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TRABAJO FIN DE MASTER EN INGENIERÍA BIOMÉDICA

DEVELOPMENT OF FUNCTIONAL HYDROGELS FOR CONTROLLED ION RELEASE

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RESUMEN

El boro es un microelemento traza, esencial en el metabolismo de los organismos vivos. Sin embargo, su papel exacto en las células de mamíferos y el modo preciso de acción a nivel molecular no han sido bien definidos hasta el momento. Los logros científicos recientes han descrito una nueva actividad del boro relacionada con la diferenciación miogénica y la vascularización. Este trabajo fin de máster tiene como objetivo optimizar la ingeniería de sistemas de materiales basados en hidrogeles capaces de liberar boro de forma sostenida para aplicaciones en ingeniería de tejidos. Se han probado dos tipos diferentes de hidrogeles. Los sistemas materiales basados en hidrogeles estan compuestos por polímeros altamente biocompatibles, alginato y polietilenglicol (PEG), que posteriormente fueron cargados con dos concentraciones diferentes de boro. Las pruebas de liberación confirmaron que los hidrogeles creados eran capaces de liberar boro. Además, el análisis termomecánico (TMA) y el análisis termogravimétrico (TGA) demostraron que el boro aumentó las propiedades mecánicas y actúa como un reticulador dentro de la red del polímero de hidrogel. Este trabajo es el primer paso para la optimización de sistemas de materiales basados en hidrogeles para la liberación de boro y nos brinda una nueva herramienta para diseñar microambientes celulares con aplicaciones en medicina regenerativa.

Palabras Clave: Hidrogeles, Ingeniería Tisular, Boro, Alginato, Polietilenglicol

RESUM

El bor és un microelement traça, essencial en el metabolisme dels organismes vius. No obstant això, el seu paper exacte en les cèl·lules de mamífers i el mode precís d'acció a nivell molecular no han sigut ben definits fins al moment. Els èxits científics recents han descrit una nova activitat del bor relacionada amb la diferenciació miogénica i la vascularització. Este treball fi de màster té com a objectiu optimitzar l'enginyeria de sistemes de materials basats en hidrogels capaços d'alliberar bor de forma sostinguda per a aplicacions en enginyeria de teixits. S'han provat dos tipus diferents d'hidrogels. Els sistemes materials basats en hidrogels estan compostos per polímers altament biocompatibles, alginat i polietilenglicol (PEG) , que posteriorment van ser carregats amb dos concentracions diferents de bor. Les proves d'alliberament van confirmar que els hidrogels creats eren capaços d'alliberar bor. A més, l'anàlisi termomecànica (TMA) i l'anàlisi termogravimétric (TGA) van demostrar que el bor va augmentar les propietats mecàniques i actua com un reticulador dins de la xarxa del polímer d'hidrogel. Este treball és el primer pas per a l'optimització de sistemes de materials basats en hidrogels per a l'alliberament de bor i ens brinda una nova ferramenta per a dissenyar microambients cel·lulars amb aplicacions en medicina regenerativa.

Paraules clau: Hidrogels, Enginyeria Tissular, Bor, Alginat, Polietilenglicol

ABSTRACT

Boron is a trace microelement essential in the metabolism of living organisms. Yet its exact role in mammalian cells and the precise mode of action at the molecular level has not been well defined. Recent scientific achievements have described a novel activity of boron related to myogenic differentiation and vascularisation. This master thesis aims to optimise the engineering of hydrogel-based material systems capable of sustained boron-release for Tissue Engineering applications. Two different types of hydrogels have been tested. The hydrogel-based material systems were composed of highly biocompatible polymers, alginate and Polyethylenglycol (PEG), and they were loaded with two different concentrations of boron. The release tests confirmed that the created hydrogels were capable of boron-delivery. Further, thermomechanical analysis (TMA) and thermogravimetric analysis (TGA) demonstrated that boron increased mechanical properties and acts as a cross-linker within the hydrogel polymer network. This work is the first step for hydrogel-based material systems optimization for boron-release and provides us with a new tool to engineer microenvironments with applications in regenerative medicine.

Keywords: Hydrogels, Tissue Engineering, Boron, Alginate, Polyethylenglycol

STRESZCZENIE

Bor jest śladowym mikroelementem niezbędnym w metabolizmie organizmów żywych. Do tej pory jego dokładna rola w komórkach ssaków oraz dokładny sposób działania na poziomie molekularnym nie zostały dobrze zdefiniowane. Ostatnie osiągnięcia naukowe wykazały, że bor ma wpływ na miogenezy oraz waskularyzacji. Celem tej pracy magisterskiej jest zoptymalizowanie hydrożelowych materiałów, do zastosowań w inżynierii tkankowej, zdolnych do kontrolowanego uwalniania jonów boru. Dwa różne rodzaje hydrożeli zostały przetestowane. Materiały hydrożelowe wykonane zostały z wysoce biokompatybilnych polimerów, alginianu sodu oraz poli(glikolu etylenowego) (PEG), do których dodano dwa różne stężenia boru. Przeprowadzone pomiary spektrofotometryczne potwierdziły zdolność wytworzonych hydrożeli do uwalniania jonów boru. Ponadto analiza termomechaniczna (TMA) i analiza termograwimetryczna (TGA) wykazały, że bor poprawia właściwości mechaniczne i działa jako środek sieciujący w sieci polimerów hydrożelowych. Tę pracę magisterską należy traktować jako pierwszy krok w kierunku optymalizacji materiałów hydrożelowych zdolnych do uwalniania jonów boru oraz zapewnia nowe narzędzie do projektowania mikrosystemów z zastosowaniem w medycynie regeneracyjnej.

Słowa klucze: Hydrożele, Bor, Inżynieria Tkankowa, Alginian, Polietylen glikol

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Introduction

1.1. Context Tissue Engineering

Tissue engineering seeks to mimic the natural extracellular matrix (ECM) using materials and bioactive factors to control cellular responses.

Tissue engineering is a dynamic interdisciplinary scientific area of research based on the combination of biology, material engineering and bioactive factors with the aim to regenerate growing tissues that could be implanted into patients in regenerative medicine applications. The final purpose of Tissue Engineering is the synthetic production of three-dimensional cell scaffolds, possessing morphological and functional properties of native tissue, with the aim to promote and reproduce tissue regeneration *in vivo* conditions after their implantation. [1]. The most important components used in Tissue Engineering are cells, biomaterials and biomolecules/bioactive factors.

In most of the cases, the cells used in Tissue Engineering applications are stem cells that can be modified using specific culture media to obtain specific types of tissues, as stem cells possess unique properties of self-renewal and differentiation in multiple cell lineages. [2]

There are a broad variety of biomolecules/bioactive factors employed for Tissue Engineering applications such as growth factors, hormones, peptides, components of the extracellular matrix, vitamins or ions, all of them promoting cell proliferation and/or differentiation. [1]. Concerning material engineering, scaffolds should be biocompatible, biodegradable, highly porous with a large surface/volume ratio, mechanically strong, and malleable. Scaffolds need to possess good mechanical properties in order to mimic the natural niche of the cells. For example, a good scaffold for bone Tissue Engineering should be 3D shaped, highly porous with a large surface/volume ratio and stiff enough to mimic the bone. Biodegradability of the scaffolds is a property that has to be balanced with a dependence of the final application, as the premature degradation of the scaffold might lead to the collapse of the tissues. [3].

Types of materials used for scaffolds:

- Ceramics
- Polymers (both synthetic and natural)
- Metals
- Composites [4]

In the past decade, hydrogels have gained popularity among biomaterials used as 3D scaffolds for tissue regeneration. The combination of their unique properties like biocompatibility, high water content, permeability, hydrophilicity, physical properties and chemical structure made them ideal candidates for different biomedical applications in tissue regeneration. [5]. We can find examples of hydrogels for tissue engineering of cartilage, liver, bladder, nerve, skin, bone, ligament etc. [3].

As the topic of this master thesis is related to hydrogels, they will be described in more detail in the next chapter.

Despite the efforts realised during the last decades, material-based approaches still lack the potential to promote tissue regeneration due to the complexity of interactions and molecules involved and the incapacity of recapitulating the totality of biochemical and physical cues governing key cellular processes.

1.2. Hydrogels

Definition

Hydrogels are three dimensional, water-swollen polymeric networks. They are characterised by their high degree of flexibility and their capability to swell as well as retain big amounts of water within their structure and yet not dissolving themselves in water. Responsible for those properties are the hydrophilic groups that are normally present in the polymeric network, that after contact with the water molecules they become hydrated. The ability of not dissolving in water is guaranteed by the presence of cross-links in the polymer chains [6]. The hydrogel property of flexibility makes them very similar to the natural tissues, making them ideal tuneable candidates to be employed in biomedical applications.

Classification of hydrogels

In the literature, we can find a variety of hydrogel classifications based on their source, physical properties, technique of preparation, the method of swelling, their response to stimuli, their ionic charges, biodegradation rate and crosslinking mechanisms. An example of this variety is represented in Figure 1:

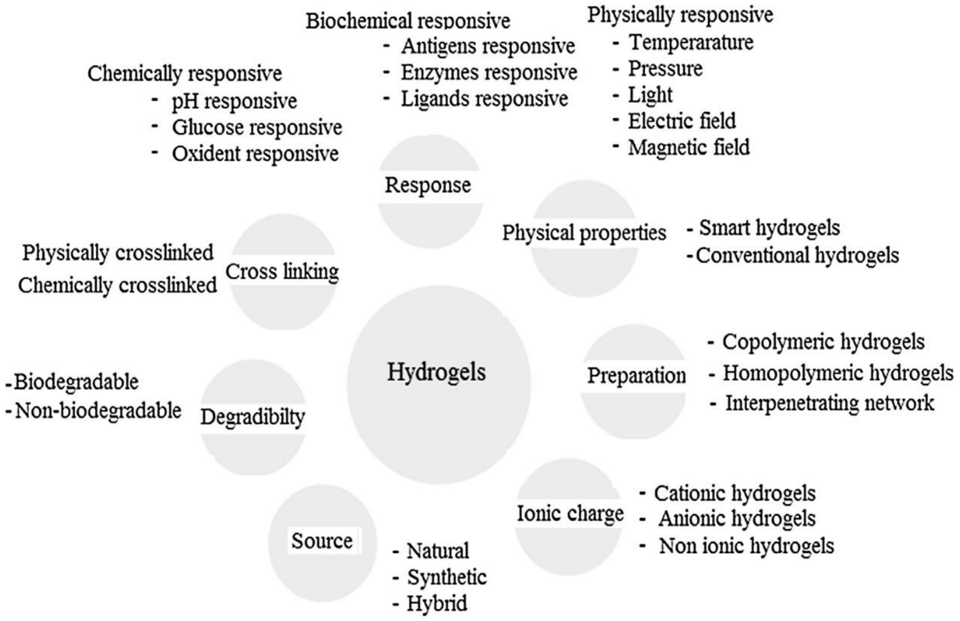


Figure 1. Classification of the hydrogels based on their properties. [7]

We can distinguish 3 types of hydrogels based on their origin:

- Natural
- Synthetic
- Hybrid

Early hydrogel studies focused on natural hydrogels such as collagen, hyaluronic acid, alginate and chitosan. However, with the advances of the material technology, synthetic and composite hydrogels have been developed, since they are more tuneable and modifiable, specifically regarding some of their properties such as the degradation rate. Examples of synthetic hydrogels are Poly(vinyl alcohol) (PVA), Polyethylene glycol (PEG), Poly(acrylic acid) (PAA) or Poly(methyl methacrylate) (PMMA).[8]

Another type of classification of hydrogels is the method of preparation. There can be classified as:

- Homopolymers – composed of one type of monomer
- Copolymers – composed of two or more types of monomers that give raise a random network
- Multipolymer interpenetrating polymeric hydrogels – composed of two separate cross-linked polymers that are combined into one network. [9]

Consecutively there is a classification based on their structure:

- Amorphous
- Semicrystalline
- Crystalline [6]

Classification based on the crosslinking method:

- Chemically cross-linked – connected by chemical bonds
- Physically cross-linked – physical interactions, hydrogen bonds, van der Waals forces, hydrophobic interactions [6]

Classification based in the electrical charge:

- Non-ionic (neutral),
- Ionic (including anionic or cationic),
- Amphoteric electrolyte (ampholytic) composed of acidic and basic groups,
- Zwitterionic (polybetaines) composed of anionic and cationic groups in each structural repeating unit [10]

Polymerisation methods

Hydrogels exist in various shapes like microspheres, films or matrices and this state is conditioned by the polymerisation method employed. Natural based polymers are polymerised using different proteins (collagen and gelatine) or polysaccharides (starch, alginate, and agarose) whereas the synthetic hydrogels require chemical methods to polymerise.

The most common polymerisation techniques are:

- Bulk polymerisation
- Solution polymerisation/cross-linking
- Suspension polymerisation or inverse-suspension polymerisation
- Grafting to a support
- Polymerisation by irradiation [11]

Hydrogels must meet the following features:

- Good biodegradability
- Mechanical properties adequate to the implanted tissue
- Appropriate porosity both open and closed pores
- High absorption capacity
- High absorbency under load
- Low cost of material and production
- Re-wetting ability
- Non-toxicity
- Chemical stability
- Infinitesimal content of residual monomers
- Easily sterilisable [10]

Advanced hydrogels

In the last years, a group of innovative hydrogels has been developed. These hydrogels are composed of superabsorbent polymers (SAPs) with an enhanced water holding capacity. They can absorb up to 1000 times of their original weight or volume in a short period of time, which has great applications in agriculture and hygienic products industry. Next, superporous hydrogels (SPHs) emerged. They swell immediately after contact with water as they possess an open cellular porous structure. Pores in superporous hydrogels are much bigger than those in SAPs. [12]

Smart materials

Smart hydrogels provide a specific response after receiving a stimulus. Their response consists of changes in their phyisico-chemical properties such as structure, shape, surface, morphology, stiffness, degradation rate, drug release, swelling or micellisation.[13]

The stimuli can be divided into two main categories:

- Physical – like pressure, light, temperature, and magnetic or electric fields
- Chemical - ionic strength, pH and ions
- Biological – glucose, growth factors, enzymes, antibodies proteins, peptides

Smart hydrogels have the widest and most promising applications in drug delivery systems. Since the detection of the stimuli, they can start -providing therapeutic agents and delivering drugs in a sustained and controlled manner- continuously and uniformly. Moreover, the release of the drugs is focused on a target area of interest.[14]

Applications

Those materials have a broad spectrum of usage. They can be found in hygienic products, agriculture, in the food industry as additives, as artificial snow and in coal mining as coal dewatering agents. They can be used in biomedical applications like pharmaceuticals, contact lenses, drug delivery systems, wound dressings, tissue engineering, regenerative medicine, biosensors, materials for biomolecules separation and diagnostics.

