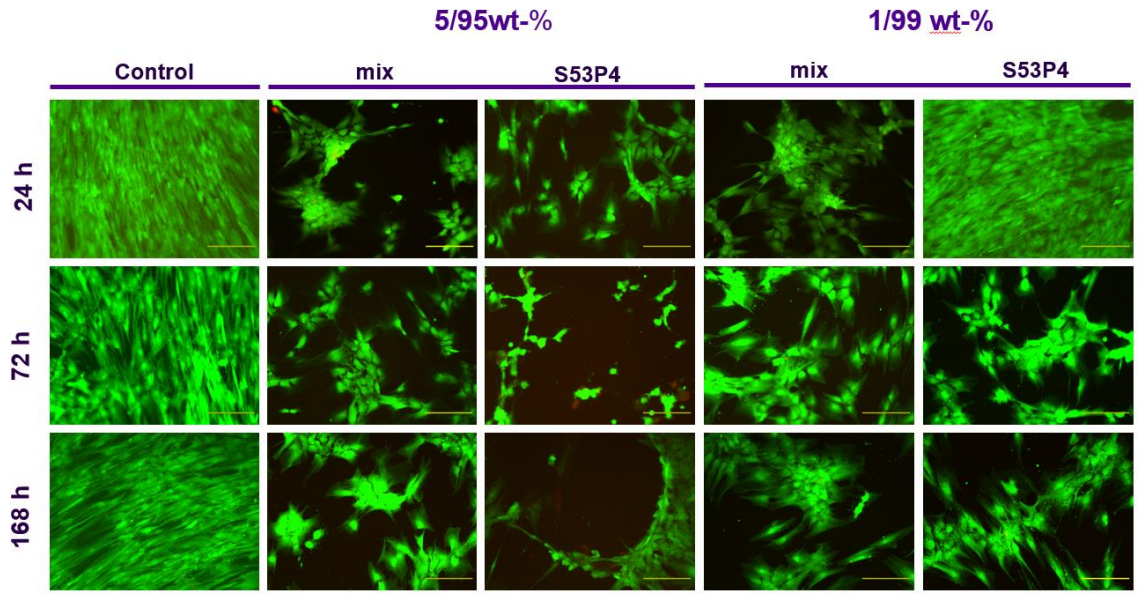


Figure 39. *hBMSC viability when cultured with hybrid extracts. Scale bar 200 µm.*

For all other composition than S53P34 1/99 wt-%, decrease in cell proliferation and attachment is detected already with 24h immersion extract. This is seen as changes in cell morphology and clear areas on the well plate, where attachment is lost.

In addition to Live/Dead assay, the ion release from hybrids during pre-incubation phase and during cell culturing when immersed in cell culturing medium was also studied with ICP-OES.

Table 11. *Ion concentrations (µg/ml) after 72h pre-incubation in medium*

	S53P4 15/85	mix 15/85	S53P4 5/95	mix 5/95	S53P4 1/99	mix 1/99
B	-	41.23 ± 3.27	-	11.17 ± 2.23	-	4.20± 0.50
Ca	147.40 ± 20.85	66.53 ± 11.14	58.17 ± 8.43	66.50 ± 9.20	64.73 ± 4.13	84.25 ± 0.49
Mg	14.03 ± 2.25	40.70 ± 2.00	13.70 ± 3.50	23.87 ± 2.12	13.50 ± 2.50	18.34 ± 1.85
P	10.97 ± 0.81	14.90 ± 5.67	17.97 ± 1.33	33.60 ± 3.63	30.53 ± 1.70	37.03 ± 6.44
Si	483.12 ± 29.44	302.40 ± 25.32	576.37 ± 200.97	453.40 ± 79.97	1043.90 ± 22.34	766.20 ± 34.65
Sr	-	166.53 ± 7.18	-	64.63 ± 15.73	-	23.87± 2.54

Overall the measured ion concentrations after 72h pre-incubation are much higher than ion concentrations measured from cell cultures after pre-incubation (Fig. 40-44). Especially Si concentrations are much higher also when compared to ICP-OES results from SBF dissolution test (Fig. 28)

ICP-OES results from cell cultures at certain time points are shown below: (Fig. 40-44).

.

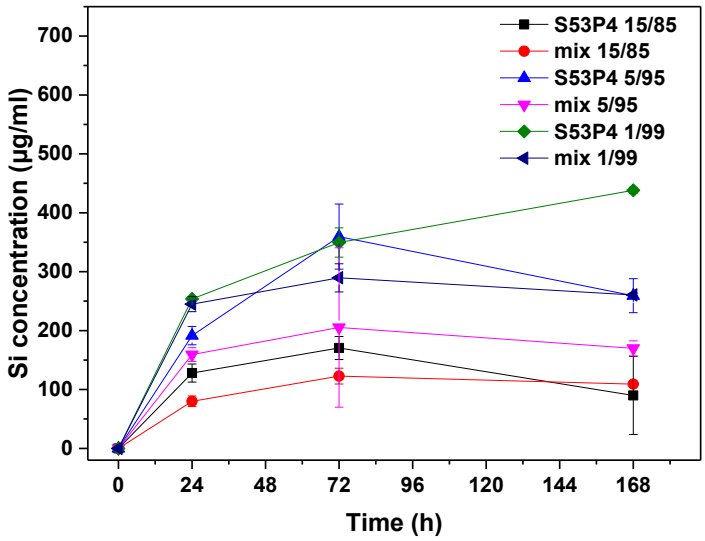


Figure 40. *Si concentration as a function of medium immersion time*

As seen in Figure 40, highest Si release was detected for 1/99 hybrids. Also during 72h pre-incubation, 1/99 hybrids possess the highest Si concentrations. In general, the Si concentration can be seen to increase with decreasing glass content of the hybrid.

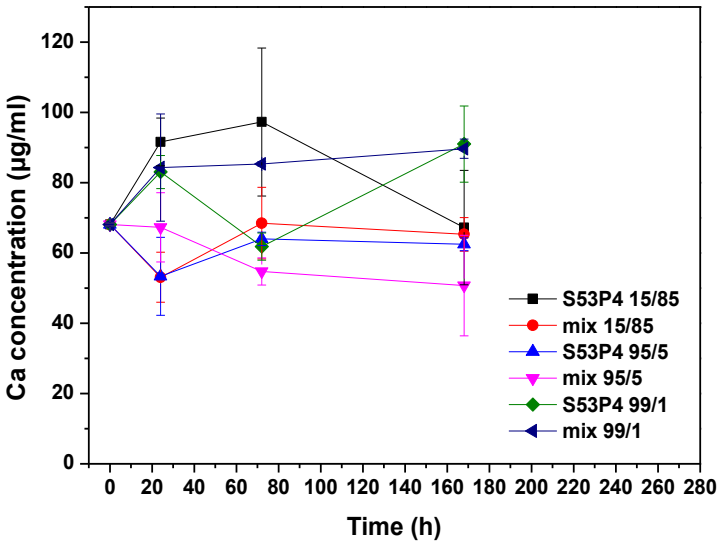


Figure 41. *Ca concentration as a function of medium immersion time*

As seen in Figure 41, Ca concentration is seen to stay quite constant upon medium immersion without big differences between hybrids.

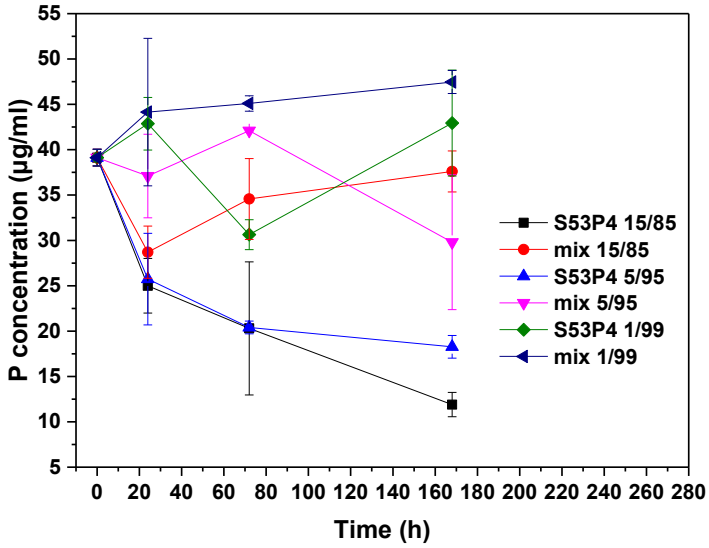


Figure 42. *P concentration as a function of medium immersion time*

As seen in Figure 42, P concentration is decreasing upon immersion quite drastically, which would suggest high reactivity and rapid HA layer precipitation.

