DEVELOPMENT OF BIOCOMPATIBLE POLYMER BASED FIBROUS MATERIALS FOR MEDICAL AND BIOLOGICAL USE

PhD Thesis

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1 Introduction

One of the most challenging tasks in medicine is the regeneration or complete replacement of damaged tissues. One of the solutions, or in other words, strategies, is the preparation of artificial matrices with embedded cells (preferably obtained by biopsy from the patient) and implantation of these matrices to the area of interest, where local regeneration might be induced. This field is called scaffold based tissue engineering or regenerative medicine. There are numerous requirements an artificial matrix must fulfill to mimick the structure of native tissues, such as biocompatibility, biodegradability and suitable chemical and last but not least physical structure.

Electrospinning enables the preparation of fibrous membranes with fiber diameters in the range of collagen fibers in natural tissues. Due to their inherent biocompatibility an obvious choice for basic materials would be the natural ones found in native tissues, such as collagen and hyaluronic acid. However, most of them are water soluble, and thus they tend to dissolve upon contact with biological fluids in the body. Although there are numerous non-water soluble polymers available, there is an emerging need for techniques making water soluble polymers available for electrospinning and finally for regenerative medicine. One solution can be the cross-linking of polymers, where in the case of water soluble polymers, a hydrogel is created, which tend to take up water and swell.

Hydrogel fibrous membranes resemble to the structure of native soft tissues especially when the

appropriate polymer is used for their preparation. However, the available techniques for the preparation of hydrogel-fibers based on electrospinning have to be broadened, since there is no general strategy for the preparation of such fibers. This is the main motivation of the research behind the thesis, which is based on a synthetic poly(amino acids) and their derivatives.

2 Objectives

The main objective of the research was the development of poly(amino acid) based fibrous membranes, which resemble to the native soft tissue and can be applied in biomedical fields. The development revolved around three major tasks as follows:

- Development of techniques and synthesis routes for the preparation of poly(aspartic acid) based fibrous hydrogel membranes based on electrospinning: it was in my interest to develop methods that can be applied not just in the preparation of cross-linked poly(aspartic acid) fibers but could be used as general strategies for cross-linked fiber preparation;
- Assessment of the physico-chemical properties of the hydrogel membranes: different techniques were developed for the preparation of hydrogel-fibers it was in my aim to compare these techniques and the resulting fibrous membranes. The most important parameters I was interested in were:
    - fibrous structure and fibrous property;

- productivity and basic characteristics (batch-to-batch or continuous fiber preparation) of the different techniques developed;
- since poly(aspartic acid) hydrogels show pH sensitive swelling properties in bulk forms, the pH sensitivity fibrous membranes was also an important property to be characterized;

- Evaluation of the biocompatibility and biodegradability of the membranes. Although polysuccinimide can be easily turned into poly(aspartic acid) in mild alkali medium, this transformation have never been shown to happen *in vivo* before. Therefore I wanted to prove the presumed hydrolysis and see the biological response to the polysuccinimide based membranes by implanting them into albino rats.

3 Methods

3.1 Synthesis of polysuccinimide (PSI) and thiol modified polysuccinimide (PSI-CYSE)

Polysuccinimide (PSI) was synthesized by the thermal poly-condensation of L-aspartic acid at 180 ^{o}C in vacuum in the presence of phosphoric acid catalyst (**Figure 1**). After the 7 hours long synthesis the polymer was dissolved in DMF then precipitated in distilled water and finally filtered. The product was washed with distilled water till the supernatant became pH neutral. The molecular weight of PSI was characterized based on viscometry (Sine-wave Vibro Viscometers (SV-10),

A&D Company, Limited, Japan) and the Kuhn-Mark-Houwink equation [1-3].

The succinimide repeating units of PSI react with primary amines at room temperature without any catalyst (**Figure 1**).