Smart hydrogels can be used not only in direct applications as hydrogels with drug delivery systems but also as model systems for investigation of dynamic interactions between cells, between cells and extra-cellular matrices, and between drug delivery and biological systems. [15]

1.3. PEG

Polyethylene glycol (PEG) is a synthetic polymer. It is neutral polyether, perfectly soluble in water and in most of the organic solvents. It is commonly used in Tissue Engineering as an artificial scaffold especially in central nervous system applications[16]–[18].

PEG chains of any length can be easily synthesised by the controlled polymerisation of ethylene oxide or ethylene glycol in aqueous solution. It belongs to the group of highly biocompatible polymers, however, it is not biodegradable although it can be easily modified for directed protease degradation.

For PEG functionalisation various biomolecules can be easily attached to the PEG hydrogel networks, mono-, di- or multivalent reactive groups, such as acrylate, amine, thiol, azide, maleimide and biotin/streptavidin.

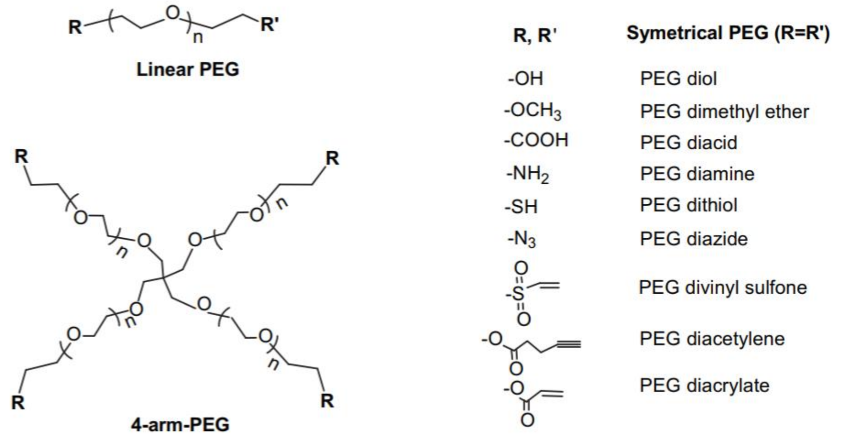


Figure 2. Structures of linear PEG and 4-arm-PEG with various functional modifiable groups.[19]

In order to mimic the natural extracellular matrix and provide bioactive properties, hydrogel branched form can be further functionalised [20]. The most common molecules used for functionalization are bioactive molecules such as cell-adhesion peptide domains, enzyme-sensitive peptides, and growth factors.

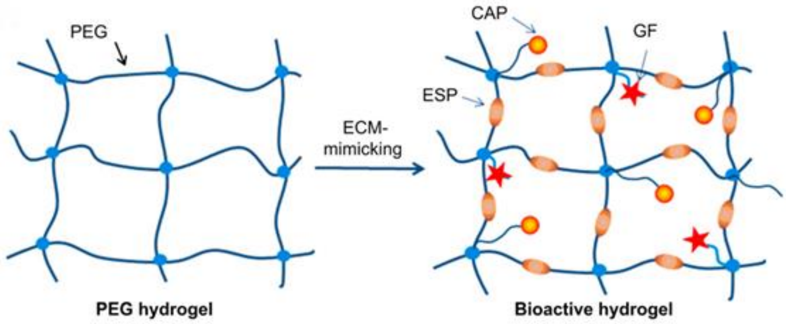


Figure 3. Biological functionalization of PEG hydrogels with bioactive molecules, such as cell-adhesive peptide (CAP), enzyme-sensitive peptide (ESP), and growth factor (GF). [19]

1.4. Alginate

Alginate hydrogels are copolymers of natural origin. Due to their high biocompatibility, relatively low price, big accessibility, simple manufacturing process and structural similarity to the natural extracellular matrix, these materials are successfully used in many branches of tissue engineering and regenerative medicine. Normally they are used as absorbent dressings to minimise bacterial infections and to facilitate wound healing, carriers of bioactive agents and pharmaceuticals, and as cellular scaffolds for potential applications in cartilage regeneration, retinal tissue regeneration and treatment of Type 1 Diabetes. [21] [22]

Characteristics and properties of sodium alginate

Sodium alginate is a naturally existing polysaccharide derived from brown marine algae including *Laminaria hyperborea*, *Laminaria digitata* and *Laminaria japonica* species, or extracellularly produced by selected bacterial strains such as *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, *and Pseudomonas fluorescens.* [23]

Alginate is a linear copolymer consisting of α-L-guluronic acid residues (G-monomer) and β-D-mannuronic monomer (M-monomer), linked by β-1-4 glycosidic linkage. [24]. Structure of sodium alginate is presented in Figure 3.

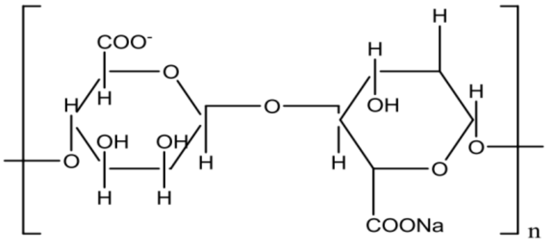


Figure 3. Chemical structure of sodium alginate [25]

In an environment containing divalent cations, particularly Ca^{2+}, sodium alginate is cross-linked to form biodegradable and biocompatible polymeric scaffolds characterised by high water content. The cross-linking reaction leads to a characteristic structure, called eggs in a box, in which divalent cations are positioned between homopolymer G blocks that form three-dimensional, well-organized forms (Figure 4).

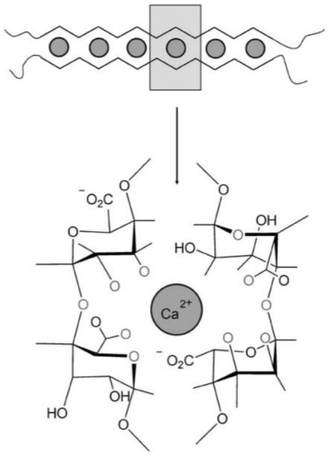


Figure 4. Egg-in-box structure of sodium alginate [26]

The interactions between cations and alginate are driven only by G blocks of the polymer. Thus, the ratio of M blocks to G blocks, their distribution along the main chain and the length of G blocks are the key factors that influence the physico-chemical properties of alginate and the resulting hydrogels. Molecules with a predominance of glucuronic acid form rigid and dense hydrogels, while the addition of mannuronic acid affects their greater flexibility, porosity and better solubility.

Another critical factor determining the physico-chemical properties of the hydrogels is the average molecular weight of the copolymer. The molecular weight of commercially available sodium alginates ranges from 32.000 to 400.000 g/mol. Better mechanical properties of alginate hydrogels are usually obtained by increasing the amount and length of the G blocks or the molecular weight of the alginate. The properties of alginate hydrogels depend to a large extent on the type and conditions of the cross-linking, e.g. selection of the cross-linker, its concentration and the concentration of sodium alginate itself. By increasing the concentration of sodium alginate, the mechanical properties of hydrogels are improved.

Selection of the cross-linker affects the gelation time when they are cross-linked using divalent cations. It allows to control the homogeneity of the structure and the strength of the hydrogel. Slower gelation gives the possibility for obtaining more homogeneous structures, which are characterised by greater mechanical integrity [27].

The most popular method used for alginate hydrogel preparation is the ionic cross-linking, where the aqueous alginate solution is combined with the ionic cross-linking agents such as divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+} or Pb^{2+}). One of the most commonly used compounds are $CaCl_2$, $CaSO_4$ and $CaCO_3$. However, $CaCl_2$ is easily soluble and leads to rapid gelation. For this reason, $CaSO_4$ and $CaCO_3$ are used instead of $CaCl_2$ as they dissolve longer in hydrogel solution providing more gradual gelation, which is extremely important for injectable hydrogels.

Hydrogels obtained after ionic crosslinking methods are characterised by their limited stability under physiological conditions. This is due to the release of divalent ions to the surrounding environment through the exchange reaction with monovalent cations, which leads to biodegradation. Depending on the application, it can be a desirable or undesirable attribute. Free radical polymerization or cellular cross-linking can be also used to obtain alginate hydrogels for Tissue Engineering applications.

1.5. Boron

Boron is a metalloid placed in the group 13 in the periodic table with atomic number 5, atomic mass 10.811 g/mol and the chemical symbol B. Boron has various isotopes, the most stable of which are 10B and 11B. In nature, boron does not exist as a single element; instead, it forms sodium and oxygen organoboronate complexes, which are the physiologically significant forms of boron in humans. Compared with hydrogen, carbon, nitrogen, or oxygen it has a low abundance on Earth, but it can be found as a part of rocks, soil, and water. In physiologic conditions the majority of boron is present as an uncharged boric acid $B(OH)_3$ and as a small amount of borate anion $B(OH)_4^-$.[28]

Borax

One of the most frequent sources of boron is the salt of boron acid, sodium borate. There are two types of borax: borax decahydrate ($Na_2B_4O_7*10H_2O$) and borax pentahydrate ($Na_2B_4O_7*5H2O$). In the industry, it is used as a flux material in metallurgy, and in the production of high-quality glasses, ceramic materials and enamels. In the first half of 20th century sodium borate and boric acid were used as a preservative in food. [29].

Borax is frequently used as a cross-linking agent, due to its high reactivity with hydroxyl groups [30] [5]. The cross-linking complex creation is illustrated in Figure 5.

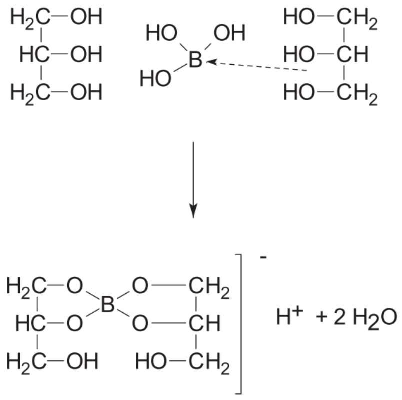


Figure 5. Scheme of the chemical reaction between borate acid and glycerol (high content of hydroxyl groups). [31]

This complex is created when borate oxide is present with all polyhydroxy compounds. [31]. In the present work, I have used as a polymeric material for hydrogel synthesis sodium alginate and polyethylenglycol, both polyhydroxy compounds, thus I anticipate a chemical crosslink reaction between borax and the hydrogels.

Boron in plants

The function of boron was first discovered t in plants and a great number of information exist regarding its activity at the physiological and molecular level. In flora, boron has important roles in nucleic acid metabolism, carbohydrate and protein metabolism, cell wall synthesis, cell wall structure, membrane integrity and function, and phenol metabolism. It cross-links pectins in plant cell walls.

Deficiency of boron results in an abnormal cell wall formation, modified cytoskeletal polymerisation, and alterations in plasma membrane permeability. Furthermore, the expansion of the leaves, root elongation, flower and fruit growth are inhibited. [32]

Boron in mammals

Boron plays a key role in mammalian cells in several metabolic pathways and in combination with other ions such as Ca^{2+} and Mg^{2+} as well as steroid hormones. It has been reported that boron deprivation interferes with male and female frog's reproductive system and, further, has its impact in embryos development. [33] In zebra fish fed with a boron-depleted diet, alterations in embryogenesis occurred. [34] In chicks boron deprivation affected gross bone abnormalities [35] and in rats decreased bone volume fraction and trabecular thickness [36]. Another study in rats provided data that combination of boron and 17b-estradiol improved the absorption of calcium, phosphorus and magnesium, however, these results were obtained only in combination, boron alone did not provide similar results [37].

Research conducted on pigs proved that diet supplemented with boron induced high levels of serum triiodothyronine.[38] Tests performed on rats and chicks suggested that boron may reduce the amount of insulin needed to maintain plasma glucose levels.[39]

Despite all the described information, little is known about boron homeostasis and function in mammalian cells at a molecular level.

Boron mechanism of action

Until recently, it was believed that boron was transported into cells by passive diffusion through the cell membrane as occurs for the boric acid. However, since the publication of Park et al., [40] the boron transporter, NaBC1, was characterised for the first time in mammalian cells as the homologous boron transporter first described in *Arabidopsis Thaliana*. NaBC1 possess a unique characteristic with a specific double function: in the presence of borate works as an electrogenic Na^{+}-coupled borate cotransporter, but in absence of borate, it conducts Na^{+} and OH^{-} (H^{+}). The main function of this transporter is to maintain boron homeostasis [40].

Only recently in the group of Rico et al. activity of boron related to myogenic differentiation has been described. For the first time, the uniqueness effects of B (acting as a bioactive ion) released from films of poly lactic acid (PLLA)-based system, in cell differentiation using murine myoblasts. It has been established that a potential mechanism of action includes cooperation with Ca^{2+}, other ion playing an essential role in the activating of myogenic transcription factors and myogenesis as well as migration and fusion of myotubes.[41] After this discovery, the group leader considered the use of boron-loaded materials to trigger muscle differentiation mechanisms, as a new tool to engineer microenvironments with applications in regenerative medicine.

This research group has also recently shown that delivery of boron enhances vascularisation. They proposed a 3D molecular model for the ion-channel NaBC1 (Figure. 6).

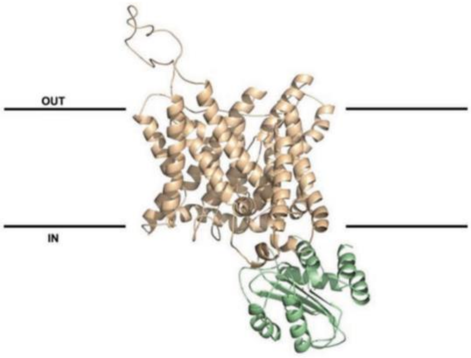


Figure 6. Structural model of NaBC1 transporter [42].