Boron and Strontium concentration from borosilicate hybrids is seen below:

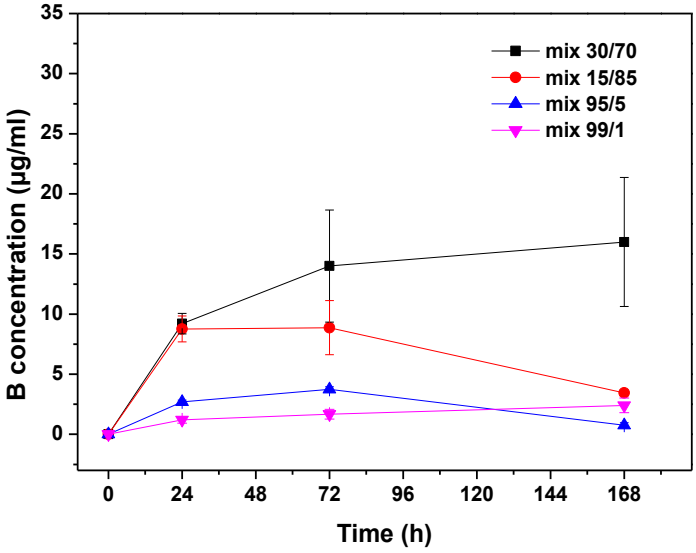


Figure 43. *B concentration as a function of medium immersion time*

When comparing different mix hybrid compositions, B concentration depends unsurprisingly on the BAG content: the higher the BAG content, the higher in the release of B.

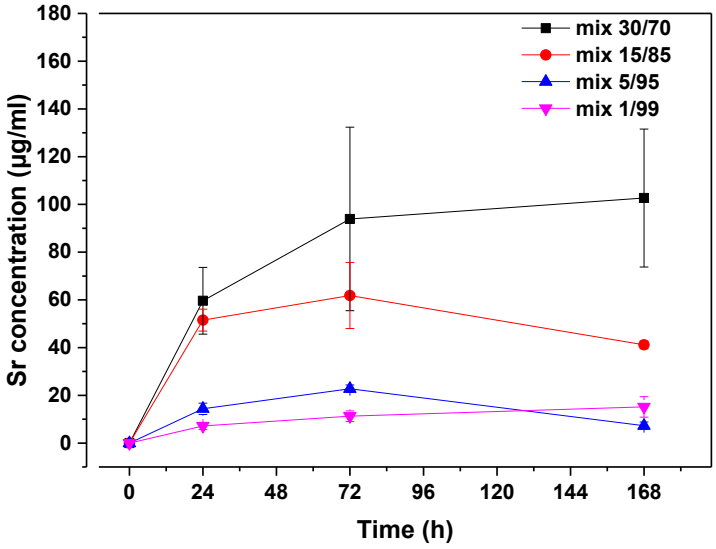


Figure 44. *Sr concentration as a function of medium immersion time*

Similar trend for Sr ions is seen as for B ions: higher Bag content indicates higher release of Sr ions.

Rest of ICP-results for each element individually are found in the Appendix.

7. DISCUSSION

7.1 pH measurements

Assessing how the different hybrid samples affect the pH-value of surrounding solution is important in order to determine whether material would be suitable for cell culturing. The transient local microenvironment due to scaffold resorption might cause big effects on cell proliferation and function. For example, Monfoulet et al. found that excessive alkalinisation (pH > 7.90) in the microenvironment of hBMSCs inhibits osteogenic differentiation (Monfoulet, Becquart et al. 2014)

Overall, both hybrids were found to show pH increase. Borosilicate –containing hybrids were found slightly more basic. The most logical explanation to this behaviour is the alkalinisation effect by bioactive glasses. Bioactive glasses consume protons during the ion exchange reactions, and release alkaline ions when resorbing, which increases the pH. In addition, during the gelation and hybrid synthesis process multiple not well-known chemical reactions occur between glass, gelatin and GPTMS, which might contribute to this pH increase. As discussed earlier it has been shown that the pH also affects to the hybrid coupling reactions. For instance, too acidic pH value during hybrid synthesis would lead to degradation of polymer, and too basic pH value would inhibit the epoxy ring opening of GPTMS. (Gabrielli, Russo et al. 2013)

However, measuring pH using pH paper would give only a rough idea of the pH value. In addition, pH meter available has quite wide error of measurements (± 0.02). When synthesizing hybrids, gelation point and complete transition from viscous liquid to viscous solid can be seen. This limits the possibilities to measure pH value of gelating hybrid solution. In addition, pH values were measured only from composition with 30/70 wt-% between glass and gelatin. Studying the pH-value changes for other compositions would have given valuable information about the level of pH during hybrid synthesis.

7.2 Thermogravimetric (TGA) analysis

TGA analysis can be used to evidence successful grafting of GPTMS to both gelatin and bioactive glass. Also, in order to calculate the actual organic-inorganic ratio in the hybrid materials the results of TGA analysis are useful.

As seen in the calculations of the actual ratios between the organic and inorganic phases, the 30/70 hybrids are quite accurately representing the theoretical ratios. However, when designing our hybrid compositions, it would have been useful to take the weight of the GPTMS into account as well. Now in the results the residual mass represents the inorganic part of GPTMS and the glass, while all the organic material is burned away. Because over half of the weight of our hybrids actually comes from the GPTMS, now the labeling 30/70, 15/85, and so on only based on the ratio gelatin and BAGs is slightly misleading.

Several other groups have made similar measurements earlier to hybrid materials. (Greenhalgh, Ambler et al. 2017, Lao, Dieudonné et al. 2016, Ghorbani, Zamanian et al. 2018, Mondal 2018) Mondal measured for similar weight ratio 30/70 between inorganic and organic coupled with GPTMS residual mass ~55%, which is quite similar to our results. Furthermore, the curve shapes displayed similar behavior than in our results. The highest drop is observed between 300-400 °C, and the overall shape of the curve is very steep.

7.3 In vitro dissolution

For this study, dissolution was analysed by the means of SBF bioactivity measurement and enzymatic degradation study by collagenase. *In vitro* characteristics of BAGs, such as their dissolution rate and conversion to HA layer, are dependent primarily on glass composition and the microstructure of scaffold. (Rahaman, Day et al. 2011) The bioactivity and dissolution of BAGs is commonly studied by SBF immersion. In the case of hybrid materials, also the organic part with the coupling agent, and the range of covalent bonding between them plays an important role to determine the dissolution behaviour of the whole hybrid material.

Mass loss of the hybrid samples measured upon immersion provides a measure of their degradation rate. Usually biomaterial scaffold is designed to degrade at same rate than tissue formation occurs at the implantation site. Benefit of degradable scaffold is that no foreign material stays in the body, biomaterial slowly disappears, and ultimately newly formed tissue fills out the space. (Rahaman, Day et al. 2011)

The mass loss was found to be significantly higher for hybrids containing the silicate glass compared to the one (with similar glass content) containing the borosilicate bioactive glass. This is due to the dissolution of the samples, but probably also due to the washing effect of unreacted compounds. Also the higher mass loss for hybrid with

higher BAG content indicates that there are probably more free particles not grafted with GPTMS dissolving into the solution. However, the mass loss might be partially compensated by the HA precipitation.

Detected increase in pH is due to the release of alkali metal (Na), and alkaline earth metal (Mg, Ca, Sr) ions, and due to the consumption of protons from the solution. This type of behaviour is typical in *in vitro* dissolution of both silicate and borosilicate bioactive glasses. When they dissolve, ions from the glass network are released to the solution. Contrary to pH results done during hybrid synthesis, in SBF immersion silicate glass S53P4 containing hybrids expressed higher pH than borosilicate hybrids.

Similar results have been observed from earlier SBF dissolution studies made with same composition borosilicate glass. As shown before for same borosilicate B12.5 –Mg 5 –Sr 10, also when this glass is a part of hybrids, it leads to pH increase in SBF immersion. (Tainio 2016, Tainio, Salazar et al. 2020) In the case of mass loss results, in earlier studies alginate silica hybrids with highest GPTMS content have been found to display highest mass loss for hybrids. (Vueva, Connell et al. 2018) In our case mass loss was found to be quite drastic for all hybrid samples. However, the impact of the GPTMS amount to mass loss is difficult to see because the same C factor was used for all hybrid samples.

Si ions released by glass dissolution are known to play an essential role in bone formation. The concentration of Si ions increases upon SBF immersion as a function of time due to breaking of silica layers in the glass network. However, since our hybrids also included Si-containing coupling agent GPTMS, at least part of Si ion release comes from the GPTMS as well. Especially in the case of hybrids with less BAG, and therefore having fewer binding sites for GPTMS, the Si release is highest. This indicates the release of unreacted GPTMS molecules contributing to increasing Si concentration upon SBF immersion.

HCA layer involves formation of silica-rich layer that act as nucleation site for CaP layer. Furthermore, the decrease of P concentration suggests CaP layer formation. From our results decrease of the concentrations of Ca and P ions is seen to all hybrids, which might indicate CaP layer formation. However, in order to ensure that, for example SEM images of the hybrid surfaces would give valuable information.