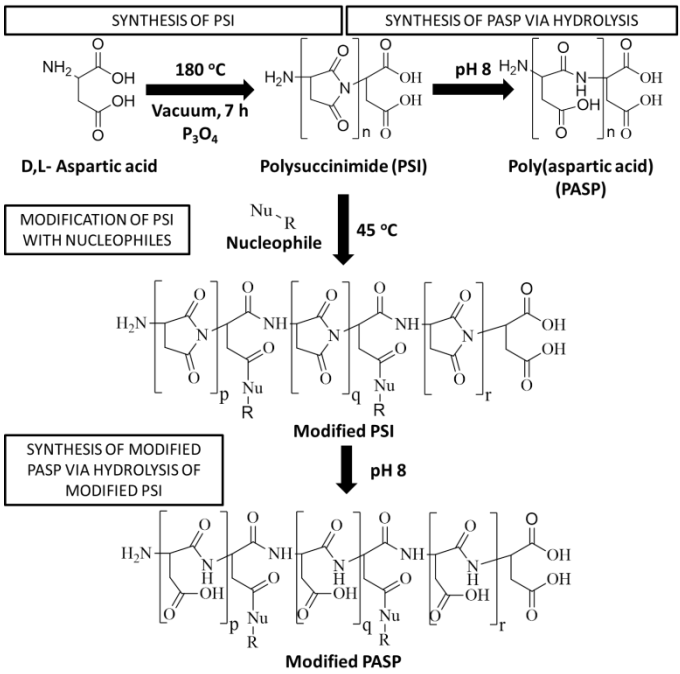

Figure 1 Synthesis and modification of PSI and hydrolysis of PSI to PASP

Consequently, PSI chains can be cross-linked by using bi- or multifunctional amines. PSI was grafted with cysteamine (CYSE), which has both amine and thiol functional groups. Since in the presence of oxygen the thiol groups of cysteamine can react with each other (forming disulfide bonds), the reaction was performed

under nitrogen or argon atmosphere. The amount of thiol groups on the main polymer chain was adjusted by the molar ratio between the repeating units and cysteamine [3-4]. The chemical structure of the polymers was investigated by FT-IR, NMR spectroscopy.

3.2 Preparation of polysuccinimide based fibers by standard, reactive and coaxial electrospinning

For the preparation of fibrous membranes, a home-made electrospinning instrument was used consisting of a glass syringe (Fortuna Optima 7.140-33) with a metal Hamilton tip (blunt and G21), a syringe pump (KD Scientific KDS100). The positive electrode was attached to the metal tip whereas the negative electrode (ground) was attached to the collector, made of tinfoil in front of the needle in a well determined distance of 15 cm (**Figure 2**). The voltage applied between 6 and 20 kV was provided by a DC power supply (GENVOLT 73030P) [2-3]. PSI concentration was varied between 15 w/w% and tip to collector distance between 15 and 25 cm to find the optimal setup for the preparation of PSI fibers. Cross-linked fibrous membranes were prepared by two distinct strategies. In post electrospinning methods, first PSI based membranes were prepared then those were immersed into the solution of 1,4-diaminobutane (DAB) or 2,2,4(2,4,4)-trimethyl-1,6-hexanediamine (THD) for different amounts of times ranging from minutest to several hours [1]. In reactive electrospinning, either the thiol modified PSI (PSI-CYSE) was electrospun to yield disulfide cross-linked PSI fibers (PSI-CYS) or in a coaxial needle (needle-in-needle) PSI was pumped in the

shell and the cross-linker (THD) was pumped in the core to yield cross-linked PSI (PSI-THD or by the addition of poly(ethyleneoxid) PSI-THD-PEO) [1]. For the preparation of fibrous membranes with real 3D network, magnetite particles were mixed in different concentration to the electrospinning of PSI-CYSE. Fibrous properties and average fiber diameters were investigated by light, atomic force and scanning electron microscopy [1, 3].