The use of boron is simple and have the potential to open up the way to new therapeutic strategies for vascularisation using ions instead of growth factors. They report a novel molecular mechanism by which boron induces angiogenesis *in vitro* and *in vivo* via simultaneous NaBC1 and VEGFR activation using ultra-low doses of soluble VEGF (< 5 ng cm^{-2}, compared to e.g. 72 µg cm^{-2} administered in clinical trials) (Figure 7). [42]

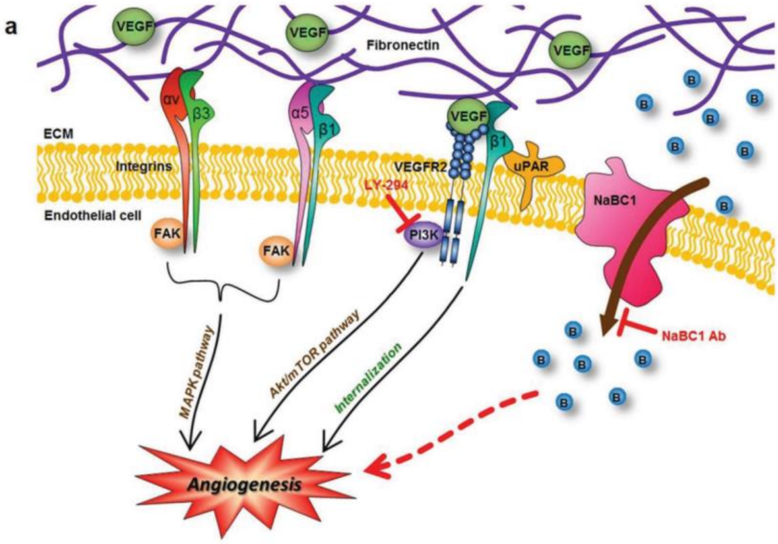


Figure 7. Schematic picture of events representing the role of boron and NaBC1 transporter, as well as other membrane receptors, in the regulatory crosstalk promoting vasculogenesis [42].

Boron in tissue engineering and medicinal chemistry

In terms of tissue engineering and biomedical applications, boron has been used for the optimisation of biomaterials especially in the bioactive glass research area in which new bioactive glasses based on borate and borosilicate compositions have shown the ability to enhance new bone formation when compared to silicate bioactive glass. Indeed, borate-based bioactive glasses also have controllable degradation rates, providing improved conditions for this type of scaffolds with applications in hard and soft tissue regeneration. [43].

The unique characteristics of boron have been exploited by synthetic chemists focusing on important medicinal molecules which production includes the application of organoborane chemistry. Particular mention deserves the Suzuki coupling reaction [44] which has made the synthesis of several pharmaceuticals significantly easier. Multiple boron-containing molecules are currently undergoing preclinical and clinical development to treat various disease conditions, including inflammation, diabetes, cancer, and many others.

2. Aim of the work

The aim of this work is to optimise the engineering of hydrogel-based material systems capable of sustained boron-release.

The material substrates selected for hydrogel preparation, Alginate and Polyethylenglycol, are well-known biodegradable and non-biodegradable materials respectively, approved by the Food and Drug Administration (FDA) for several biomedical applications with remarkable properties for tissue engineering such as excellent biocompatibility and processability.

Since boron related myogenic differentiation and vascularisation have been recently described, I have selected boron as a bioactive element to be released from the hydrogel systems, avoiding the use of other bioactive elements such as growth factors, cytokines or hormones. The strategies based on growth factor delivery normally require the use of high concentrations and they can also play an essential role in tumour growth and cancer. Therefore, the use of boron as a bioactive factor represents a simple, cost-effective, safe and a robust alternative for regenerative medicine applications.

3. Materials and methods

3.1. Experimental design

Based on the previous results obtained in the group, PEG and alginate hydrogels will be loaded with two concentrations of boron that were proven to be optimal for cellular stimulation. These concentrations are 0.59 mM (Boron low) and 1.47 mM (Boron high). After hydrogel loading with boron, a long-term release assay will be conducted to assess the boron delivery from hydrogel systems. The aim of the experimental design is to decipherate the relation between the hydrogel composition and the boron-delivery, hydrogel characterisation and optimisation of hydrogel production. Liberated boron from hydrogels will be assessed by colorimetric techniques measuring absorbance using azomethine reaction that occurs specifically with boron. Mechanical (TMA) and thermogravimetric analysis (TGA) will be used to investigate the effects of boron in the mechanical and thermal properties of the hydrogels.

3.1.1. PEG hydrogels preparation

In order to prepare PEG hydrogel PEG-4MAL (20kDa, Laysan Bio, Item 4arm-PEG-MAL-20K-5g) as a polymer and PEG-dithiol (3.4kDa, Laysan Bio, Item SH-PEG-SH-3400-1g) as a crosslinker was used. It has to be stressed out that PEG-4MAL has 4 Maleimide groups and PEG-dithiol has 2 thiol groups, thus a molar ratio 1:1 of these two compounds were used in order to accomplish the complete crosslink of the network.

In all experiments sodium tetraborate decahydrate, borax 10 mol (Borax) ($Na2B4O7*10H2O$) (Borax España S.A) was used as a source of boron to load hydrogels. It will be named Borax later on.

Three types of PEG hydrogels were designed: PEG 3% (30 mg/ml), PEG 5% (50 mg/ml) and PEG 10% (100 mg/ml) and three different types of samples: control sample with no boron concentration (B0), low boron content (0.59 mM, BL) and high boron content (1.47 mM, BH) samples. Stoichiometric proportions for preparation of 9 samples of each type of hydrogels are presented in the following table:

Table 1. Stoichiometric proportions of reagents for PEG hydrogels.

Type of hydrogel	PEG 3%	PEG 5%	PEG 10%
Amount of PEG-4MAL	15 mg	25 mg	50 mg
Volume of DPBS used to dissolve PEG-4MAL	360 µl	360 µl	360 µl

Amount of PEG-dithiol	5.1 mg	8.5 mg	17 mg
Volume of DPBS used to dissolve PEG-dithiol	90 µl	90 µl	90 µl
Final volume	450 µl	450 µl	450 µl

PEG-4MAL and PEG-dithiol solutions were mixed using vortex for about 1 min to make sure that reagents were dissolved. To obtain cylindrical hydrogel shapes, syringes were used as a mould. First, 40 µl of PEG-4MAL solution was placed in the syringe. After that, 1 µl of the borax solution was added (from a solution of 90 µg/µl) for BL samples, and 1 µl of the borax solution was added (from a solution of 225 µg/µl) for BH samples. After boron addition, 10 µl of PEG-dithiol was added, as a cross-linker agent. The gelation process occurs extremely fast. The samples were triplicated to obtain significant statistical data. Schematic representation of this process is represented in Figure 8.

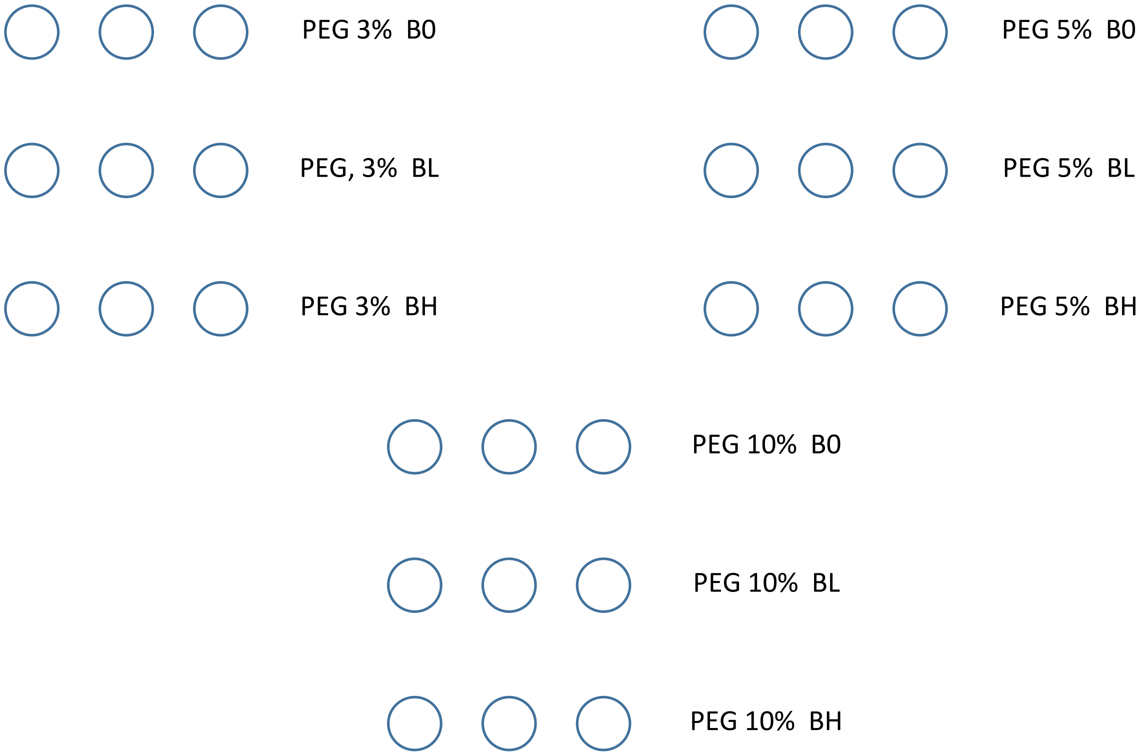


Figure 8. Schematic representation of PEG samples preparation. Hydrogels with three different concentrations of PEG were prepared. Each of these hydrogels was also produced with different concentrations of boron: control sample with no boron (B0), sample with low boron content (BL) and sample with high boron content (BH).

3.1.2. Alginate

Alginate sodium hydrogels were prepared based on the method described by Albert Espona-Nogueraa et al. [21].

Ultrapure sodium alginate (FMC Biopolymer, 75–200 KDa and Guluronate/Mannuronate ratio ≥1.5), was dissolved in 1% (w/v) D-mannitol (Sigma-Aldrich) with a concentration of 1.5%. A combination of Na_2HPO_4 0.3 M and $CaSO_4$ 1.22 M (Sigma-Aldrich) was used as a gelation agent. Borax solutions dissolved in alginate were also prepared ($Na_2B_4O_7 * 10H_2O$) (Borax España S.A).

Solutions of Mannitol 1%, $CaSO_4$ in mannitol, Na_2HPO_4 in mannitol, sodium alginate in mannitol, low borax content solution and high borax content solution both in mannitol were prepared and left over night to dissolve at room temperature.

Table 2. Stoichiometric proportions of reagents for Alginate hydrogels.

Type of hydrogel	AL B0	AL BL	AL BH
Volume of Na_2HPO_4	60 µl	60 µl	60 µl
Volume of $CaSO_4$	60 µl	60 µl	60 µl
Amount of sodium alginate 1,5%	0,60 g	0,60 g	0,60 g
Volume of mannitol 1%	40 ml	40 ml	40 ml
Amount of Borax	0 g	2,4 g	6,0 g

All of the solutions were filtered through a 0.22 µm pore Minisart Syringe Filter (Sartorius).

The system was set using 2 syringes and a Fluid Dispensing Connector (Braun). In one syringe sodium alginate was placed, in the second one the combination of Na_2HPO_4 and $CaSO_4$. Both solutions were mixed 15 times until their complete homogenization.

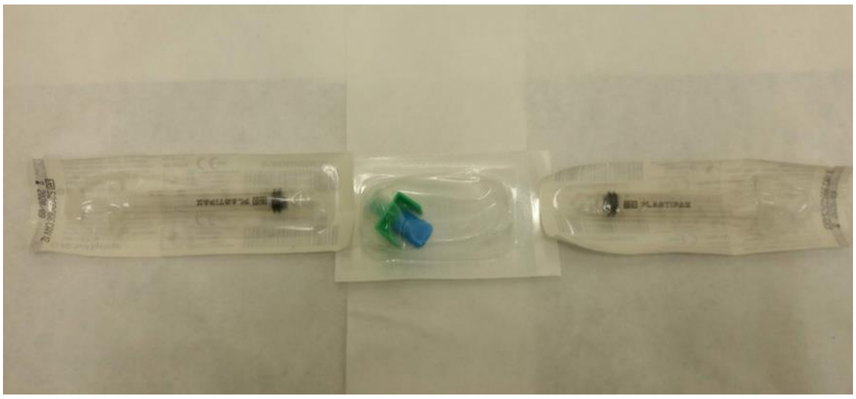


Figure 9. Set of syringes and fluid dispenser used for mixing solutions.

Control alginate samples were pressed out from syringe (100 µl) and left to gelify. Gelification process took about 12 min. Interestingly, we observed that alginate samples loaded with boron remained liquid for long period of time, thus we have obtained a long-time (more than a month) injectable hydrogel-based biomaterial very useful for biomedical applications. All samples were tested in triplicate.

3.2. Release tests

3.2.1. PEG samples

Newly produced hydrogels were placed into a 24-well plate. To each well 1 ml of DPBS (with Ca and Mg) was added. After that, plates were incubated in a Thermo Fisher Scientific 131-3111 cell incubator (conditions 5% CO2, 20% O2, 95% humidity and 37°C). The release test was noncumulative, thus the DBPS+ was changed and replaced for a fresh one every day. The different aliquots were preserved for further boron detection. Measurements were taken on days: 1, 2, 5, 7, 9, 13, 15.

3.2.2. Alginate samples

Control samples were placed in 24-well plate using spatula and samples containing boron were directly injected in the plate. Hydrogels were incubated in exactly the same condition as PEG ones. With one difference, that amount of placed hydrogel was 100 µl.

It was impossible to conduct this study for 15 days because after the first 24 h samples were totally dissolved. For this reason, measurements were taken only days 1 and day 2.

3.3. Absorbance measurements

In order to calculate the concentrations of the boron-released the absorbance measurements have been referenced to a standard curve set for samples with known concentrations of boron. All the tests were performed using azomethine (Sigma-Aldrich) in acid medium (KAc/HAc buffer pH 5.2) originating a colorimetric reaction measured at 405 nm in a Victor III (Perkin Elmer) device. Standard curves for calibration were prepared at concentrations of 0, 0.1, 0.25, 0.5, 1, 2.5,5, 10, 25, 50 and 500 mg/l of boron. As the amounts of boron inserted into hydrogels were big and the amount expected value of liberated ions was unknown a broad range of concentrations was used for the preparation of the standard curve.