However, the accuracy of SBF as a representative of *in vivo* conditions has been questioned (Kokubo, Takadama 2006, Bohner, Lemaitre 2009). SBF itself contains both calcium and phosphate ions, so therefore, in SBF solution hydroxyapatite can form solely from the solution. (Varila, Fagerlund et al. 2012) Especially issues when measuring Ca

and P concentrations might occur since SBF is already supersaturated towards the precipitation of HA.

In addition, by comparing the thermogravimetric (TGA) results from samples before and after SBF immersion it is possible to get an idea of the possible changes in hybrid composition and stability upon immersion. However, the interpretation of these results is challenging due to the ungrafted GPTMS releasing into the solution upon SBF immersion. Therefore, the residual consists not only of BAG left but also the attached GPTMS. In addition to remained GPTMS and BAG, possible HA precipitation is also contributing to the residual, making the interpretation even more challenging.

If gelatin is not properly functionalized by GPTMS, and GPTMS not linked to BAG phase, rapid and uncontrolled dissolution can occur, indicating instability of the hybrid. Covalent coupling is necessary to control the rate of degradation and mechanical properties of hybrid materials. Some idea of the degradation behaviour mimicking in vivo conditions can be assessed by using collagenase enzymatic degradation assay. However, in vitro degradation assays will always fail to completely imitate the complexity of in vivo context, where various cells and enzymes are present. Based on our enzymatic degradation results, very rapid and degradation of samples was detected for both hybrids with not much difference between them. However, if compared to gelatin alone, hybrids act as more stable gels, because they don’t dissolve to 37 °C solution immediately.

7.4 Rheological properties

Rheological properties of hybrids were assessed in order to evaluate the gel point and other viscoelastic properties of the hybrids. The rheological measurements were challenging to perform due to very narrow time window prior full gelation of the hybrids. It was possible to detect very quick transformation from transparent liquid to white and much less transparent viscous solution, and ultimately to a solid gel. This issue has been discussed previously with similar silica/gelatin hybrids (Nelson 2016). Also, due to observed shrinking of hybrids during their gelation, it was impossible to keep the axial force of the geometry positive after gelation point. If the axial force is lost (negative values), it indicates lost contact between the geometry and sample, leading to unreliable results.

Due to the time-responsivity of GPTMS crosslinking, it was impossible to assess the viscosity as a function of temperature without gelation at certain time point. In order to accurately determine whether temperature changes affected hybrid viscosity, all the

temperature ramp measurements were carried out without adding the coupling agent GPTMS. This way it was possible to exclude the time-dependent viscosity increase caused by GPTMS. However, therefore no information about the real hybrid gelation as a function of temperature was obtained. The results show only the effect of the different BAG on the viscosity of the gels in different temperatures.

In literature coupling agents are usually added afterwards due to the very fast gelation time also observed in this study. (Gao, Rahaman et al. 2013) Gao et al. also reported the use of GPTMS as coupling agent to lead to rapid formation of stiff gel, which was not suitable for bioprinting.

In order to use hybrids for example as a bioink, it would need to fulfil criteria such as sufficient rheological properties: it needs to be fluid enough to be able to pass through a nozzle but also retain three dimensional shape and not collapse as layers are added. This bioink developing and optimizing stage is very time consuming, since lots of rheological measurements and mathematical modelling is needed to calculate appropriate “window of printability”. (Paxton, Smolan et al. 2017) In our case, it was evident that due to the relatively narrow viscosity window for printing, this type of hybrid, at least in the test composition 30/70 with C-factor 1000, would not be very effective as a bioink.

7.5 Hybrid cytotoxicity

Glass composition influences its ability to support proliferation of cells *in vitro*. In the case of bone tissue engineering applications, hBMSCs are often chosen based on the rationale that they have been well established as an ideal source of cell-based therapy for bone tissue engineering applications. (Montoro, Wan et al. 2014) In order to determine whether the BAG/gelatin GPTMS-coupled hybrids would support hBMSC proliferation and attachment Live/Dead staining were performed to gain qualitative insight of the cell morphology, proliferation and attachment.

As indicated by Live/Dead assay results, it is clearly seen that the hybrid materials are inhibiting the proliferation and attachment of hBMSCs both when culturing in contact with the samples, and with hybrid dissolution product extracts indirectly. The most essential question to assess is whether this inhibition depends on the inorganic part of the hybrid, referring to the BAG, or on the organic content, referring to the gelatin functionalized with GPTMS.

In the case the inhibition is due to the BAGs, possible reasons include their too high reactivity. This would be shown as drastic pH changes, and high ion release levels, indicating rapid HA precipitation and BAG dissolution. In general, BAGs tend to induce alkalinization of the external medium, due to leaching of ions to surrounding aqueous solutions. Contrary to glass discs or other larger size particles, here <38 μm BAG particles were used in the hybrid samples. Small size particles have a large reactive surface area, which correspond to the reactivity of the BAGs. However, as shown in the SBF test, the reactivity of the used BAGs is probably not the main reason for the inhibition of the cell proliferation.

The effect of Boron on osteogenic differentiation of hBMSCs is studied previously, and it was found that concentration higher than 1000 ng/ml inhibits the cell proliferation after 4 days (Ying, Cheng et al. 2011) As shown in the ICP-OES results, the levels of B in cell culturing medium exceed that value, which might be one reason for inhibition of proliferation. Also, Ca ion levels have been shown to affect cell proliferation. (McCullen, Zhan et al. 2010) However, in our case the hBMSCs are simultaneously exposed to several different ions, and therefore it is not straightforward to specify the effect of individual ions alone. Furthermore, S53P4-based borosilicates have been previously cultured with human adipose stem cells (hASCs) expressing similar ion release profiles, which would suggest that the toxic level of ion release is not only option to proliferation-inhibition effect of hybrids. (Ojansivu, Mishra et al. 2018)

In addition to toxic level of ions and pH increase, it is possible that unreacted excessive GPTMS contributes to low cell proliferation. It is probable that from the hybrids unreacted GPTMS is released into the medium, which is seen as high Si concentrations, when ICP-OES measurements were conducted to culturing medium. Despite of the washing effect of unreacted compounds in 72h sample pre-incubation, there might be still unreacted GPTMS left.

In literature polymer functionalization with GPTMS is found to be most effective in mildly acidic conditions, which favours GPTMS epoxy ring opening and reaction with polymer. (Connell, Gabrielli et al. 2017) In our case, adding of GPTMS and bioactive glass were carried out simultaneously, which might lead to insufficient reaction of GPTMS, leaving unreacted molecules to solution. As discussed earlier, the role of pH in GPTMS chemistry is crucial, which might also have an effect here. In addition, usually hybrids are synthesized with TEOS with much higher silica content, this would in theory yield more attachment sites for GPTMS than what BAG particles used here would. (Mahony, Tsigkou et al. 2010)

For these reasons, it would have been interesting to experiment with other possible coupling agents, such as (3-Aminopropyl)triethoxysilane (APTES). APTES is possible to graft with BAGs (Stanić 2017), and if it would be found to be able to graft also with gelatin, it would be one attractive option to try in the future experiments.

Decrease in cell attachment might be also due to changes in the material matrix elasticity, surface chemistry or not suitable topography of the samples. This can be studied in direct contact but with indirect culturing in extracts this is not considered. Mahony et al. showed that BMSC cell attachment and morphology varied between hybrids, seeming that the C-factor affects. Higher C-factor was found to lead to an enhanced cell attachment and morphology, possibly due to improved rigidity and stability of hybrid (Mahony, Tsigkou et al. 2010) Due to this findings it would be interesting to experiment with ways to produce more rigid hybrid gels.

In general, reactive materials such as BAGs are difficult to evaluate in *in vitro* conditions. This is due to the changes in environment: ion concentrations in the culturing medium, and pH changes as materials degrade. Engineering is needed to optimize the composition that releases suitable amount of therapeutic ions, and with not too dramatic pH changes. In vitro cell cultures are static, and therefore smaller ion amount can show material to be toxic to cells, but in reality, in *in vivo* conditions there is more dynamic flow, replenishment of body fluids would distribute ions. This can lead to false interpretations. For example, in an earlier work by Fu et al. 13-93-3b borate bioactive glass was found toxic to cells *in vitro*, but not in *in vivo* rat model. (Fu, Rahaman et al. 2010) Perhaps it would be beneficial to experiment with more *in vivo* -like conditions, such as culturing with more dynamic microenvironment. For example, gentle intermittent rocking of well plates might prevent local high concentrations of ions.