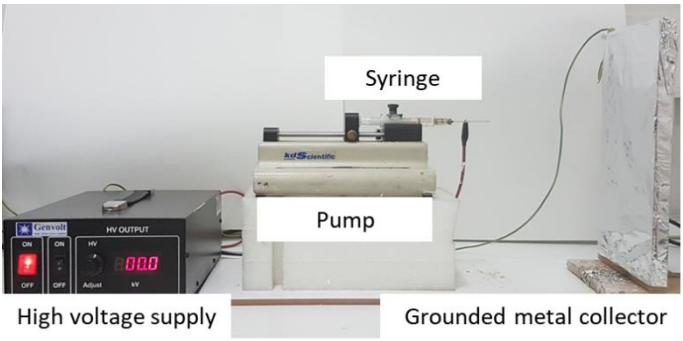

Figure 2 Setup of electrospinning machine

3.3 Preparation and properties of poly(aspartic acid) based gel fiber membranes

Poly(aspartic acid) based fibrous membranes were prepared by the mild alkaline hydrolysis of PSI based membranes (**Figure 1**). PASP based networks are denoted by changing the PSI part in the original sample name to PASP. For example, PSI-CYS after hydrolysis is denoted as PASP-CYS, etc. PASP based membranes show pH dependent swelling properties, which was investigated by immersing THD and DAB cross-linked membranes into pH 3 and pH 8 buffer solutions for

several cycles. After the swelling or de-swelling has finished the size of the membranes were measured by a caliper [1].

3.4 *In vivo* biocompatibility and biodegradability study of 1,4-diaminobutane and disulfide cross-linked polysuccinimide fiber membranes

Biocompatibility and biodegradability of electrospun PSI-CYS and PSI-DAB samples were investigated on 24 male Wistar rats (250 g), 12 for each sample type. Pretreated samples (pressed and wetted by physiological saline) were implanted sub cutan into the nuchal region of the animals and fixed by one stitch. Termination and sample retrieval was performed after 3 days (Group A) and 7 days (Group B). Samples were then preserved in formaldehyde and sent off for histological evaluation, whereas in the case of PSI-DAB, SEM micrographs were taken from retrieved and freeze-dried samples. The experimental protocol adhered to rules laid down by the Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes and was approved by the Semmelweis University's Institutional Animal Care and Use Committee. The accreditation number of the laboratory is 25/2/2015.

For histopathology samples were gathered for both PSI-DAB and PSI-CYS at 3 and 7 days after implantation. For the evaluation the standard preparation and hematoxylin-eozin staining was applied.

4 Results

The synthesis of polysuccinimide yielded a white grayish powder. According to the measurements, the average molecular weight of PSI varied between 23000 and 31000 g/mol. The chemical structure was identified by both ^{1}H-NMR and FT-IR spectroscopies [3-4].

4.1 Preparation of polysuccinimide fibers by electrospinning (T1)

At lower concentrations than 22.5 w/w% PSI solution only electrospraying, thus particle preparation was obtained, whereas at 22.5 w/w% fibers with beads and finally at 25 w/w% smooth fibers were prepared with according to SEM an average fiber diameter of 911 ± 41 nm (**Figure 3a and b**). The collector distance did not affect fiber diameter, however it affected the deposition area of fibers. The higher the distance was, the bigger was the area where the fibers were deposited [3].