Figure 10. Multi-task plate reader Victor III (Perkin Elmer)

All the tests were conducted with a use of 96 well-plate. On every well-plate, the standard samples and samples of unknown concentrations were triplicated in order to minimise statistical mistakes. Scheme of 96 well-plate looks as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0,1	0,25	0,5	1	2,5	5	10	25	50	500	
B	0	0,1	0,25	0,5	1	2,5	5	10	25	50	500	
C	0	0,1	0,25	0,5	1	2,5	5	10	25	50	500	
D	a	a	a		a	a	a		a	a	a	
E	b	b	b		b	b	b		b	b	b	
F	c	c	c		c	c	c		c	c	c	
G	d	d	d		d	d	d		d	d	d	
H	e	e	e		e	e	e		e	e	e	

Figure 11. Scheme of location of the standard and tested samples on 96 well-plate.

In rows A, B, C wells are placed standard samples with known boron concentrations (mg/l) and consecutively in rows E-H, wells are set with samples of unknown concentration (a-e).To each well on the 96-well plate 40 µl of sample, 40 µl of potassium acetate buffer and 20 µl of azomethine solution was added. The plate was incubated at room temperature for 1h. After that, absorbance measurements were performed.

Solution of azomethine requires to be done every time before the measurements as it possesses the ability to oxidise. Azomethine solution is prepared as follows:

- 9 g/l azomethine
- 20 g/l ascorbic acid
- Dissolved in miliQ ultrapure water.

Absorbance measurements of known concentrations allowed for preparation of standard curve presented on the Figure 12.

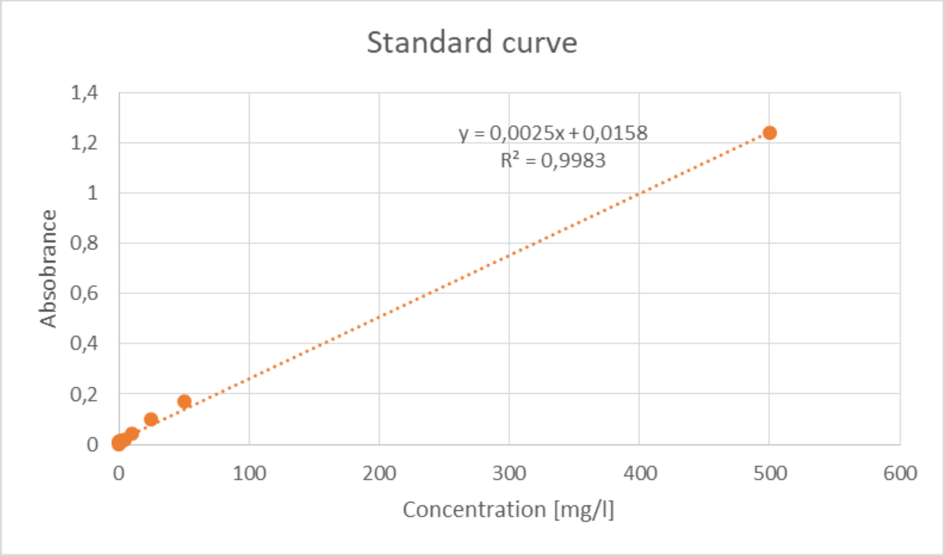


Figure 12. Standard curve for known concentrations of boron.

Linear equation of the standard curve of boron concentration led to the calculation of unknown concentrations of boron. A control standard curve was included in every plate to avoid internal error measurements intrinsic of Victor spectrophotometer device. For this experiment, 14 well-plates were used.

Using linear function equation

$$y = ax + b$$

The concentration of boron was calculated as follows:

$$y - absorbance$$
$$x - concentration$$
$$x = \frac{y - b}{a} \left[\frac{mg}{ml}\right]$$

In this example it was

$$x = \frac{y - 0{,}0158}{0{,}0025}$$

3.4. Thermomechanical analysis

Static compression tests were performed using Seiko TMA/SS6000 device.

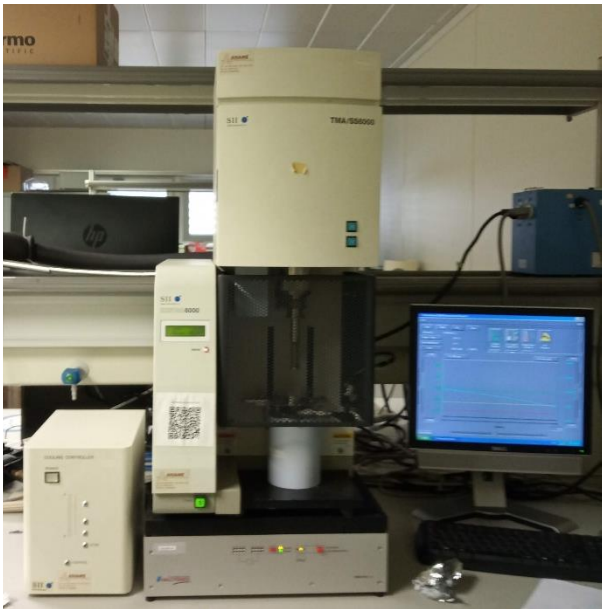


Figure 13. Seiko TMA/SS6000 device.

Thermomechanical analysis (TMA) can measure linear or volumetric changes in the dimensions of a material as a function of temperature, time and force. To obtain the results control mode with constant velocity of 100 µm/min was used. The test lasted 30 min and the temperature was constant and equal to 19°C.

From raw data ε - strain and σ - stress values were calculated, which allowed to create the stress-strain graphs.

$$\varepsilon = \frac{L - L_0}{L_0} \ [dimensionless]$$

$where$

$$L - original\ length\ of\ the\ sample\ [\mu m]$$
$$L_0 - change\ in\ length\ of\ the\ sample\ [\mu m]$$

$$\sigma = \frac{F}{A}\ [MPa]$$

$where$

$F -\ applied\ force\ [N]$

$A - surface\ area -\ cylinder's\ surface\ area\ 19{,}63\ mm^2, as\ sample\ had\ biger\ surface\ area$

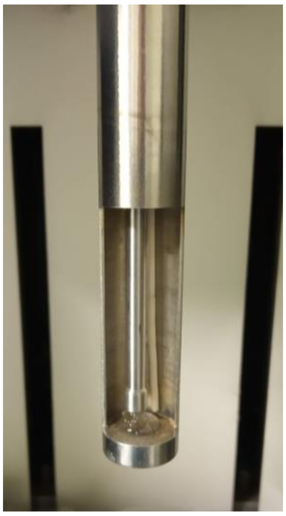


Figure 14. Sample colocation in TMA analysis.

Young modulus calculations

Based on the stress-strain curves of the hydrogels Young modulus were calculated for PEG hydrogels.

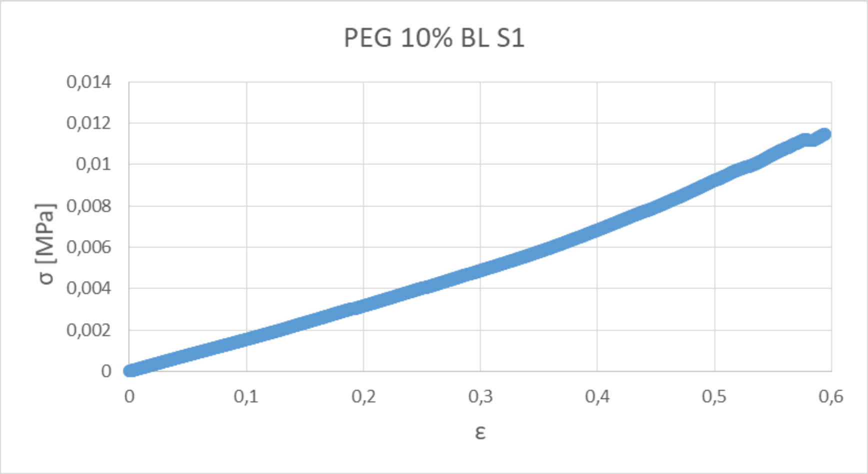


Figure 15. Stress-strain curve for a PEG 10% BL sample number 1

Young modulus of the samples was calculated, using the "method of least squares". In order to perform this calculation, the region that seemed the most linear from point 0 to 0,15 was marked on the x-axis which was 25% of the initial deformation. Then the curve was plotted and a linear trend line was made.

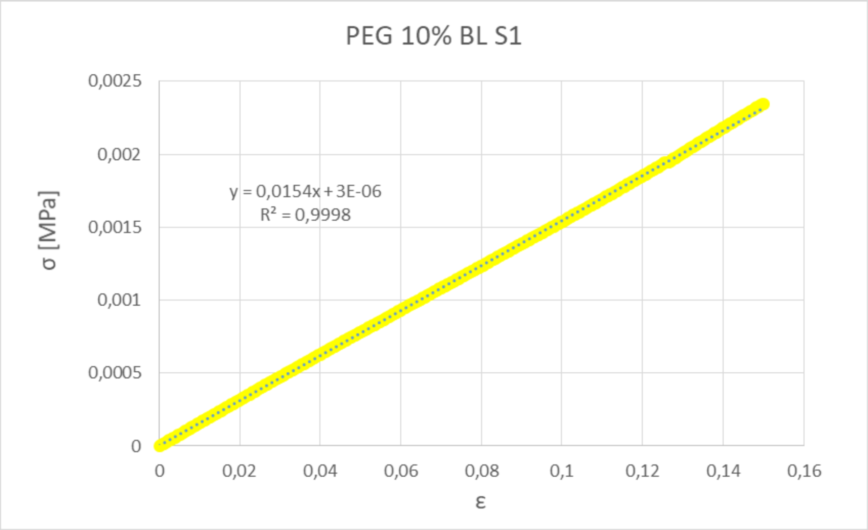


Figure 16. Stress-strain curve representing 25% of graph of PEG 10% BL S1

Where $y = ax + b$

$$a - slope - Young\ modulus\ of\ the\ given\ sample$$

All the results of Young modulus are represented in the results chapter.

3.5. Thermogravimetric analysis

This method can be described as, thermogravimetric analysis (TGA) or thermogravimetry (TG). It is a technique of thermal analysis, where the mass of the sample is measured versus temperature or time. TGA allows for identification of the components of the sample, phase transitions and comparison of thermal stability. [45] In case of this master thesis it was used to compare the thermal properties of different hydrogels and calculation of the mass residue.

Figure 17. TGA/DSC 1 star system METTLER TOLEDO on which TGA measurements were taken.

Conditions for measurements for both PEG and alginate samples were equal. The sample holder was made of platinum with a volume of 70 µl, start temperature 30°C, end temperature 700°C, heating rate 10 °C/min. The gas used in this case was nitrogen with a gas flow of 50 ml/min.

Basing on TG and DTG curves of samples mean temperature of sample decomposition were established. Thanks to weight loss derivative I was able to mark on curves onset and endset temperatures of the mass drop and after that I calculated mean values.

$$T_d = \frac{T_o + T_e}{2}$$

$$\begin{array}{l} where \\ T_d - decomposition\ temperature\ [°\mathrm{C}] \\ T_o - onset\ temperature, when\ \ the\ mass\ drop\ started\ [°\mathrm{C}] \\ T_e - endset\ temperature, when\ the\ mass\ drop\ stopped\ [°\mathrm{C}] \end{array}$$

TGA technique also was used for computation of residual mass. I calculated the percentage of residual mass using this equation:

$$M_r = \frac{(M_i - M_e)}{M_i} * 100\%$$

$$\begin{array}{l} where \\ M_r - residual\ mass\ [\%] \\ M_i - initial\ mass\ [mg] \\ M_e - end\ mass\ [mg] \end{array}$$

4. Results and discussion

4.1. Images of the samples

4.1.1. PEG

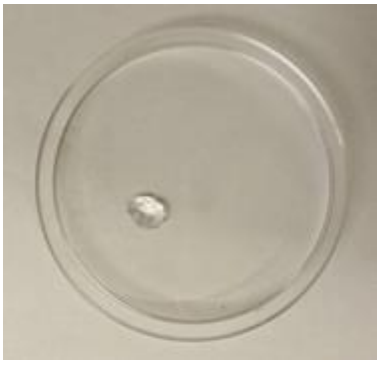
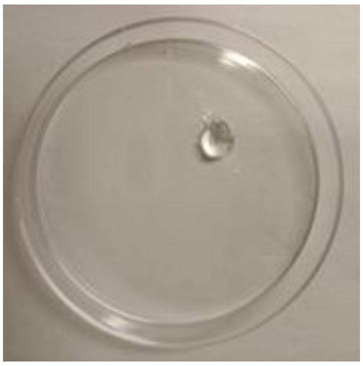
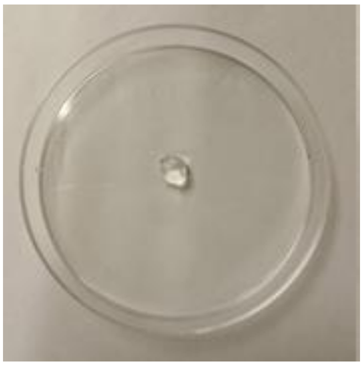
<table>
<tr><td colspan="3">PEG 3% B0</td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td colspan="3">PEG 3% BL</td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td colspan="3">PEG 3% BH</td></tr>
<tr><td></td><td></td><td></td></tr>
</table>