8. CONCLUSIONS

Hybrid biomaterials, combining bioactive glasses (BAG) and natural polymers, such as gelatin, are potential alternatives for bone tissue engineering applications. In this thesis work GPTMS –coupled bioactive glass/gelatin hybrid biomaterials were synthesized and characterized *in vitro* with various methods. Firstly, the hybrid stability in aqueous solutions, such as Simulated Body Fluid (SBF) were demonstrated with multiple BAG/gelatin ratios. In addition, the hybrids showed controlled ion release upon SBF and cell medium immersion, suggesting HA layer precipitation indicating bioactivity. From enzymatic degradation studies hybrids were also found to be more resistant to enzymatic degradation than gelatin alone. Some preliminary rheological studies also gave some idea of the viscoelastic properties of the hybrids. Hybrids were found to gelate rapidly as a function of time with increasing G' and G'' moduli. BAG/gelatin gels without GPTMS were also found to exhibit higher viscosity as a function of decreasing temperature.

However, some concerns are raised by the inhibitory effect of hybrid samples on human bone marrow –derived mesenchymal stem cell (hBMSC) proliferation. Cells are able to proliferate and attach to some extent for 24 hours, but after 72 hours strong inhibition on proliferation is detected for all compositions. This is probably due to the release of ungrafted GPTMS from the hybrids. Further optimization of the hybrid composition and more cell studies are needed to confirm the suitability of these hybrid biomaterials as a biocompatible bone tissue engineering scaffold material. For example, experimenting with more dynamic culturing methods, different C-factors or even different coupling agents such as APTES would be viable options for future trials. Ultimately, more optimal and biocompatible inorganic/organic hybrids would find multiple applications in bone tissue engineering due to their tailorable properties and shaping possibilities.

REFERENCES

AMINI, A.R., LAURENCIN, C.T. and NUKAVARAPU, S.P., 2012. Bone Tissue Engineering: Recent Advances and Challenges. *Critical Reviews™ in Biomedical Engineering,* **40**(5), pp. 363-408.

ANDRZEJEWSKA, A., LUKOMSKA, B. and JANOWSKI, M., 2019. Concise Review: Mesenchymal Stem Cells: From Roots to Boost. *STEM CELLS,* **37**(7), pp. 855-864.

BIANCHI, G., BANFI, A., MASTROGIACOMO, M., NOTARO, R., LUZZATTO, L., CANCEDDA, R. and QUARTO, R., 2003. *Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Experimental Cell Research,* ***287****(1), pp. 98-105.*

BIGI, A., COJAZZI, G., PANZAVOLTA, S., ROVERI, N. and RUBINI, K., 2002. *Stabilization of gelatin films by crosslinking with genipin. Biomaterials,* ***23****(24), pp. 4827-4832.*

BIGI, A., COJAZZI, G., PANZAVOLTA, S., RUBINI, K. and ROVERI, N., 2001. *Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking. Biomaterials,* ***22****(8), pp. 763-768.*

BOHNER, M. and LEMAITRE, J., 2009. *Can bioactivity be tested in vitro with SBF solution?* Biomaterials, **30**(12), pp. 2175-2179.

BOSETTI, M. and CANNAS, M., 2005. *The effect of bioactive glasses on bone marrow stromal cells differentiation. Biomaterials,* ***26****(18), pp. 3873-9.*

BRYDONE, A.S., MEEK, D. and MACLAINE, S., 2010. Bone grafting, orthopaedic biomaterials, and the clinical need for bone engineering. *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine,* **224**(12), pp. 1329-1343.

CAMPANA, V., MILANO, G., PAGANO, E., BARBA, M., CICIONE, C., SALONNA, G., LATTANZI, W. and LOGROSCINO, G., 2014. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. *Journal of Materials Science: Materials in Medicine,* **25**(10), pp. 2445-2461.

CONNELL, L.S., GABRIELLI, L., MAHONY, O., RUSSO, L., CIPOLLA, L. and JONES, J.R., 2017. Functionalizing natural polymers with alkoxysilane coupling agents: reacting 3-glycidoxypropyl trimethoxysilane with poly(γ-glutamic acid) and gelatin. *Polymer Chemistry,* **8**(6), pp. 1095-1103.

DHANDAYUTHAPANI, B., YOSHIDA, Y., MAEKAWA, T. and SAKTHI, K.D., 2012. International Journal of Polymer Science: Polymeric scaffolds in tissue engineering application: a review. *Cellular Polymers,* **31**(1), pp. 51.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F.C., KRAUSE, D.S., DEANS, R.J., KEATING, A., PROCKOP, D.J. and HORWITZ, E.M., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* **8**(4), pp. 315-317.

DONATI, D., ZOLEZZI, C., TOMBA, P. and VIGANÒ, A., 2007. Bone grafting: historical and conceptual review, starting with an old manuscript by Vittorio Putti. *Acta Orthopaedica,* **78**(1), pp. 19-25.

DZONDO-GADET, M., MAYAP-NZIETCHUENG, R., HESS, K., NABET, P., BELLEVILLE, F. and DOUSSET, B., 2002. Action of boron at the molecular level: Effects on transcription and translation in an acellular system. *Biological trace element research,* **85**(1), pp. 23-33.

FU, Q., RAHAMAN, M.N., BAL, B.S., BONEWALD, L.F., KUROKI, K. and BROWN, R.F., 2010. Silicate, borosilicate, and borate bioactive glass scaffolds with controllable degradation rate for bone tissue engineering applications. II. In vitro and in vivo biological evaluation. *Journal of Biomedical Materials Research Part A,* **95A**(1), pp. 172-179.

GABRIELLI, L., RUSSO, L., POVEDA, A., JONES, J.R., NICOTRA, F., JIMÉNEZ-BARBERO, J. and CIPOLLA, L., 2013. Epoxide Opening versus Silica Condensation during Sol-Gel Hybrid Biomaterial Synthesis. *Chemistry (Weinheim an der Bergstrasse, Germany),* **19**(24), pp. 7856-7864.

GAO, C., RAHAMAN, M.N., GAO, Q., TERAMOTO, A. and ABE, K., 2013. Robotic deposition and in vitro characterization of 3D gelatin-bioactive glass hybrid scaffolds for biomedical applications. *Journal of biomedical materials research.Part A,* **101**(7), pp. 2027-2037.

GENTLEMAN, E., FREDHOLM, Y.C., JELL, G., LOTFIBAKHSHAIESH, N., O'DONNELL, M.D., HILL, R.G. and STEVENS, M.M., 2010. *The effects of strontium-substituted bioactive glasses on osteoblasts and osteoclasts in vitro. Biomaterials, **31**(14), pp. 3949-56.*

GHANBARI, H. and VAKILI-GHARTAVOL, R., 2016. Bone Regeneration: *Current Status and Future Prospects, Advanced Techniques in Bone Regeneration,* Alessandro Rozim Zorzi and Joao Batista de Miranda, IntechOpen.

GHORBANI, F., ZAMANIAN, A., BEHNAMGHADER, A. and DALIRI JOUPARI, M., 2018. A novel pathway for in situ synthesis of modified gelatin microspheres by silane coupling agents as a bioactive platform. *Journal of Applied Polymer Science,* **135**(41), pp. 46739-n/a.

GREENHALGH, R.D., AMBLER, W.S., QUINN, S.J., MEDEIROS, E.S., ANDERSON, M., GORE, B., MENNER, A., BISMARCK, A., LI, X., TIRELLI, N. and BLAKER, J.J., 2017. Hybrid sol–gel inorganic/gelatin porous fibres via solution blow spinning. *Journal of Materials Science,* **52**(15), pp. 9066-9081.

HENCH, L.L., SPLINTER, R.J., ALLEN, W.C. and GREENLEE, T.K., 1971. Bonding mechanisms at the interface of ceramic prosthetic materials. *Journal of Biomedical Materials Research,* **5**(6), pp. 117-141.

HENCH, L., 2006. The story of Bioglass. *Journal of Materials Science: Materials in Medicine,* **17**(11), pp. 967-978.

HENCH, L.L., 1998. Bioceramics. *Journal of the American Ceramic Society,* **81**(7), pp. 1705-1728.

HENDRIKX, S., KASCHOLKE, C., FLATH, T., SCHUMANN, D., GRESSENBUCH, M., SCHULZE, F.P., HACKER, M.C. and SCHULZ-SIEGMUND, M., 2016. Indirect rapid prototyping of sol-gel hybrid glass scaffolds for bone regeneration – Effects of organic crosslinker valence, content and molecular weight on mechanical properties. *Acta Biomaterialia,* **35**, pp. 318-329.

HOPPE, A., GÜLDAL, N.S. and BOCCACCINI, A.R., 2011. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. *Biomaterials,* **32**(11), pp. 2757-2774.

JONES, J.R., 2013. Review of bioactive glass: From Hench to hybrids. *Acta Biomaterialia,* **9**(1), pp. 4457-4486.

JONES, J.R., 2012. *Bio-glasses: An Introduction.* Chichester, West Sussex: Wiley.