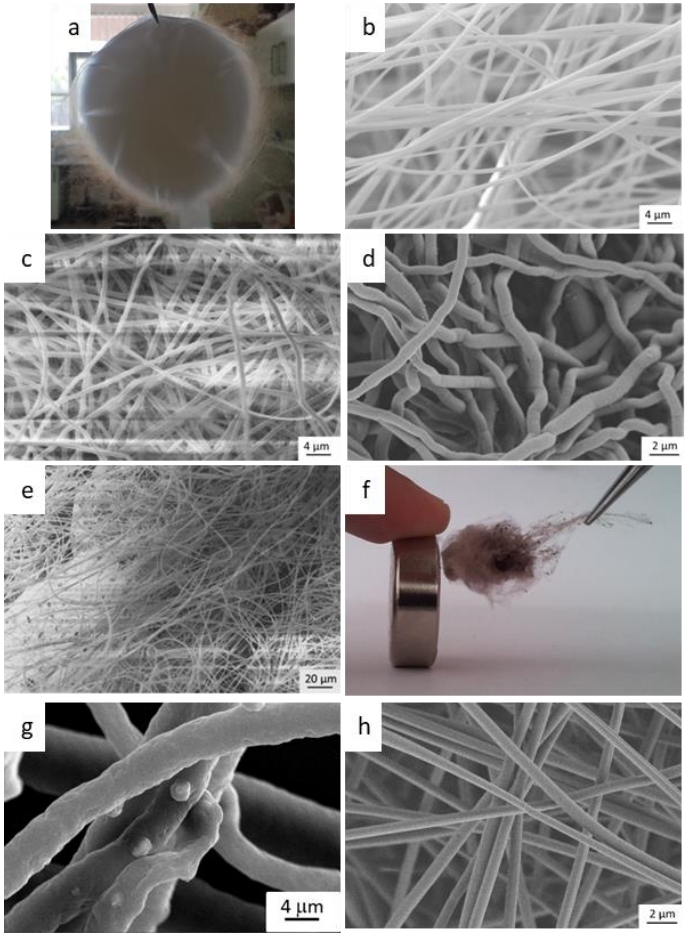

Figure 3 Electrospun PSI fiber membrane (a) fibers (b); PSI-DAB fibers (c), PSI-THD fibers (d), PSI-CYS fibers (e), magnetic particle loaded PSI-CYS fiber membrane attracted by magnet (f) and a SEM micrograph of the same sample (g) PSI-THD-PEO fibers (h)

4.2 Cross-linked polysuccinimide fibers prepared by post-electrospinning cross-linking (T2)

The fast heterogeneous reaction between the succinimide rings in the PSI fibers and DAB (**Figure 3c**) or THD (**Figure 3d**) yielded smooth defectless fibers with average diameters close to that of the original PSI fibers' before cross-linking. For the optimal dipping time (reaction time) in the case of DAB it was found, that a minimum of 5 minutes is necessary for the preparation of enough cross-links for the membranes not to dissolve and 10 to maintain fibrous properties. However at least 1 hour was necessary to obtain completely stable, cross-linked fibrous membranes. In the case of THD a minimum of 3 hours was necessary to obtain defectless fibers [1].

4.3 Reactive electrospinning based on thiol-disulfide chemistry (T3)

After careful optimization of the reaction time in the preparation of PSI-CYSE was found to be 50 minutes, which enabled the electrospinning of smooth fibers for an hour (**Figure 3e**). Although the synthesis was carried out in sealed container the cross-linking of thiol groups started in the syringe used in electrospinning, inevitably leading to the constant raising of solution viscosity and finally to gelation. After an hour the solution of PSI-CYSE gelated and blocked the nozzle. It was found, that at the beginning of electrospinning, fibers with diameters around 90 ± 30 nm

are prepared, while at the end of electrospinning with diameters around 850 ± 40 nm [3].

4.4 Fibrous membranes with real 3D structures prepared based on the thiol-disulfide reactive electrospinning and the addition of magnetic particles (T4)

The addition of magnetic particles into the electrospinning of PSI and PSI-CYSE led to the preparation of magnetic particle doped fibrous membranes. Due to the presence of magnetic particles, the color of the fibers turned from whitish to black. Furthermore, the viscosity and fiber diameter risen significantly (1.43 ± 0.5 μm) in the case of mixing PSI and the particles. Magnetite had a huge impact on the electrospinning of PSI-CYSE. When both magnetite and thiol concentration was high, the mixtures gelated even before electrospinning. However, at lower concentrations for both, membranes with real 3D structure could be prepared with responsivity to external magnetic field (**Figure 3f and g**) [2].

4.5 Continuous cross-linked fiber preparation developed based on coaxial reactive electrospinning (T5)

Continuous preparation of cross-linked fibers was developed based on a coaxial needle setup and the rapid cross-linking reaction between PSI and THD. With the addition of PEO into the solution of THD, smooth fibers were prepared with an average fiber diameter of 320 ± 42 nm (**Figure 3h**) [1].