Figure 18. Pictures of PEG 3% samples

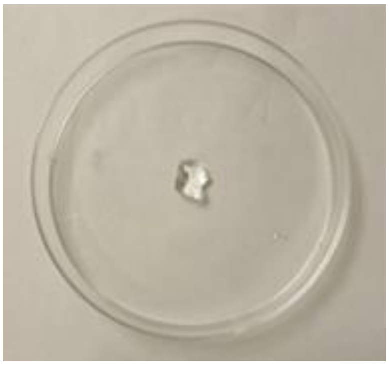
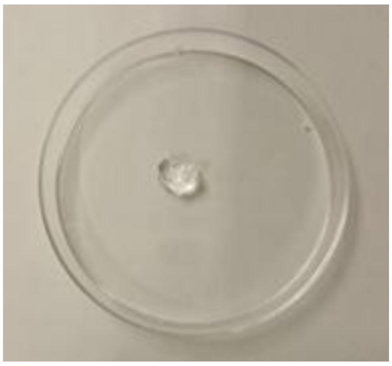
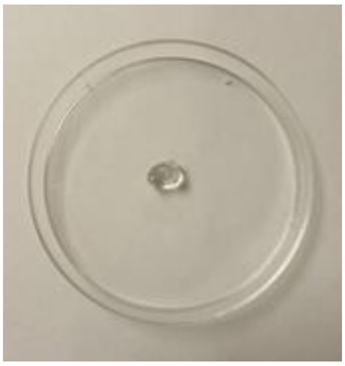
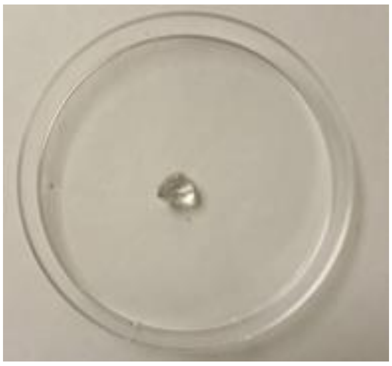
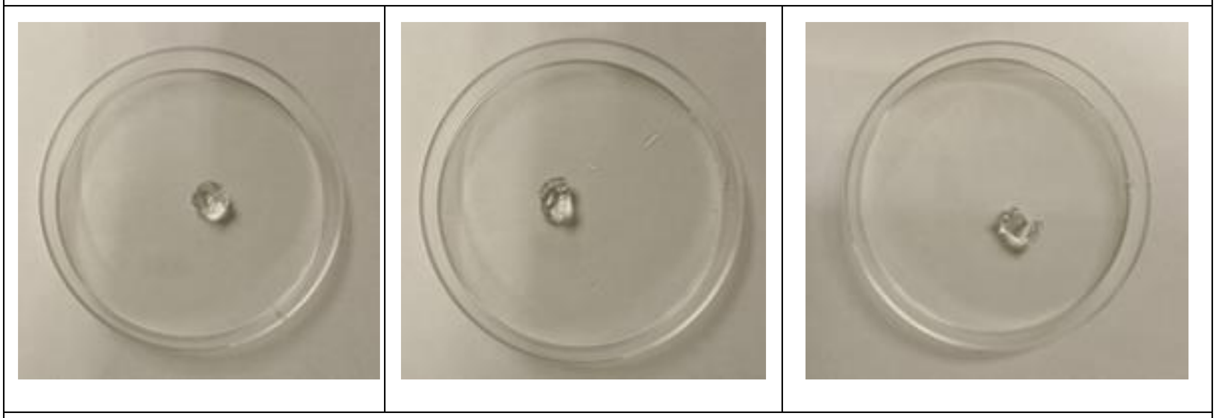
PEG 5% B0		
		
PEG 5% BL		
		
PEG 5% BH		
		

Figure 19. Pictures of PEG 5% samples

PEG 10% B0

PEG 10% BL



PEG 10% BH

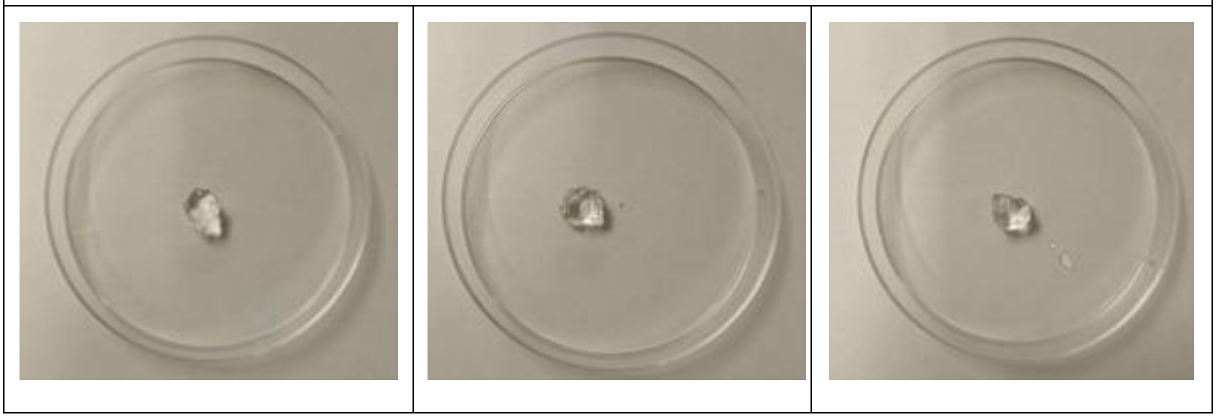


Figure 20. Images of PEG 10% samples

All of the hydrogels were transparent, colourless and odourless with a cylindrical shape. It was observed that the hydrogel stiffness increased monotonically with the content of boron. This was a simple observation detected in gel manipulation. However, hydrogels were resistant to damage using the spatula. To confirm those assumptions and receive reliable data further TMA tests were conducted.

4.1.2. Alginate

Alginate B0%

Alginate BL on the left and BH on the right

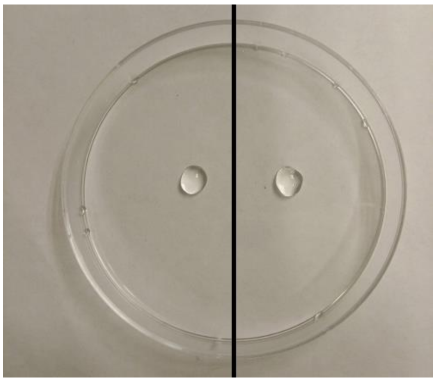


Figure 21. Images of Alginate samples

Alginate hydrogels were transparent, colourless and odourless. They possessed a droplet shape. The difference in stiffness among different hydrogels was detected. Only alginate control samples remained in a solid state after 12 minutes of gelation time. Interestingly, boron-loaded alginate hydrogels (BL and BH) remained in a liquid form, and furthermore, they remained injectable for more than a month with the syringe even though they could stay as semi-gelifyied hydrogels when deposited in the plate. All of the samples were easily destroyed during manipulation and for this reason no mechanical tests were performed on alginate hydrogels.

4.2. Boron release measurements

4.2.1. PEG

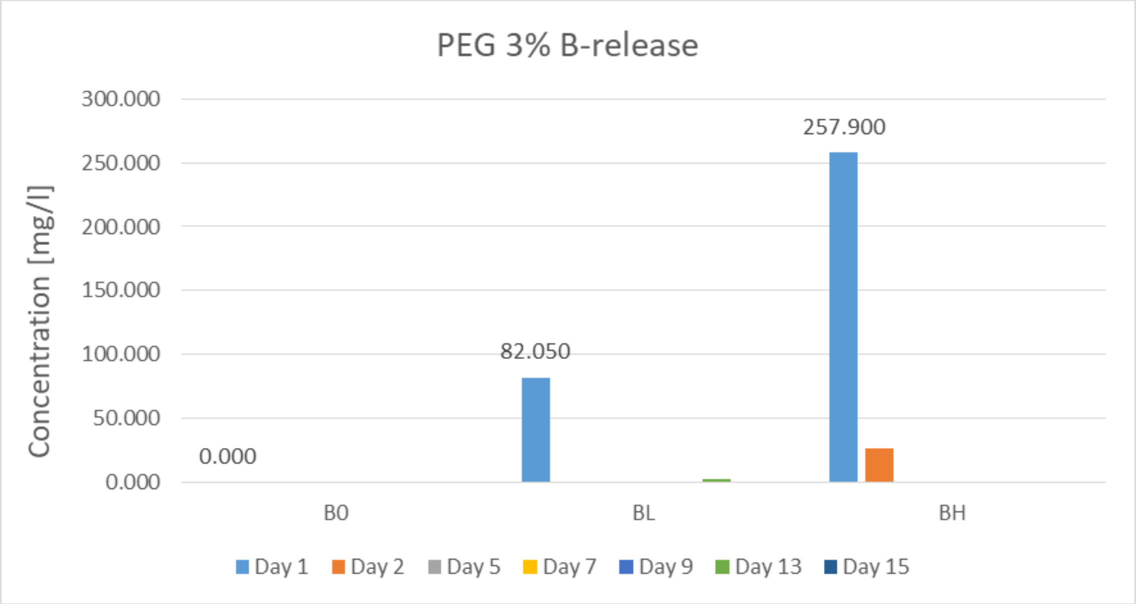


Figure 22. Boron-release from PEG 3% hydrogel samples.

Table 3. Boron-release from PEG 3% hydrogels.

PEG 3%							
Concentration [mg/l]	**Day 1**	**Day 2**	**Day 5**	**Day 7**	**Day 9**	**Day 13**	**Day 15**
B0	0,000	0,000	0,000	0,000	0,000	0,000	0,000
BL	82,050	0,000	0,000	0,000	0,000	2,146	0,000
BH	257,900	26,226	0,000	0,000	0,000	0,000	0,000

The results showed that almost all the boron is released after one day immersed in DPBS. For the sample PEG 3% BH, there exist a lower amount of boron-release on the second day. The assay was performed during 15 days but the boron-release during this period was undetectable. It can be concluded that the boron-release of PEG 3% hydrogels increases as the concentration of boron does in the sample.

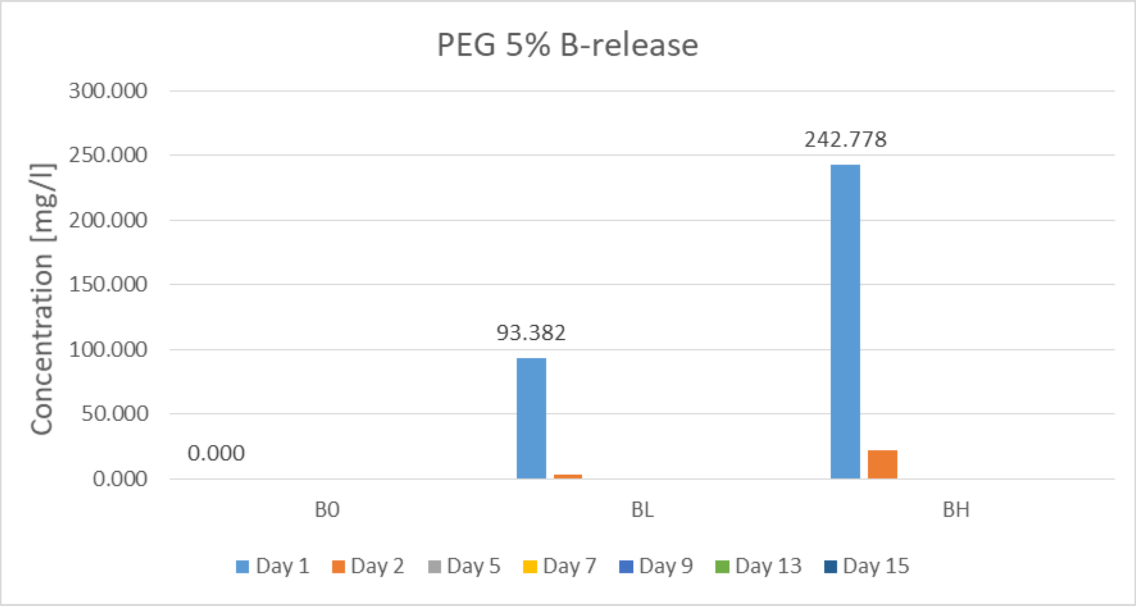


Figure 23. Boron-release from PEG 5% hydrogels.

Table 4. Boron-release from PEG 5% hydrogels

	PEG 5%						
Concentration [mg/l]	**Day 1**	**Day 2**	**Day 5**	**Day 7**	**Day 9**	**Day 13**	**Day 15**
B0	0,000	0,000	0,000	0,000	0,000	0,000	0,000
BL	93,382	2,755	0,000	0,000	0,000	0,000	0,000
BH	242,778	21,973	0,000	0,000	0,000	0,000	0,000

The results for PEG 5% hydrogels are in concordance with those obtained in PEG 3% hydrogels. It is visible that almost all the boron is released after one day immersed in DPBS. For the samples PEG 5% BL and BH, there is still a lower amount of boron-release on the second day. The assay was performed during 15 days but the boron-release during this period was undetectable. It can be concluded that the boron-release from PEG 5% hydrogels increases as the concentration of boron does in the sample.

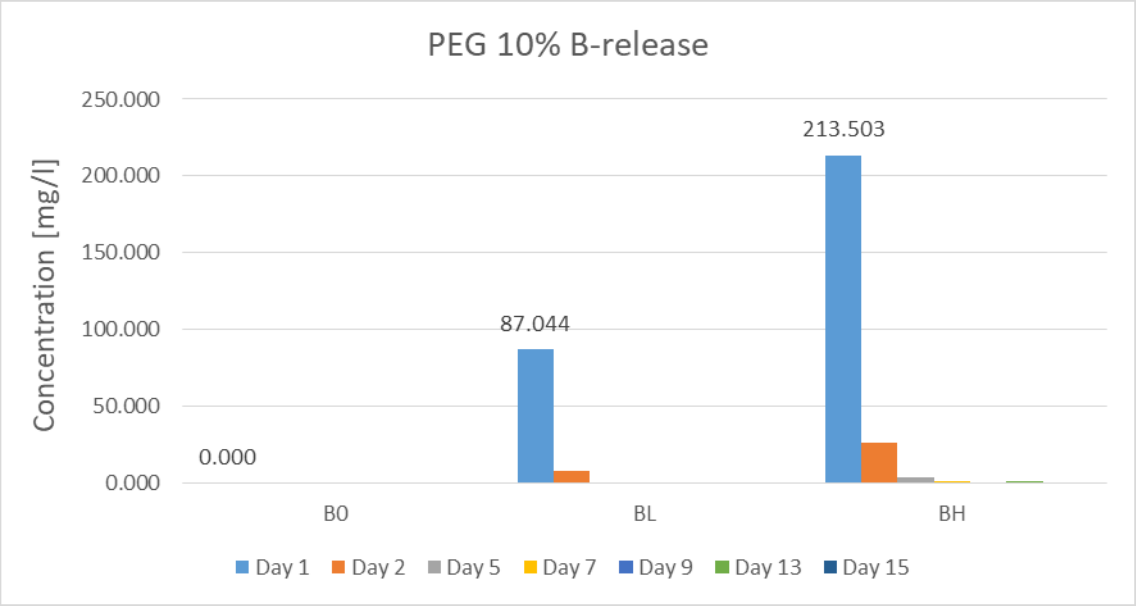


Figure 24. Boron-release from PEG 10% hydrogels.