KICKELBICK, G., 2006. Introduction to Hybrid Materials. In: G. Kickelbick, ed., *Hybrid Materials: Synthesis, Characterization, and Applications.* Weinheim: Wiley-VCH, pp.1-46.

KOKUBO, T., KUSHITANI, H., SAKKA, S., KITSUGI, T. and YAMAMURO, T., 1990. Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W. *Journal of biomedical materials research,* **24**(6), pp. 721-734.

KOKUBO, T. and TAKADAMA, H., 2006. *How useful is SBF in predicting in vivo bone bioactivity? Biomaterials,* ***27****(15),* pp. 2907-2915

LANGER, R. and VACANTI, J.P., 1993. Tissue engineering. *Science,* **260**(5110), pp. 920-926.

LAO, J., JALLOT, E. and NEDELEC, J.-., 2008. Strontium-delivering glasses with enhanced bioactivity: A new biomaterial for antiosteoporotic applications? *Chemistry of Materials,* **20**(15), pp. 4969-4973.

LAO, J., DIEUDONNÉ, X., FAYON, F., MONTOUILLOUT, V. and JALLOT, E., 2016. Bioactive glass–gelatin hybrids: building scaffolds with enhanced calcium incorporation and controlled porosity for bone regeneration. *Journal of Materials Chemistry B,* **4**(14), pp. 2486-2497.

MAHONY, O., TSIGKOU, O., IONESCU, C., MINELLI, C., LING, L., HANLY, R., SMITH, M.E., STEVENS, M.M. and JONES, J.R., 2010. Silica-Gelatin Hybrids with Tailorable Degradation and Mechanical Properties for Tissue Regeneration. *Advanced Functional Materials,* **20**(22), pp. 3835-3845.

MAHONY, O., YUE, S., TURDEAN-IONESCU, C., HANNA, J., SMITH, M., LEE, P. and JONES, J., 2014. Silica–gelatin hybrids for tissue regeneration: inter-relationships between the process variables. *Journal of Sol-Gel Science and Technology,* **69**(2), pp. 288-298.

MARIE, P.J., AMMANN, P., BOIVIN, G. and REY, C., 2001. Mechanisms of action and therapeutic potential of strontium in bone. *Calcified tissue international,* **69**(3), pp. 121-129.

MARTIN, I., MURAGLIA, A., CAMPANILE, G., CANCEDDA, R. and QUARTO, R., 1997. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology,* **138**(10), pp. 4456-4462.

MASSERA, J., HUPA, L. and HUPA, M., 2012. Influence of the partial substitution of CaO with MgO on the thermal properties and in vitro reactivity of the bioactive glass S53P4. *Journal of Non-Crystalline Solids,* **358(**18-19), pp. 2701-2707.

MASSERA, J., CLAIREAUX, C., LEHTONEN, T., TUOMINEN, J., HUPA, M. and HUPA, L., 2011. Control of the thermal properties of slow bioresorbable glasses by boron addition. *Journal of Non-Crystalline Solids,* **357**(21), pp. 3623-3630.

MASSERA, J., FAGERLUND, S., HUPA, L., HUPA, M. and PINCKNEY, L., 2012. Crystallization Mechanism of the Bioactive Glasses, 45S5 and S53P4. *Journal of the American Ceramic Society,* **95**(2), pp. 607-613.

MCCULLEN, S.D., ZHAN, J., ONORATO, M.L., BERNACKI, S.H. and LOBOA, E.G., 2010. Effect of Varied Ionic Calcium on Human Adipose-Derived Stem Cell Mineralization. *Tissue engineering. Part A,* **16**(6), pp. 1971-1981.

MEUNIER, P.J., SLOSMAN, D.O., DELMAS, P.D., SEBERT, J.L., BRANDI, M.L., ALBANESE, C., LORENC, R., PORS-NIELSEN, S., DE VERNEJOUL, M.C., ROCES, A. and REGINSTER, J.Y., 2002. Strontium ranelate: Dose-dependent effects in established postmenopausal vertebral osteoporosis - A 2-year randomized placebo controlled trial. *Journal of Clinical Endocrinology and Metabolism,* **87**(5), pp. 2060-2066.

MEZGER, T.G., 2012, Theœ Rheology Handbook [Homepage of Vincentz Network], [Online]. Available: https://doi.org/10.1515/9783748600367.

MONDAL, D., 2018. *Covalently Crosslinked Organic/Inorganic Hybrid Biomaterials for Bone Tissue Engineering Applications.* Western University

MONFOULET, L., BECQUART, P., MARCHAT, D., VANDAMME, K., BOURGUIGNON, M., PACARD, E., VIATEAU, V., PETITE, H. and LOGEART-AVRAMOGLOU, D., 2014. The pH in the Microenvironment of Human Mesenchymal Stem Cells Is a Critical Factor for Optimal Osteogenesis in Tissue-Engineered Constructs. *Tissue Engineering Part A,* **20**(13-14), pp. 1827-1840.

MONTORO, D.T., WAN, D.C. and LONGAKER, M.T., 2014. *Chapter 60 - Skeletal Tissue Engineering.* Boston: Academic Press. In: ROBERT LANZA, JOSEPH VACANTI and ROBERT LANGER, eds, *Principles of Tissue Engineering*

MOURIÑO, V., VIDOTTO, R., CATTALINI, J.P. and BOCCACCINI, A.R., 2019. Enhancing biological activity of bioactive glass scaffolds by inorganic ion delivery for bone tissue engineering. *Current Opinion in Biomedical Engineering, 10,* pp.23-34.

MURATA, H., 2012. *Rheology - Theory and Application to Biomaterials.* InTech.

NELSON, M., 2016. *3D Silica-Gelatin Hybrid Scaffolds for Tissue Regeneration* , Imperial College London.

NOVAK, B.M., 1993. Hybrid Nanocomposite Materials?between inorganic glasses and organic polymers. *Advanced Materials,* **5**(6), pp. 422-433.

O'NEILL, E., AWALE, G., DANESHMANDI, L., UMERAH, O. and LO, K.W., 2018. The roles of ions on bone regeneration. *Drug Discovery Today,* **23**(4), pp. 879-890.

O'DONNELL, M.D., CANDARLIOGLU, P.L., MILLER, C.A., GENTLEMAN, E. and STEVENS, M.M., 2010. Materials characterisation and cytotoxic assessment of strontium- substituted bioactive glasses for bone regeneration. *Journal of Materials Chemistry,* **20**(40), pp. 8934-8941.

OJANSIVU, M., MISHRA, A., VANHATUPA, S., JUNTUNEN, M., LARIONOVA, A., MASSERA, J. and MIETTINEN, S., 2018. The effect of S53P4-based borosilicate glasses and glass dissolution products on the osteogenic commitment of human adipose stem cells. PLoS ONE **13**(8): e0202740.

ORYAN, A., ALIDADI, S., MOSHIRI, A. and BIGHAM-SADEGH, A., 2014. Bone morphogenetic proteins: A powerful osteoinductive compound with non-negligible side effects and limitations. *BioFactors,* **40**(5), pp. 459-481.

OZBOLAT, I.T. and HOSPODIUK, M., 2016. Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials*, 76, pp. 321-343.

PARENTEAU-BAREIL, R., GAUVIN, R. and BERTHOD, F., 2010. Collagen-Based Biomaterials for Tissue Engineering Applications. *Materials,* **3**(3), pp. 1863-1887.

PAXTON, N., SMOLAN, W., BÖCK, T., MELCHELS, F., GROLL, J. and JUNGST, T., 2017. Proposal to assess printability of bioinks for extrusion-based bioprinting and evaluation of rheological properties governing bioprintability. *Biofabrication,* **9**(4), pp. 044107.

POOLOGASUNDARAMPILLAI, G., YU, B., TSIGKOU, O., VALLIANT, E., YUE, S., LEE, P.D., HAMILTON, R.W., STEVENS, M.M., KASUGA, T. and JONES, J.R., 2012. Bioactive silica–poly(γ-glutamic acid) hybrids for bone regeneration: effect of covalent coupling on dissolution and mechanical properties and fabrication of porous scaffolds. *Soft Matter,* **8**(17), pp. 4822.

POOLOGASUNDARAMPILLAI, G., IONESCU, C., TSIGKOU, O., MURUGESAN, M., HILL, R.G., STEVENS, M.M., HANNA, J.V., SMITH, M.E. and JONES, J.R., 2010. Synthesis of bioactive class II poly(γ-glutamic acid)/silica hybrids for bone regeneration. *Journal of Materials Chemistry,* **2**(4), pp. 8952-8961.

POOLOGASUNDARAMPILLAI, G. and MAÇON, A., 2016. Organic -Inorganic Hybrid Biomaterials
. In: A.R. BOCCACCINI, D.S. BRAUER and L. HUPA, eds, *Bioactive Glasses: Fundamentals, Technology and Applications.* Royal Society of Chemistry; 1 edition, pp. 286-302.