4.6 Preparation of pH sensitive poly(aspartic acid) based gelfiber membranes (T6)

PASP based fibrous membranes were prepared by the mild alkali hydrolysis of cross-linked polysuccinimide based fibrous membranes. In all cases, the chemical transformation slightly changed the fibrous structure: the fibers were loosely interconnected and few narrow and flat sheets were found in different degrees in all of the samples. According to SEM the average diameter of non-fused PASP-DAB fibers was 940 ±60 nm (**Figure 4a**). During this chemical transformation, the volume of gels changed slightly. A macroscopic picture taken of a PASP-CYS membrane can be seen on **Figure 4b**. It is also important to note, that opposed to bulk PASP gels these fibrous membranes are very flexible and rubberlike, withstanding bending without any damage, however, their transparency is worse due to the fibrous structure. It can be clearly seen that the fibers in PASP-CYS (**Figure 4c**) and PASP-THD-PEO (**Figure 4d**) fused together forming an interconnected web, and thus determination of the fiber diameter distribution was not possible [1, 3].

The pH responsive properties of PASP based membranes were tested on PASP-DAB and PASP-THD-PEO samples by measuring the size of the fiber membranes in different buffers. It can be seen on **Figure 5** that compared to the dry membranes the samples in alkali medium swelled significantly whereas in acidic pH their size decreased to about their initial sizes before modification and hydrolysis. Compared to their original

sizes in pH 8 and pH 3, PASP-DAB samples reached the same sizes with only a small error throughout the 4 cycles in pH 8 and pH 3.

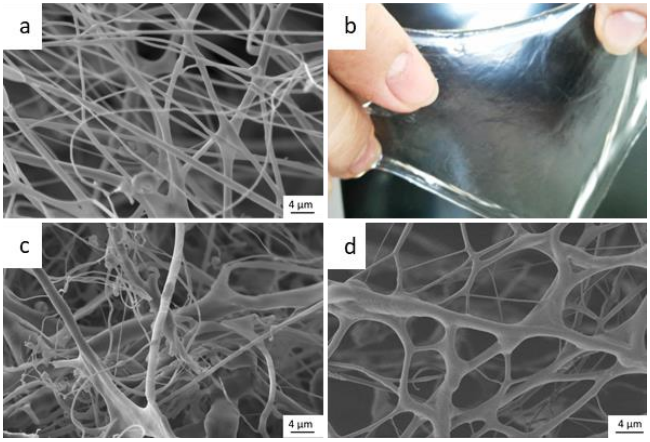

Figure 4 SEM micrograph of PASP-DAB fibers (a), picture taken of a PASP-CYS membrane (b), SEM micrographs of PASP-CYS (c) and PASP-THD-PEO fibers (d)

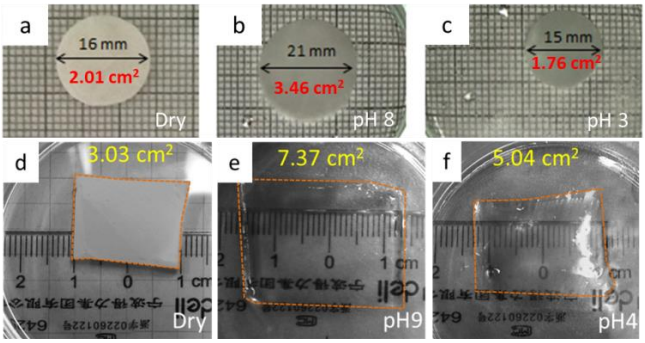

Figure 5 Dry PSI fibrous membrane (a), PSI-DAB in pH 8 buffer solution (b) and in pH 3 (c), PSI-THD-PEO membrane (d), PASP-THD-PEO membrane in pH 9 (e) and in pH 4 buffer solutions (f)