Table 5. Boron-release from PEG 10% hydrogels.

	PEG 10%						
Concentration [mg/l]	**Day 1**	**Day 2**	**Day 5**	**Day 7**	**Day 9**	**Day 13**	**Day 15**
B0	0,000	0,000	0,000	0,000	0,000	0,000	0,000
BL	87,044	7,910	0,000	0,000	0,000	0,000	0,000
BH	213,503	26,208	3,468	0,110	0,000	0,013	0,000

Similar results were obtained in PEG 10% hydrogels. Boron-release was practically completed after the two first days. The assay was performed during 15 days but the boron-release during this period was undetectable. A lower amount of boron liberation from PEG 10% hydrogels is observed only in the BH condition until day 5 of incubation. It may be indicating that PEG 10% is capable of preserving more boron within the network. The conclusion can be made that the boron-release from all the PEG hydrogel compositions increases as the concentration of boron does in the sample, and that release is independent of the percentage of the polymer in the hydrogels.

All types of PEG hydrogels are liberating around 90 mg/l for BL and around 250 mg/l for BH samples.

4.2.2. Alginate

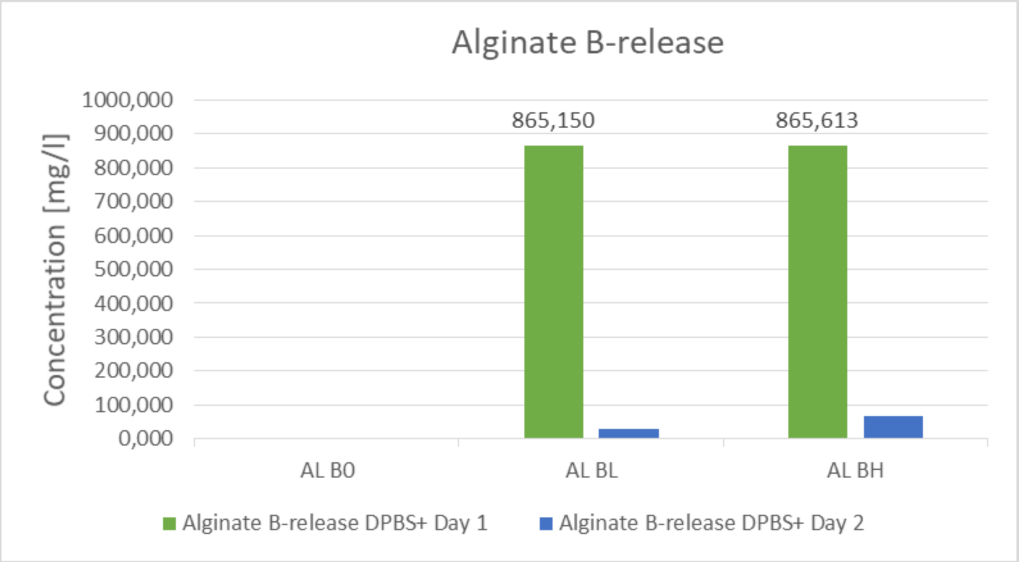


Figure 25. Boron-release from alginate hydrogels.

Table 6. Boron-release from alginate hydrogels.

	Alginate B-release	
Concentration [mg/l]	**Day 1**	**Day 2**
AL B0	0,000	0,000
AL BL	865,150	30,747
AL BH	865,613	66,747

This test was performed only for 2 days because alginate-based hydrogels dissolved themselves in DPBS after first 24 hours. It is very interesting that for both BL and BH types of hydrogels the amount of boron-released after the first day is almost the same.

4.3. Mechanical properties of PEG hydrogels

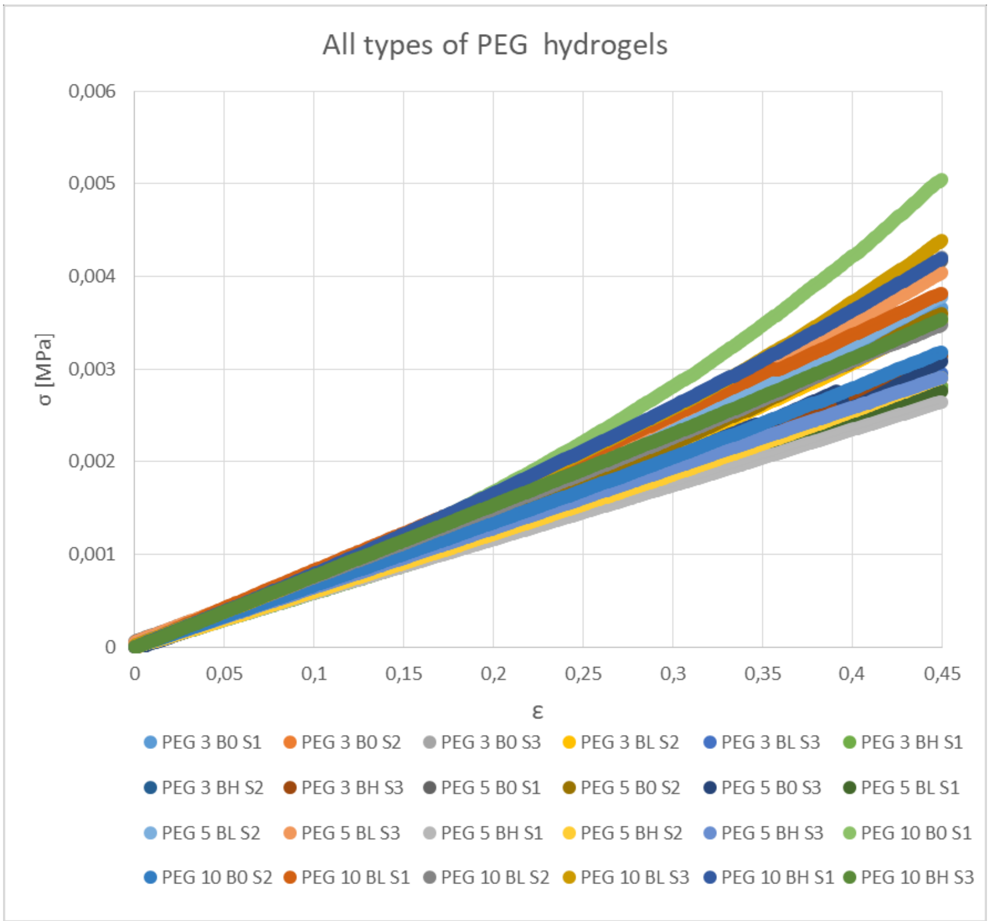


Figure 26. Stress – strain graph of all of the PEG hydrogels.

The collective graph shows curves of all samples, that have been tested with this method. Due to mechanical damages samples PEG 3% BL sample 1 PEG 10% B0 sample 3 and PEG 10% BH sample 2 were excluded from these tests. It is visible that samples with higher cross-linker content possess higher stress values.

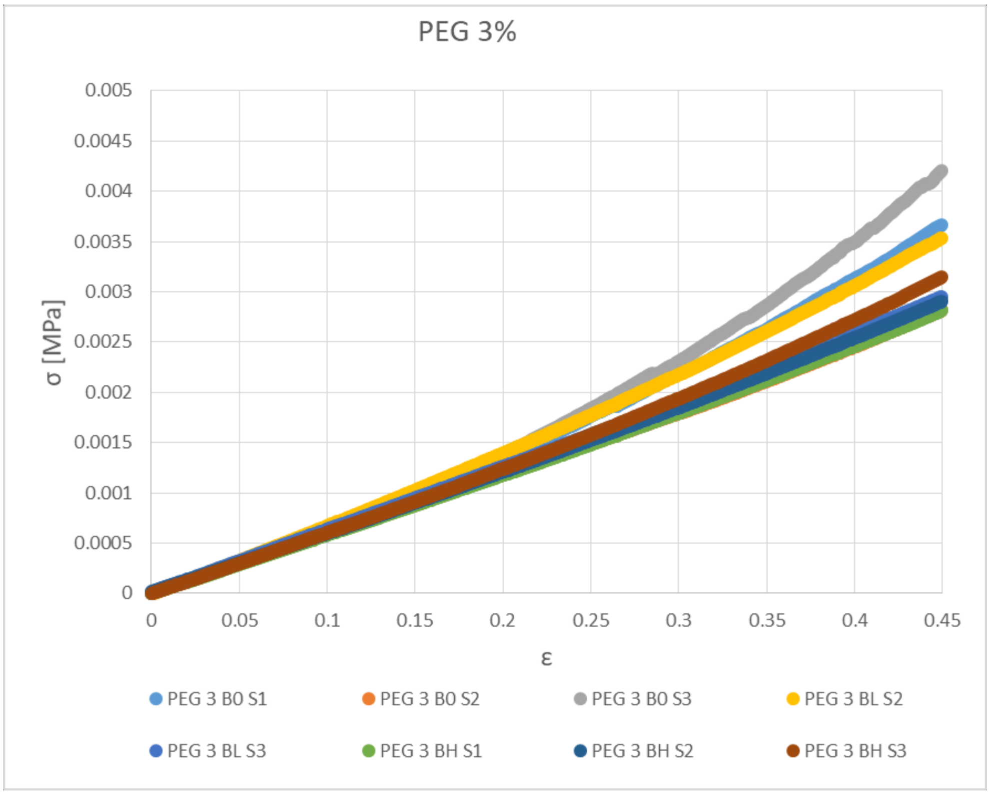


Figure 27. Stress – strain graph of PEG 3% hydrogels.

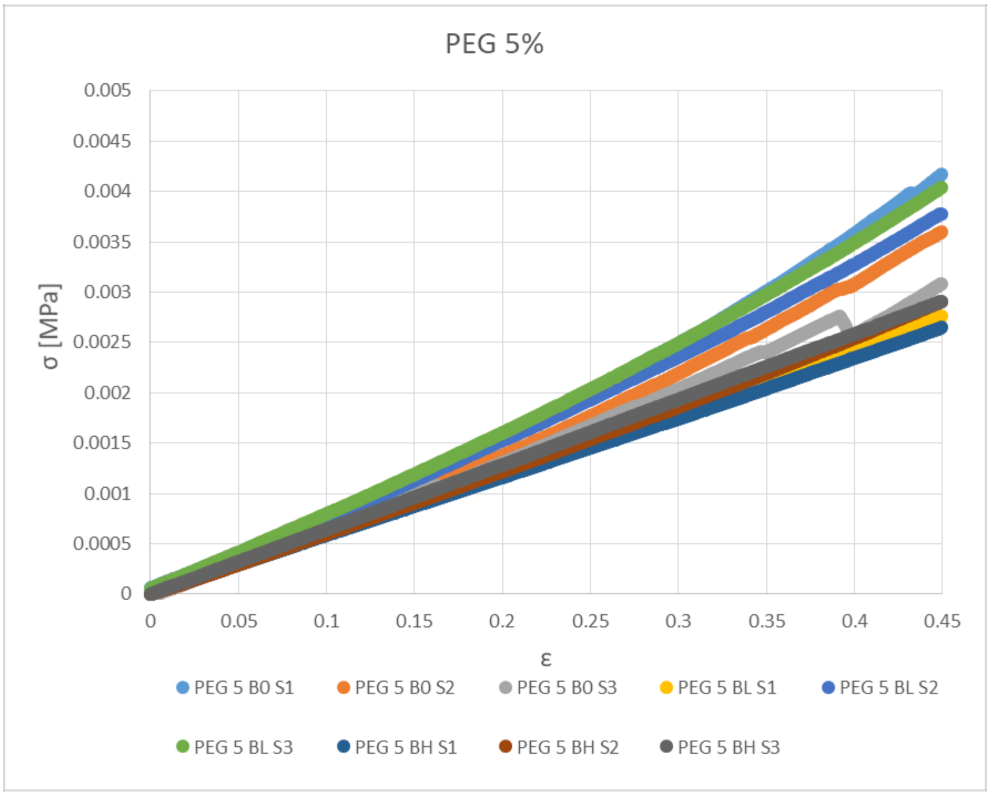


Figure 28. Stress – strain graph of PEG 5% hydrogels.

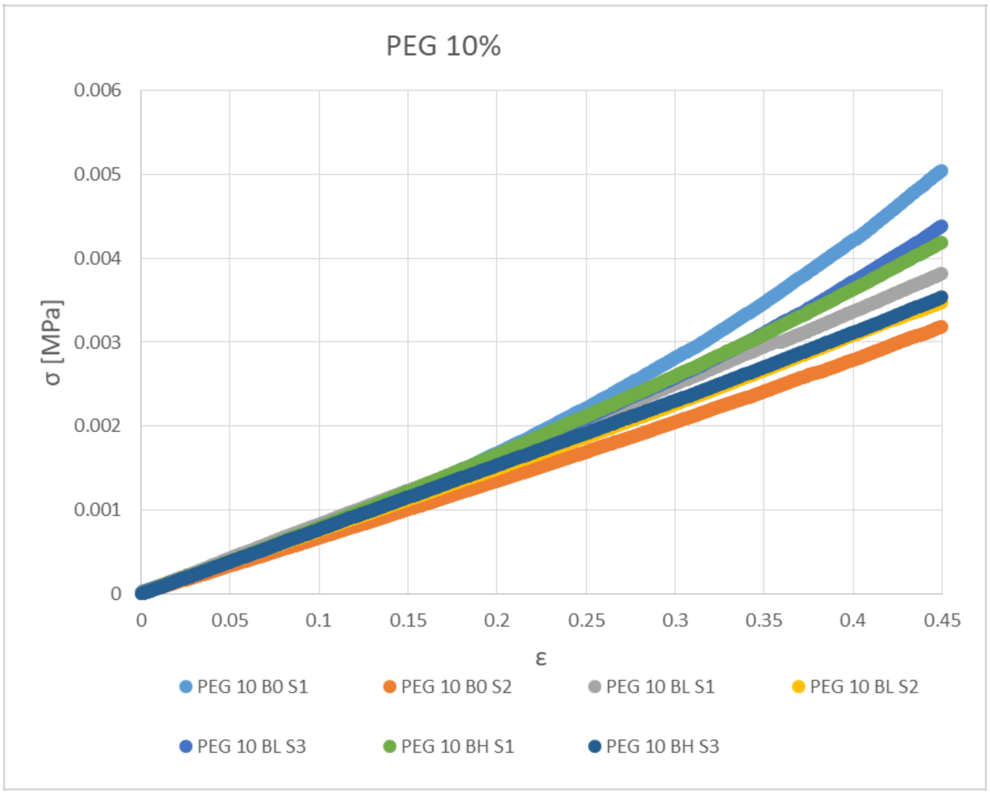


Figure 29. Stress – strain graph of PEG 10% hydrogels.