RADIN, S., REILLY, G., BHARGAVE, G., LEBOY, P.S. and DUCHEYNE, P., 2005. Osteogenic effects of bioactive glass on bone marrow stromal cells. *Journal of Biomedical Materials Research Part A,* **73A**(1), pp. 21-29.

RAHAMAN, M.N., DAY, D.E., SONNY BAL, B., FU, Q., JUNG, S.B., BONEWALD, L.F. and TOMSIA, A.P., 2011. Bioactive glass in tissue engineering. *Acta Biomaterialia,* **7**(6), pp. 2355-2373.

REN, L., TSURU, K., HAYAKAWA, S. and OSAKA, A., 2001. Synthesis and Characterization of Gelatin-Siloxane Hybrids Derived through Sol-Gel Procedure. *Journal of Sol-Gel Science and Technology,* **21**(1), pp. 115-121.

ROSS, M.H. and PAWLINA, W., 2016. *Histology.* Seventh edition, international edition edn. Philadelphia: Wolters Kluwer.

SAMADIKUCHAKSARAEI, A., LECHT, S. and POLAK, J.M., 2014. Chapter 4 - Stem Cells as Building Blocks. In: ROBERT LANZA, JOSEPH VACANTI and ROBERT LANGER, eds, *Principles of Tissue Engineering.* pp. 41-55.

SEPULVEDA, P., JONES, J.R. and HENCH, L.L., 2001. Characterization of melt-derived 45S5 and sol-gel–derived 58S bioactive glasses. *Journal of Biomedical Materials Research,* **58**(6), pp. 734-740.

SHIROSAKI, Y., OSAKA, A., TSURU, K. and HAYAKAWA, S., 2012. Inorganic-Organic Sol-Gel Hybrids. *Bio-Glasses.* Chichester, UK: John Wiley & Sons, Ltd, pp. 139-158.

SHIROSAKI, Y., TSURU, K., HAYAKAWA, S., OSAKA, A., LOPES, M.A., SANTOS, J.D., COSTA, M.A. and FERNANDES, M.H., 2009. Physical, chemical and in vitro biological profile of chitosan hybrid membrane as a function of organosiloxane concentration. *Acta Biomaterialia*, **5**(1), pp. 346-355.

SHRIVATS, A., ALVAREZ, P., SCHUTTE, L., HOLLINGER, J., 2014. Chapter 55: Bone regeneration. *Principles of Tissue Engineering.* Elsevier Inc., pp. 1201-1221.

STANIĆ, V., 2017. Variation in Properties of Bioactive Glasses After Surface Modification. *Clinical Applications of Biomaterials.* Springer, Cham, pp. 35-63.

TAINIO, J.M., SALAZAR, D.A.A., NOMMEOTS-NOMM, A., ROILAND, C., BUREAU, B., NEUVILLE, D.R., BRAUER, D.S. and MASSERA, J., 2020. Structure and in vitro dissolution of Mg and Sr containing borosilicate bioactive glasses for bone tissue engineering. *Journal of Non-Crystalline Solids*, 533.

TAINIO, J., 2016. *Impact of magnesium and strontium on dissolution and sintering of bioactive borosilicate glasses*, Tampere University of Technology.

VALLIANT, E.M. and JONES, J.R., 2011. Softening bioactive glass for bone regeneration: sol–gel hybrid materials. *Soft Matter,* **7**(11), pp. 5083.

VARILA, L., FAGERLUND, S., LEHTONEN, T., TUOMINEN, J. and HUPA, L., 2012. Surface reactions of bioactive glasses in buffered solutions. *Journal of the European Ceramic Society,* **32**(11), pp. 2757-2763.

VUEVA, Y., CONNELL, L.S., CHAYANUN, S., WANG, D., MCPHAIL, D.S., ROMER, F., HANNA, J.V. and JONES, J.R., 2018. Silica/alginate hybrid biomaterials and assessment of their covalent coupling. *Applied Materials Today,* **11**, pp. 1-12.

YAMASAKI, Y., YOSHIDA, Y., OKAZAKI, M., SHIMAZU, A., UCHIDA, T., KUBO, T., AKAGAWA, Y., HAMADA, Y., TAKAHASHI, J. and MATSUURA, N., 2002. Synthesis of functionally graded MgCO3 apatite accelerating osteoblast adhesion. *Journal of Biomedical Materials Research,* **62**(1), pp. 99-105.

YING, X., CHENG, S., WANG, W., LIN, Z., CHEN, Q., ZHANG, W., KOU, D., SHEN, Y., CHENG, X., ROMPIS, F., PENG, L. and ZHU LU, C., 2011. Effect of Boron on Osteogenic Differentiation of Human Bone Marrow Stromal Cells. *Biological Trace Element Research,* **144**(1), pp. 306-315.

YOUSEFI, A., JAMES, P.F., AKBARZADEH, R., SUBRAMANIAN, A., FLAVIN, C. and OUDADESSE, H., 2016. Prospect of Stem Cells in Bone Tissue Engineering: A Review. *Stem cells international,* **2016**, pp. 6180487-13.

ZREIQAT, H., HOWLETT, C.R., ZANNETTINO, A., EVANS, P., SCHULZE-TANZIL, G., KNABE, C. and SHAKIBAEI, M., 2002. Mechanisms of magnesium-stimulated adhesion of osteoblastic cells to commonly used orthopaedic implants. *Journal of Biomedical Materials Research,* **62**(2), pp. 175-184.

APPENDIX A: PROTOCOL FOR PREPARING SIMULATED BODY FLUID (SBF)

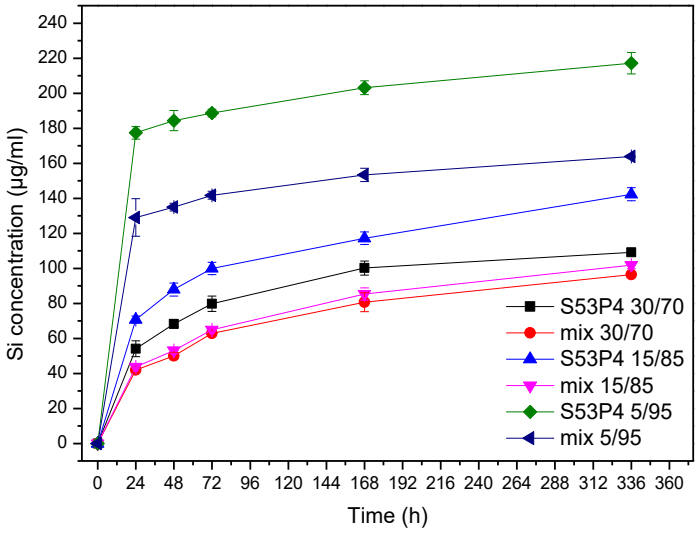
1 litre of SBF is prepared by following adjusted Kokubo protocol below. (Kokubo, Kushitani et al. 1990) Reagents are diluted one by one in given order to distilled H_2O:

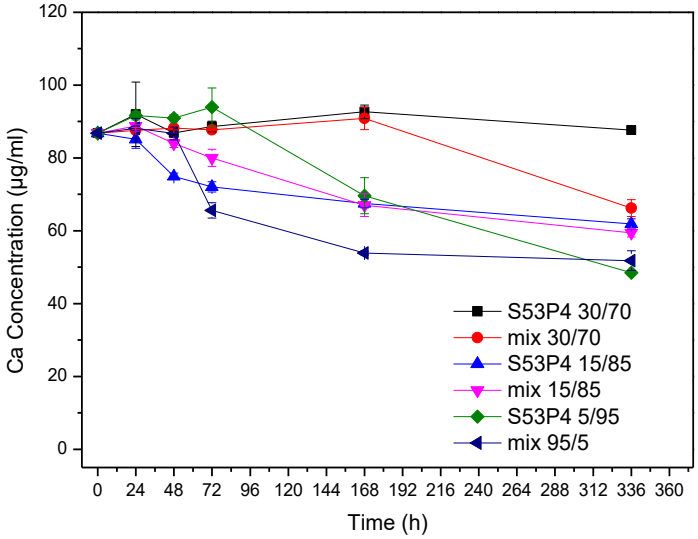
	Reagent	Amount
1	NaCl	7.996 g
2	$NaHCO_3$	0.35 g
3	KCl	0.224 g
4	$K_2HPO_4 \cdot 3\ H_20$	0.228 g
5	$MgCl_2 \cdot 6\ H_2O$	0.305 g
6	1 M HCl	~40 ml
7	$CaCl_2 \cdot 2\ H_2O$	0.368 g
8	Na_2SO_4	0.071 g
9	Trizma-base 2-Amino-2-(hydroxymethyl)-1,3-propane-diol	6.057 g

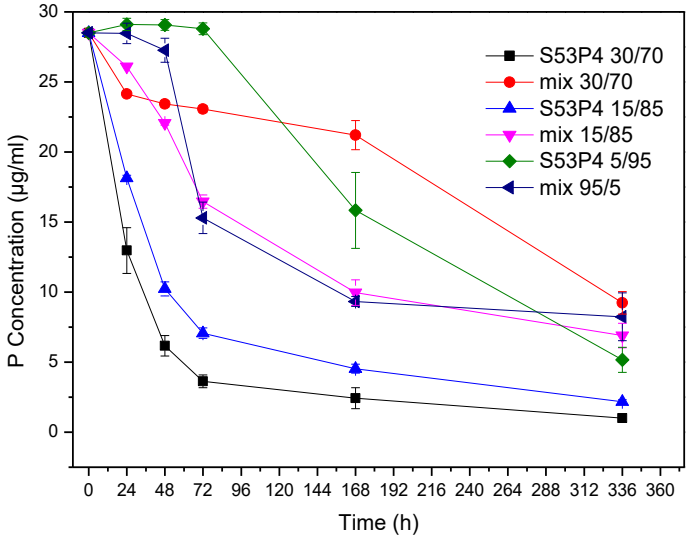
pH value is adjusted at 37.00 ± 0.02 to 7.40 ± 0.02 with 1 M HCl. SBF is stored in polyethylene bottle in fridge at 5-10 °C for maximum 30 days.