4.7 Biocompatibility and biodegradability of polysuccinimide based cross-linked fibrous membranes (T7)

Hydrolysis of polysuccinimide based membranes, and thus their transformation into poly(aspartic acid) based membranes was observed in albino rats *in vivo*. Both the disulfide and 1,4-diaminobutane cross-linked membranes went through hydrolysis during the first 3 days after implantation and caused foreign body reactions with acceptable severities without any toxicity (**Figure 6a and b** shows a PSI-DAB membrane 3 days after implantation). It is also important to note, that the size change caused by hydrolysis *in vivo* is the same as it was observed *in vitro*. 7 days after implantation PSI-CYSE membranes were either not visible macroscopically or were diminished in size and covered in new tissue (**Figure 6d**). However, they were still visible under a microscope (**Figure 6e**). PSI-DAB membranes were weaker and easily truncated by tweezers (**Figure 6c**). In the case of both types of membranes granulation tissue composed of fibroblasts and newly formed vessels had developed surrounding the membrane with numerous histiocytes present in the close vicinity without any sign of acute inflammation proving the incorporation of the membrane into the native tissue (**Figure 6e and f**). However, deep tissue invasion into PSI-DAB membranes was observable in contrast to PSI-CYSE membranes, where it could not be observed (**Figure 6f and g**).

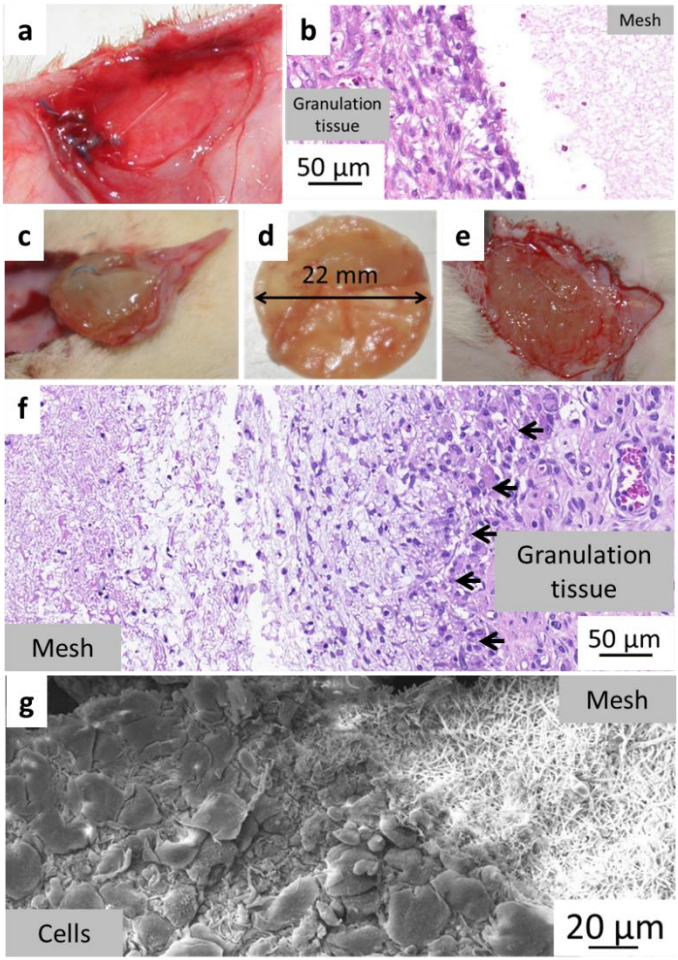

Figure 6 PSI-CYS 7 days after implantation (a) and its histopathological slide (b), PSI-DAB 3 (c, d) and 7 days (e) after implantation, histopathological slide (f) and SEM image (g) of PSI-DAB 7 days after implantation