All stress-strain curves presented below show that mechanical properties are similar and comparable to each other. As explained in the materials and methods chapter, those curves were used to calculate Young modulus of hydrogels.

Table 7. Results of Young modulus values for PEG 3% samples.

PEG 3%					
Boron content	**Sample 1**	**Sample 2**	**Sample 3**	**Mean value [kPa]**	**Standard deviation**
B 0	6,386	7,764	5,413	6,521	1,181
B L	7,385	7,708	-	7,547	0,228
B H	6,600	6,169	6,144	6,304	0,256

Table 8. Results of Young modulus values for PEG 5% samples.

PEG 5%					
Boron content	**Sample 1**	**Sample 2**	**Sample 3**	**Mean value [kPa]**	**Standard deviation**
B 0	7,766	7,140	7,203	7,370	0,344
B L	11,108	11,215	10,936	11,086	0,141
B H	9,563	8,948	11,997	10,169	1,613

Table 9. Results of Young modulus values for PEG 10% samples.

PEG 10%					
Boron content	**Sample 1**	**Sample 2**	**Sample 3**	**Mean value [kPa]**	**Standard deviation**
B 0	9,289	10,206	-	9,747	0,649
B L	15,446	12,244	10,877	12,856	2,345
B H	11,620	-	13,551	12,586	1,366

Results allow to conclude that the higher amount of polymer and cross-linker in hydrogel the higher the value of Young modulus. Also for all the PEG samples, it can be seen that the addition of Borax increased the Young modulus values for both concentrations compared to control sample (in the case of PEG 3% inconsistent values should be a mistake). Since the Borax is used in medicinal chemistry as a chemical cross-linker, it was expected to obtain higher Young modulus values with higher contents of Borax. It needs to be mentioned that more reliable tests should be done by rheometer as this device is more appropriated and designed to specifically test the mechanical properties of liquid and humid samples.

It is interesting to note that that the Young modulus obtained results are in the same order of magnitude and similar to those corresponding to real tissues such as muscle, gland or skin tissue. [46] Considering the future biomedical application of these hydrogel-based material systems, it can be concluded that these systems accomplish the standards for muscle tissue studies and importantly borax addition does not affect the stiffness of the material.

4.4. Thermogravimetric analysis results

4.4.1. Borax

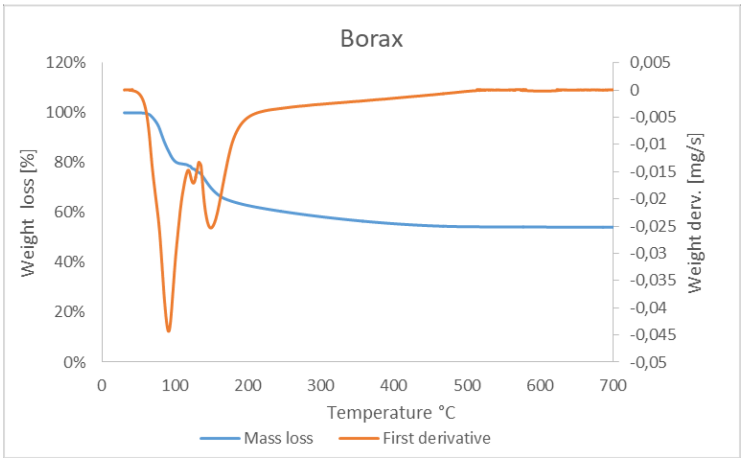


Figure 30. TGA and TGA derivative curve of Borax ($Na_2B_4O_7*10H_2O$).

TGA measurement of borax was performed to compare with TGA curves of created hydrogels. In the range of temperature up to 700°C, 54% of the initial mass is left as a residue. It suggests that created residues in case of hydrogel samples might contain boron.

4.4.2. PEG

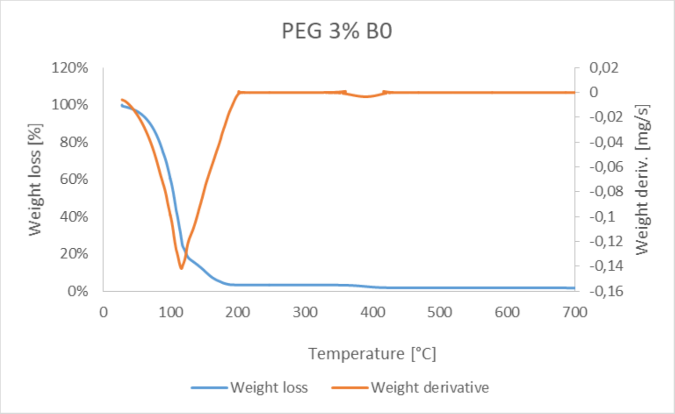


Figure 31. TGA and DTG of PEG 3% B0

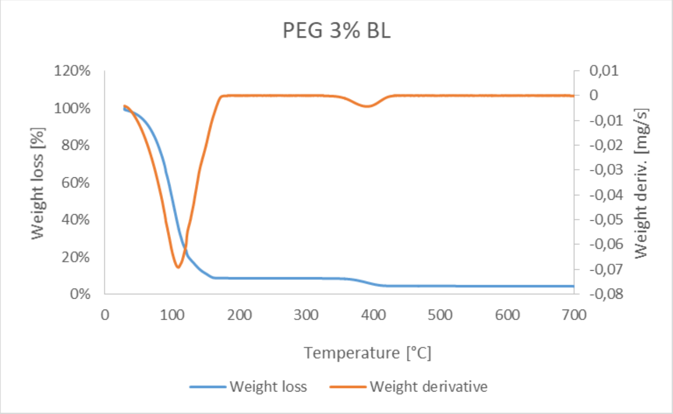


Figure 32. TGA and DTG of PEG 3% BL

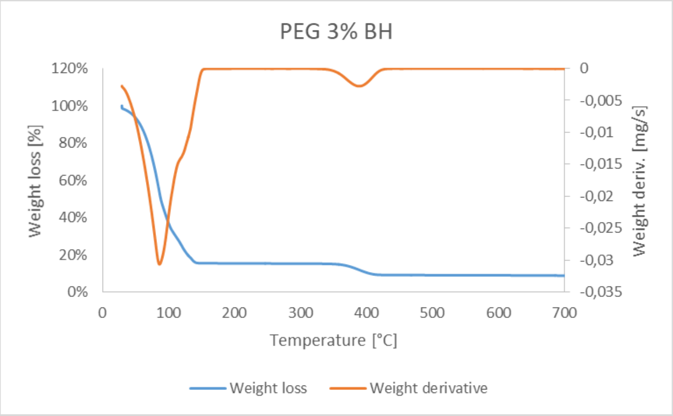


Figure 33. TGA and DTG of PEG 3% BH

As the hydrogel in majority consists of water for all samples there will be a visible peak around 100°C for mass loss and mass loss derivative. It corresponds to dehydration of the samples. Each PEG 3% sample present similarities with PEG TGA curve presented in the figure found in the literature. They possess a peak of PEG decomposition around temperature 390°C. Additionally, it can be observed that mass residue is increasing with the increase of boron concentration.

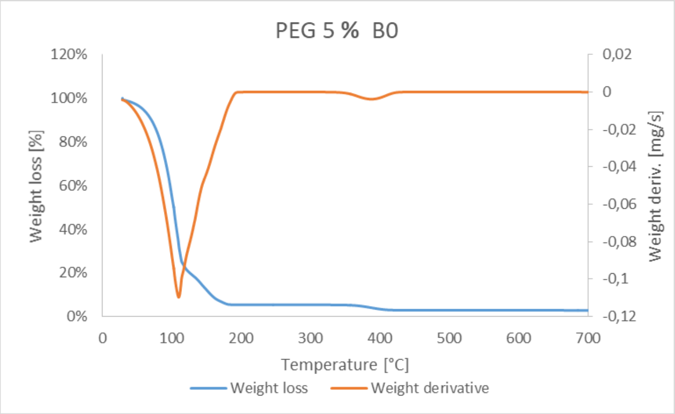


Figure 34. TGA and DTG of PEG 5% B0

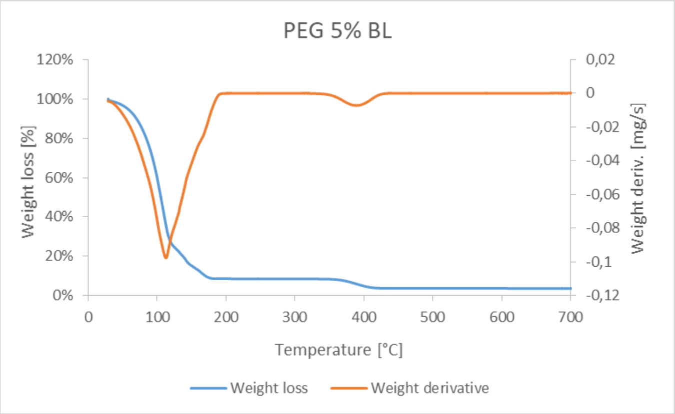


Figure 35. TGA and DTG of PEG 5% BL

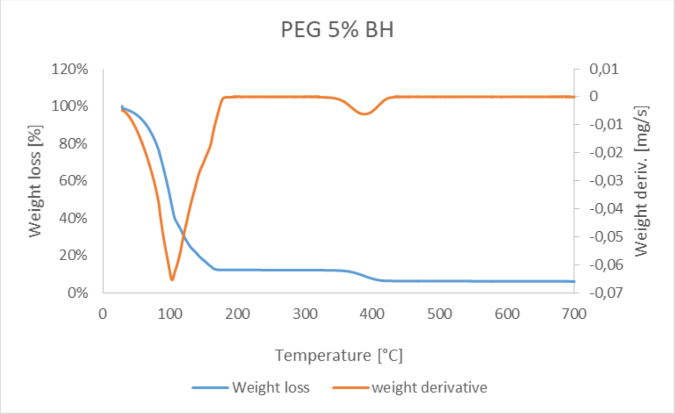


Figure 36. TGA and DTG of PEG 5% BH

Very similar observations can be taken for the samples made of PEG 5%. There are peaks corresponding to dehydration and sample decomposition at the same places as for PEG 3% samples. Similarly, the percentage of residue rises with the increase of boron content.

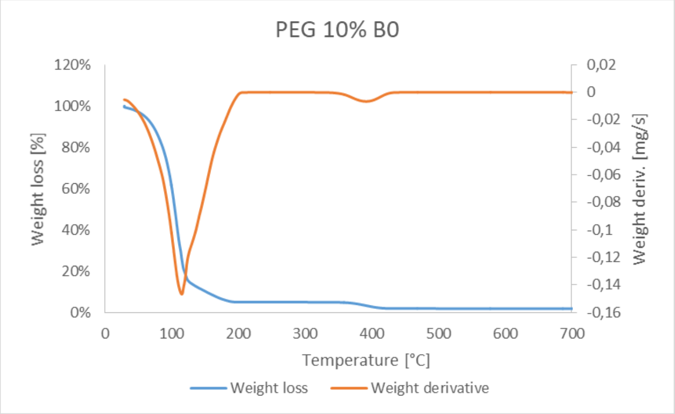


Figure 37. TGA and DTG of PEG 10% B0

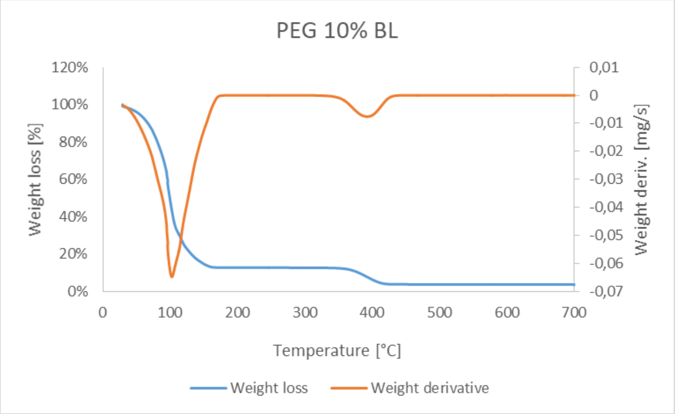


Figure 38. TGA and DTG of PEG 10% BL

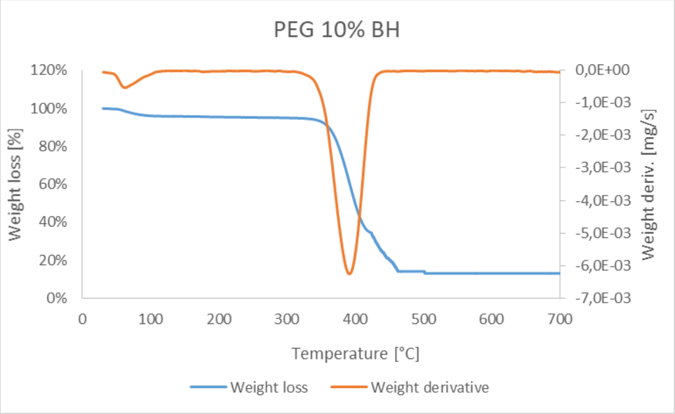


Figure 39. TGA and DTG of PEG 10% BH

Changes in mass loss and derivative of mass loss curves for sample PEG 10% BH can be explained with logistic restrictions. In fact, only 5 samples could be measured at one time. Which means that this sample has lost majority of water in room temperature, waiting for the experiment to be taken. That is why mass loss peak is much bigger compared with two other samples form PEG 10%. For samples based on PEG 10% peaks exist in the same place as for Peg 3% and PEG 5% and once again percentage of mass loss drops with increase of boron content.

All PEG hydrogels TGA curves without boron correspond to thermograms of PEG found in literature data and presented below [47]. Peak around 390°C is typical property of PEG.