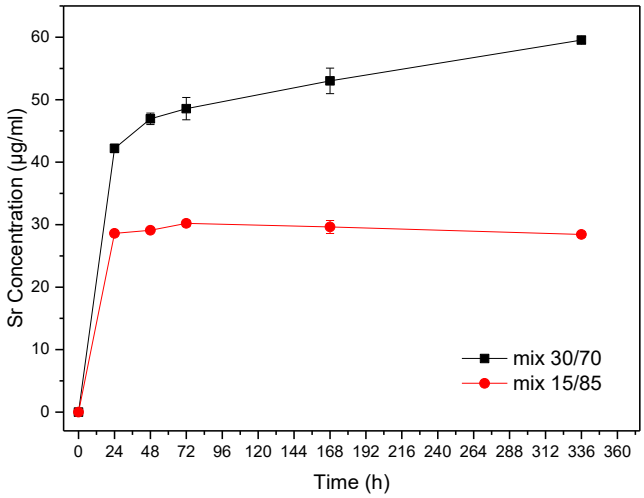
APPENDIX B: ICP-OES RESULTS

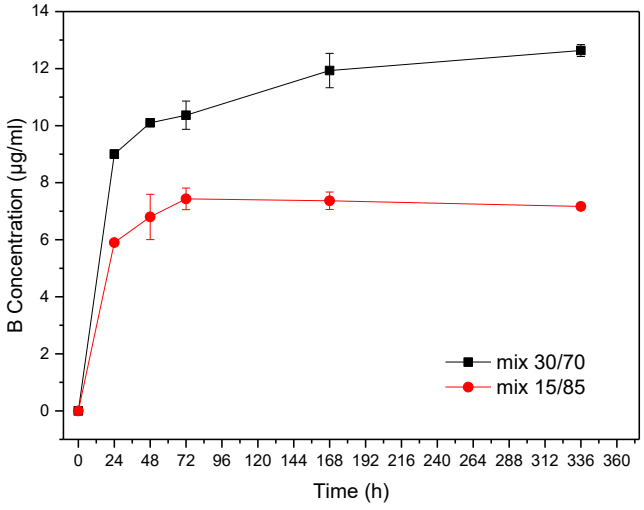
Individual ion concentrations from SBF dissolution series

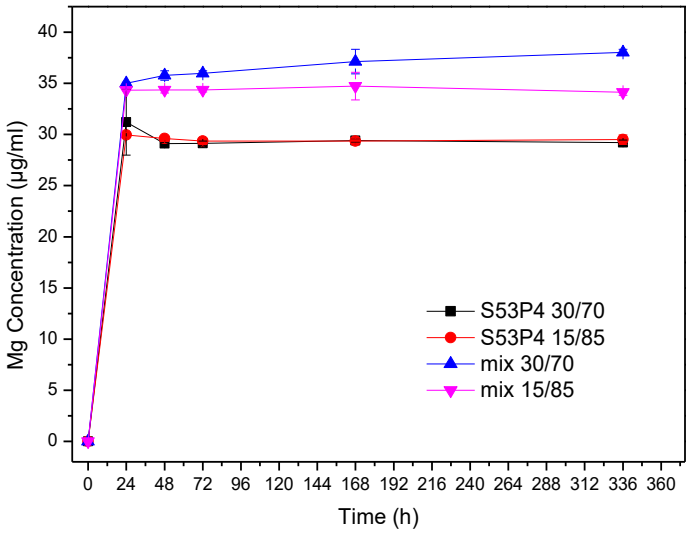


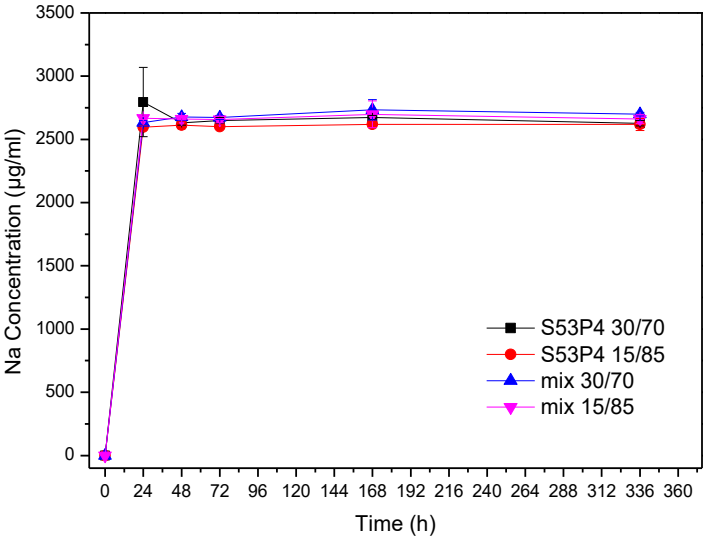



P Concentration (µg/ml)
Time (h)
S53P4 30/70
mix 30/70
S53P4 15/85
mix 15/85
S53P4 5/95
mix 95/5


Sr Concentration (µg/ml)
Time (h)
mix 30/70
mix 15/85


B Concentration (µg/ml)
Time (h)
mix 30/70
mix 15/85


Mg Concentration (µg/ml)
Time (h)
S53P4 30/70
S53P4 15/85
mix 30/70
mix 15/85


Na Concentration (µg/ml)
Time (h)
S53P4 30/70
S53P4 15/85
mix 30/70
mix 15/85

Combined ion concentration for S53P4 and mix hybrids from enzymatic degradation test