5 Conclusion

Polymeric, fibrous membranes prepared by electrospinning are emerging materials in biomedicine. However, there is still need for new polymers involved in research. Poly(amino acids) with their peptide like chemical structure predict biocompatibility and biodegradability and thus they are perfect candidates for bio-based material research. However, poly(amino acids) are water soluble, that severely hinders their application in electrospun medical implants since they immediately dissolve in contact with the aqueous based biological fluids. Therefore the main objective of my research was to develop methods -based on electrospinning- that enable the use of water soluble polymers, specifically poly(aspartic acid) and its derivatives as fibrous membranes for implantation. In this work first fiber formation of polysuccinimide was optimized (**Figure 3a and b**) and then three methods were developed for the preparation of cross-linked polysuccinimide and - by their hydrolysis - cross-linked poly(aspartic acid) based fibrous membranes: a post electrospinning method, where the previously prepared fibers were dipped into the cross-linkers solution (DAB, THD) (**Figure 3c and d**); a reactive electrospinning based, where the thiol group (CYSE) modified polysuccinimide created cross-links during electrospinning (**Figure 3e**); a continuous method based on coaxial electrospinning, where the cross-linker (THD) was fed into the core and the polymer into the shell of the needle (**Figure 3h**). Also, by the addition of magnetite particles into the electrospinning solution,

membranes with real 3D structure were created (in cm size) (**Figure 3f and h**). By mild alkaline hydrolysis of polysuccinimide based systems, poly(aspartic acid) based membranes were prepared. Due to the chemical transformation, the fibrous structure was altered, as fibers fused together in different degrees in all sample types (**Figure 4**)

The poly(aspartic acid) based cross-linked membranes showed similar pH sensitivity to bulk hydrogels of similar chemical structures: the fibrous membranes overall size got smaller in acidic pHs whereas in alkali pH they grew (through several cycles) (**Figure 5**). The biocompatibility and biodegradability of two cross-linked polysuccinimide based systems were tested *in vivo* in albino rats (**Figure 6**). These experiments showed mild foreign body reaction and tissue invasion into the membranes after one week as well as proved the hypothesis that polysuccinimide undergoes hydrolysis in biological environment.

The developed methods can be generalized for other polymeric systems broadening the available water soluble polymers for fibrous implants. Also the developed poly(aspartic acid) based fibrous membranes could be good candidates for drug delivery, implantation, cell culturing and other biomedical applications.

The new scientific accomplishments are collected in the following points:

T1. Important parameters in the preparation of polysuccinimide fibers by electrospinning were assessed.

T2. Cross-linked polysuccinimide fibers were prepared by immersing polysuccinimide fibers into the solution of cross-linker's solution post-electrospinning.

T3. Development of a reactive electrospinning strategy based on thiol-disulfide chemistry using a modified polysuccinimide with thiol side-groups.

T4. Preparation of fibrous membranes with real 3D structures were based on the thiol-disulfide reactive electrospinning and the addition of magnetic particles.

T5. Continuous cross-linked fiber preparation was developed based on coaxial reactive electrospinning.

T6. pH sensitive poly(aspartic acid) based gelfiber membranes were successfully prepared.

T7. Polysuccinimide based cross-linked fibrous membranes (-S-S- and DAB) goes through hydrolysis *in vivo* and are biocompatible.

6 Publications of the candidate

6.1 Publications related to the thesis

[1] Molnar K, Jedlovszky-Hajdu A, Zrinyi M, Jiang S, Agarwal S (2017) Poly(amino acid)-Based Gel Fibers with pH Responsivity by Coaxial Reactive Electrospinning. Macromol Rapid Commun 201700147:1700147. IF_{2016}: 4.638

[2] Jedlovszky-Hajdu A, Molnar K, Nagy PM, Sinko K, Zrinyi M (2016) Preparation and properties of a magnetic field responsive three-dimensional electrospun polymer scaffold. Colloids Surfaces A Physicochem Eng Asp 503:79–87. IF_{2016}: 2.765

[3] Molnar K, Juriga D, Nagy PM, Sinko K, Jedlovszky-Hajdu A, Zrinyi M (2014) Electrospun poly(aspartic acid) gel scaffolds for artificial extracellular matrix. Polym Int 63:1608–1615. IF_{2014}: 2.409

6.2 Publications not related to the thesis

[4] Varga Z, Molnár K, Torma V, Zrínyi M (2010) Kinetics of volume change of poly(succinimide) gels during hydrolysis and swelling. Phys Chem Chem Phys 12:12670–12675. IF_{2010}: 4.116