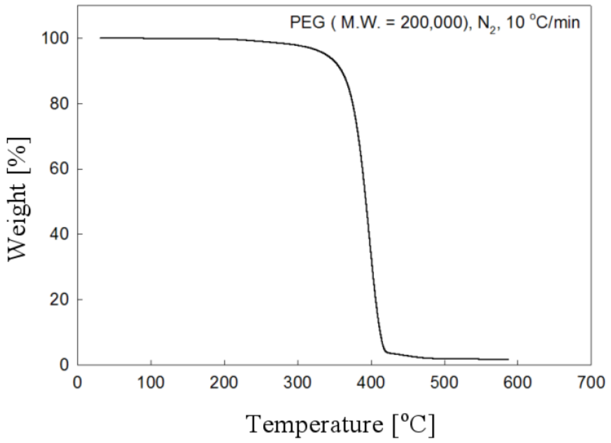


Figure 40. TGA curve for PEG [47].

The peak around 100°C is negligible as it is known that this is connected with dehydration. The decomposition temperature was stated based on the weight derivative DTG. This data was calculated to check whether the addition of boron will change the thermal properties of hydrogels. Results are presented in table 10.

Table 10. Mean decomposition temperature of PEG hydrogels.

Sample type	Mean temperature of decomposition [°C]	Standard deviation
PEG 3% B0	387,66	0,92
PEG 3% BL	388,72	0,40
PEG 3% BH	387,47	0,64
PEG 5% B0	386,66	0,80
PEG 5% BL	389,48	1,16
PEG 5% BH	387,85	0,64
PEG 10% B0	388,42	1,42
PEG 10% BL	390,60	0,65
PEG 10% BH	388,92	0,24

Based on the obtained results it can be stated that boron does not change the thermal properties of PEG hydrogels. The same conclusion can be drawn regarding the amount of polymer and cross-linker, it does not affect the thermal properties of PEG hydrogels.

Table 11. Residual mass percentage of samples and calculated percentage of boron for PEG hydrogels.

Sample type	Residual mass percentage	Mass percentage of boron
PEG 3% B0	2%	0%
PEG 3% BL	4%	2%
PEG 3% BH	9%	7%
PEG 5% B0	3%	0%
PEG 5% BL	4%	1%
PEG 5% BH	6%	3%
PEG 10% B0	2%	0%
PEG 10% BL	4%	2%
PEG 10% BH	13%	11%

For all of the PEG samples release tests were conducted before the thermogravimetric analysis was taken. Therefore residual mass percentage apply to boron cross-linked in polymer chain.

Calculated data are confirming that boron content does change the percentage of residual mass of the samples. It is an evidence that boron is building up into the PEG polymer chain.

4.4.3. Alginate

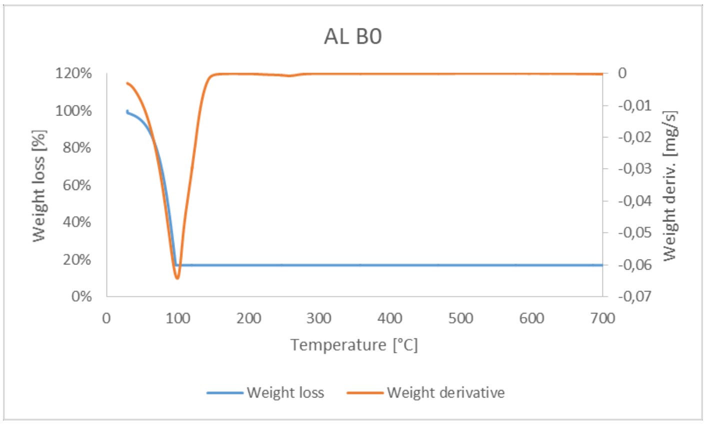


Figure 41. TGA and DTG of alginate B0

Alginate hydrogels likewise PEG hydrogels experienced problems with sample dehydration in room temperature. For alginate control sample the measurement was taken straight after placing it into TGA equipment. Due to this reason, a great percentage of this sample is water. Consequently, huge peak around temperature 100°C is visible. Close to the temperature 250°C there can be observed small peak typical for sodium alginate samples, which is comparable to the literature data presented below. The mass loss for this sample is quite big as a result of big water content.

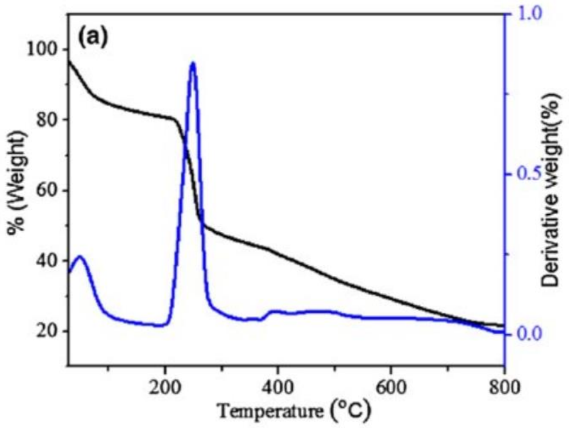


Figure 42. TGA and DTG od sodium alginate literature data [48]

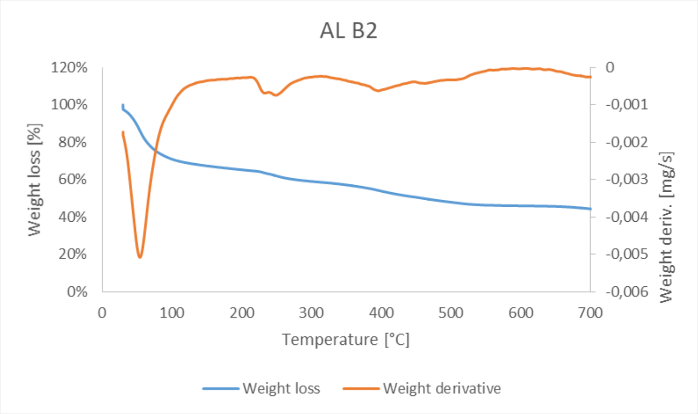


Figure 43. TGA and DTG of alginate BL

TGA curve for alginate sample with low boron content possesses more similarities to thermogram of sodium alginate from literature than the control sample. Just as in all hydrogel samples described before around temperature 100°C exists a peak corresponding to dehydration. Next on the DTG it is visible peak around temperature 250°C, it must be decomposition reaction, further on there are small changes visible around temperature 400°C. Comparing with the control sample this one is much stable as there is a higher amount of residue left.

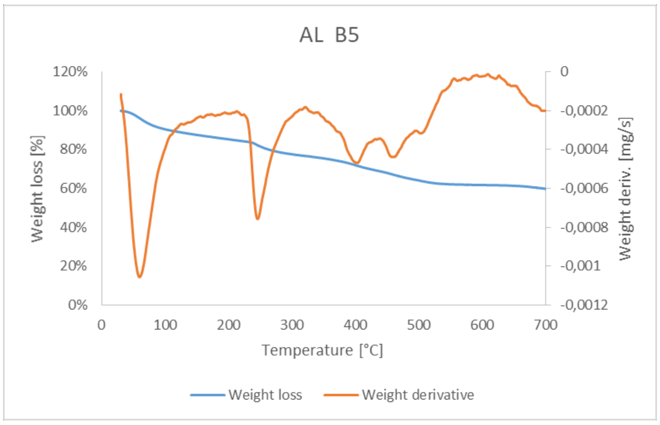


Figure 44. TGA and DTG of alginate BH

The last TG and DTG curve shows alginate with high boron content. The mass loss curve shape is similar to literature data and corresponds to the sample with low boron content. However, the amount of residue is the biggest of all. There are peaks around the temperature 250°C, 400°C, and 470 °C. First one is responsible for alginate decomposition but with the increase of the temperature there are visible peaks that were also present on low boron content sample, but samples presented on this figure are much stronger They are not corresponding either to borax nor pure alginate. Which allows for the conclusion that boron is reacting with alginate and thanks to that samples congaing boron are more thermally stable.

Table 12. Mean decomposition temperatures of alginate hydrogels.

Sample type	Mean decomposition temperature of first step [°C]	Standard deviation	Mean decomposition temperature of second step [°C]	Standard deviation	Mean temperature decomposition of third step [°C]	Standard deviation
AL B0	253,88	12,93	-	-	-	-
AL. BL	237,98	12,21	391,93	7,21	-	-
AL. BH	245,93	7,95	396,37	11,28	467,01	8,16

As already mentioned describing TGA curves, for samples containing there are two more decomposition reactions which is a confirmation that boron reacts with sodium alginate polymer chain.

Table 13. Residual mass percentage of sample and calculated percentage of boron for alginate hydrogels.

Sample type	Residual mass percentage	Mass percentage of boron
AL B0	17%	0%
AL BL	44%	27%
AL BH	60%	43%

For alginate samples, thermogravimetric analysis was taken omitting release study. Therefore residual mass percentage applies to total boron added to the sample. Calculated mass residues allows for the statement that with the increase of boron added to the hydrogel, mass residue rises as well.

5. Conclusions

The main goal of this thesis was to optimise the engineering of hydrogel-based material systems capable of sustained boron-release. Creation of the hydrogens was successful. This master thesis should be treated as the first step of development and optimisation of functionalised hydrogels for controlled boron-release.

Boron concentration measurements, TGA and TMA studies confirm that boron interacted with both hydrogels. As the quantity of boron inside the PEG hydrogel polymer chain is quite high it generates a possibility to release those ions during degradability of the sample.

Boron remains cross-linked in the PEG polymer chain independently from the concentration of PEG and its cross-linker.

Boron-loaded alginate hydrogels release similar boron concentrations. However, TGA results indicated that with the increase in the amount of boron, the percentage of residual mass increases.

Taking into consideration all performed tests it can be suspected that boron is covalently cross-linked with polymer chains.

Injectable hydrogels that remain injectable after a longer period of time (more than 1 month) were created, which is a promising tool for future in vivo applications.

As the results of performed tests are satisfying more research should be done. Suggested experiments are as follows:

- Rherological tests – to measure mechanical properties of injectable hydrogels and also repeating tests on PEG hydrogels as rheometer is designed for humid and liquid samples and might give more reliable data.
- Cell culture test – to confirm whether sodium alginate and PEG hydrogels containing boron are adequate scaffolds for tissue engineering applications.

Creation of tunable PEG hydrogels using cysteine-terminated peptides containing the cell adhesion sequences RGD or IKVAV and the VPM peptide as a cross-linker to produce protease degradable hydrogels.

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Budget

Budget estimation was bade basing on the number of materials for this Master Thesis project.

I was divided into 4 categories:

- Equipment – where pieces are given for hour of use,
- General material – in the meaning of materials that are not chemical reagents,
- Chemical reagents – prices given correspond to the amount of material used,
- Workforce

Table 14. Pricing of equipment used.

Equipment				
Number	Price per hour	Unit (h)	Name	Final price
1	1,00 €	4	Multi-task plate reader Victor III	4,00 €
2	0,95 €	270	TGA	256,50 €
3	0,15 €	2	Ultrasonic cleaner	0,30 €
	0,02 €	2160	Freezer (-80°C)	43,20 €
4	0,01 €	480	Fridge	4,80 €
5	0,50 €	10	TMA	5,00 €
6	0,75 €	432	Incubator	324,00 €
7	0,01 €	15	Balance	0,15 €
8	0,01 €	2	Vorex	0,02 €
9	0,10 €	48	Magnetic stirrer	4,80 €
10	0,10 €	15	Pipette 100-1000ul	1,50 €
11	0,10 €	15	Pipette 20-200ul	1,50 €
Total:				**645,77 €**

Table 15. Pricing of general material used.

General material				
Number	Price per unit	Unit	Name	Final price
1	5,00 €	20 units	96 well-plate	100,00 €
2	5,00 €	5 units	24 well-plate	25,00 €
3	50,00 €	1 pack	Eppendorf 1.5 ml	50,00 €
4	50,00 €	1 pack	Eppendorf 2 ml	50,00 €
5	10,00 €	1 pack	Latex Gloves	10,00 €
6	14,00 €	2 units	Borosilicate bottle 250 ml	28,00 €
7	10,00 €	2 units	Beaker	20,00 €
8	20,00 €	1 unit	Lab coat	20,00 €
9	15,00 €	1 unit	Goggles	15,00 €
10	22,00 €	1 unit	Gas mask	22,00 €
11	12,00 €	1 box	Syringe 1 ml	12,00 €
12	15,00 €	1 box	Syringe 0,5ml	15,00 €
13	8,00 €	1 box	Needle 0,5 mm	8,00 €
14	3,00 €	2 unit	Minisart Syringe Filter	6,00 €
15	10,00 €	1 unit	Fluid dispenser	10,00 €
16	12,00 €	1 unit	Spatula	12,00 €
17	15,00 €	1 unit	Laboratory magnet	15,00 €
18	35,00 €	1 pack	Pipette tips 100-1000ul	35,00 €
19	40,00 €	1 pack	Pipette tips 20-200ul	40,00 €
Total:				**493,00 €**

Table 16. Pricing of chemical reagents used.

Chemical regents				
Number	Price	Unit	Name	Final price
1	1 500,00 €	1 g	PEG-4MAL	1 500,00 €
2	294,00 €	1 g	PEG-dithiol	294,00 €
3	78,00 €	15 g	Borax	78,00 €
4	42,00 €	1 l	DPBS	14,00 €
5	34,00 €	100 g	Mannitol	34,00 €
6	10,00 €	50 g	Sodium alginate	10,00 €
7	159,00 €	5 g	Azomethine	159,00 €
8	13,90 €	25 g	L ascorbic acid	13,90 €
9	2,10 €	1 l	Mili-Q water	2,10 €
10	7,50 €	20 g	Na_2HPO_4	7,50 €
11	22,00 €	20 g	$CaSO_4$	22,00 €
12	10,05 €	500 ml	Kac buffer	10,05 €
Total:				**2 144,55 €**

Table 17. Pricing of workforce done.

Workforce				
Number	Price per hour	Unit (h)	Name	Final price
1	10 €	300	Student	3 000 €
2	17	30	Technical specialist	510
3	22	100	Supervisor	2200
Total:				**5 710,00 €**

Table 18. General pricing of Master Thesis.

Equipment	645,77 €
General material	493,00 €
Chemical regents	2 144,55 €
Workforce	5 710,00 €
All costs:	**8 993,32 €**