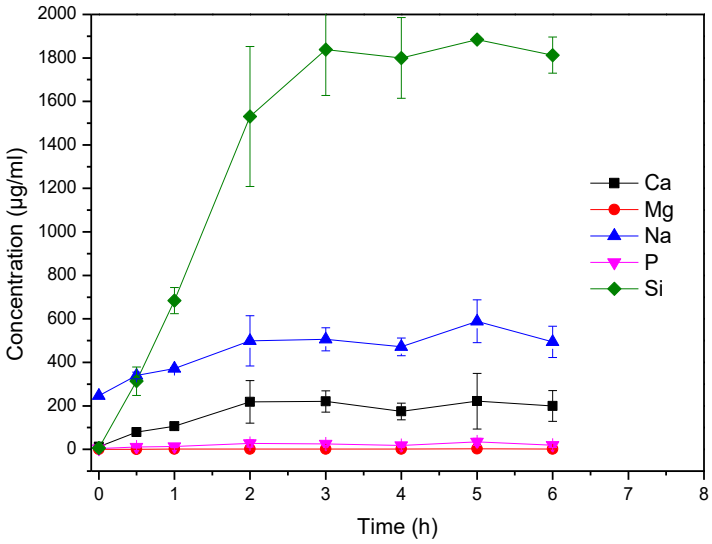


Figure 45. *30/70 S53P4 hybrid ion concentrations*

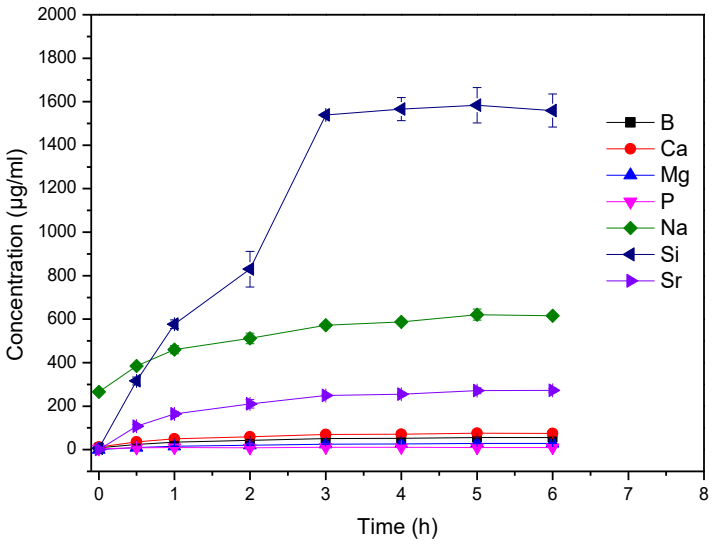
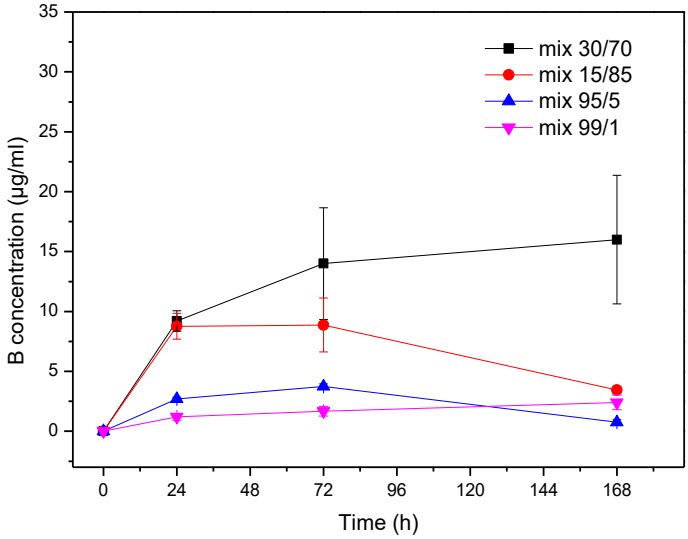
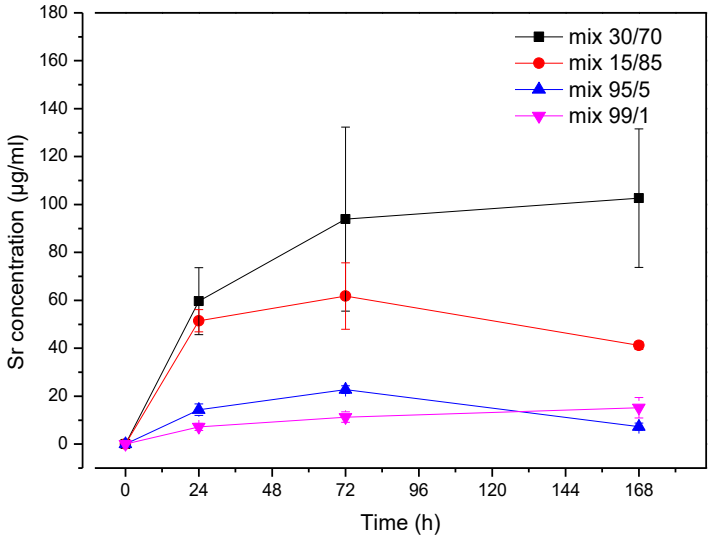
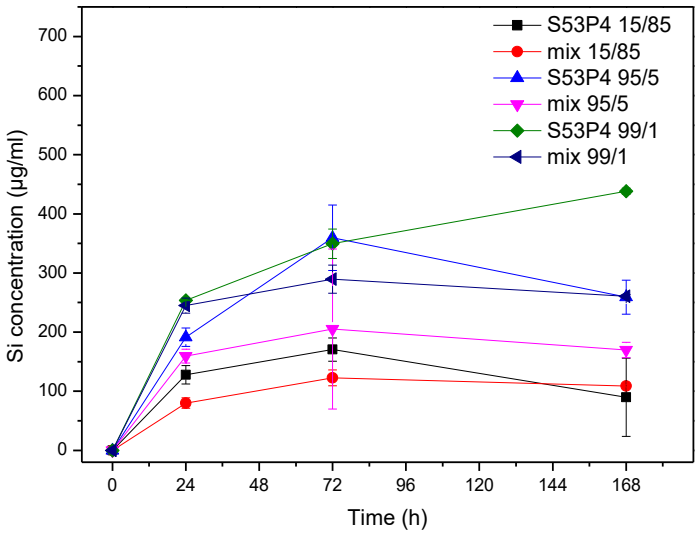


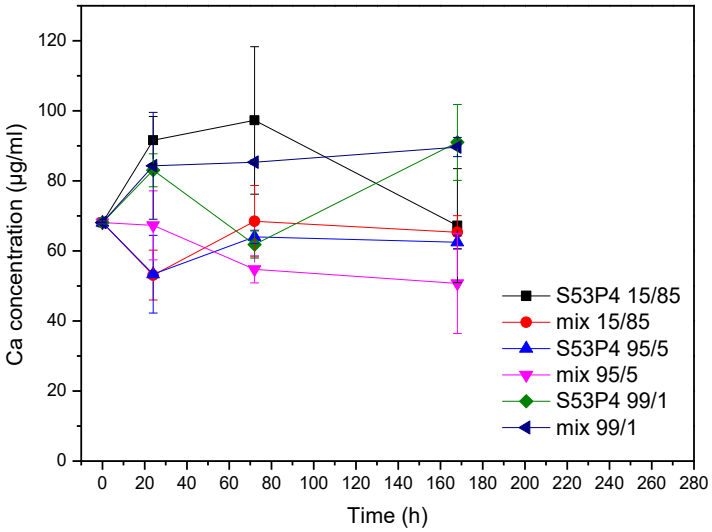
Figure 46. *30/70 mix hybrid ion concentrations*

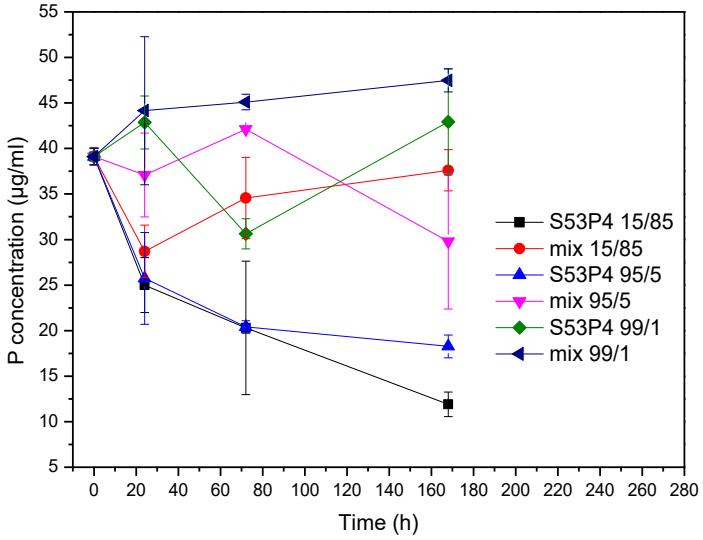
Individual ion concentrations from cell culturing medium

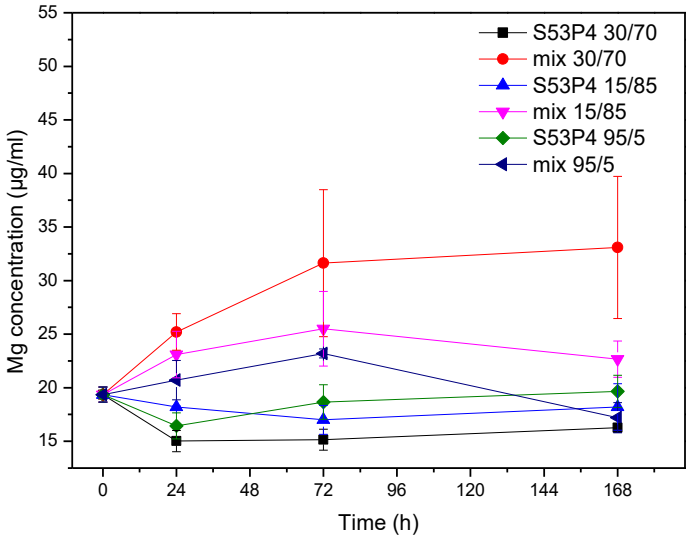





Si concentration (µg/ml)
Time (h)
S53P4 15/85
mix 15/85
S53P4 95/5
mix 95/5
S53P4 99/1
mix 99/1


Ca concentration (µg/ml)
Time (h)
S53P4 15/85
mix 15/85
S53P4 95/5
mix 95/5
S53P4 99/1
mix 99/1


P concentration (µg/ml)
Time (h)
S53P4 15/85
mix 15/85
S53P4 95/5
mix 95/5
S53P4 99/1
mix 99/1


Mg concentration (µg/ml)
Time (h)
S53P4 30/70
mix 30/70
S53P4 15/85
mix 15/85
S53P4 95/5
mix 95/5