# Porous poly(amino acid) based electrospun fibrous scaffolds for tissue engineering

*Thesis Booklet*

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## 1. Introduction

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to develop methods to restore, maintain or improve tissue functions. Most techniques use scaffolds to mimic the native extracellular matrix (ECM) by providing mechanical support for the cells that are intended to replace the injured or diseased tissue. To imitate the fibrous structure of the native ECM, the electrospinning technique is widely used. During this process, a non-woven scaffold with a diameter on a nano- or micrometer-scale can be produced from a viscous polymer solution by charging and ejecting under a high-voltage electric field.

As scaffolding materials, hydrophilic polymers that can form hydrogels through crosslinks, show increasing popularity, as they have been found to be useable in many biomedical applications. Due to their high water content, their mechanical properties are similar to that of the ECM and soft tissues. Consequently, hydrogels are considered potential materials for tissue engineering. A primary requirement for any type of successful implant is biocompatibility, indicating that neither the material nor its degradation products have cytotoxic effects and generate immunological overreaction. However, besides the advantages of hydrogels as scaffolds, they also introduce challenges concerning long-term preservation. To prevent the scaffolds from degradation and contamination during storage and to create an easy-to-store sample, freeze-drying process was also introduced in this work.

A further crucial requirement to fulfill is the porous structure of the artificial scaffolds. The adequate porosity of the scaffold enables the penetration of the cells and the sufficient diffusion of nutrients and metabolic waste products. However, electrospinning yields densely deposited mesh of randomly arrayed fibers resulting in low porosity and small pore sizes, which restricts their use in biomedical applications. To improve the porosity of the electrospun scaffolds, researchers developed

various methods. One approach is the application of ultrasonication post-electrospinning to decrease the tight structure of the fibers and improving porosity.

This thesis aimed to overcome one of the major limitations regarding the application of conventional electrospun scaffolds, namely the dense packing of fibers, that results in small pores and hereby the inhibition of cellular penetration. The other goal was to improve the storing conditions of fibrous hydrogels and extend their shelf life without degradation. To create a hydrogel scaffolding material, I used the precursor of polyaspartic acid (PASP), i.e. polysuccinimde (PSI) to produce electrospun fibers. The polymer chains were crosslinked with 1,4 Diaminobutane (DAB) and after hydrolyzation, a fibrous hydrogel network was formed (PASPDAB). The influence of ultrasonication on the chemical, mechanical, and toxicological properties was investigated, as well as the permeability of PASPDAB scaffolds for cells. Freeze-drying as an alternative storing condition and its influence on the mechanical properties were also examined.

## 2. Objectives

The main objective of the thesis was the preparation of poly(aspartic acid) based fibrous scaffolds, that can be used in tissue engineering, creating a substrate for the cells to adhere to, grow and proliferate on.

1. To prepare polysuccinimide (PSI) fibrous scaffolds by electrospinning, compress them, crosslink the PSI chains (PSIDAB), and hydrolyze the scaffolds (PASPDAB), and to investigate the cell viability on the top of the scaffolds (PSIDAB and PASPDAB) and cell attachment on their surface.

2. To use ultrasonication as a post-electrospinning technique to expand the fibrous PASPDAB hydrogel samples into the 3rd dimension.

3. To investigate the effect of ultrasonication on the fiber morphology, chemical structure, and the mechanical properties: the elongation, and the specific load capacity of the scaffolds.

4. To investigate the effect of ultrasonication on the cytotoxicity and to visualize the cellular penetration into the ultrasonicated scaffolds.

5. To examine freeze-drying as a possible storage condition and investigate the effect of freeze-drying and rehydrating on the mechanical properties.

## 3. Materials and Methods

### 3.1. Synthesis of PSI and electrospinning

PSI was synthesized through thermal polycondensation of L-aspartic acid in the presence of phosphoric acid as a catalyst at 180°C under vacuum.

To fabricate PSI-based electrospun nanofibrous scaffolds, PSI was dissolved in DMF to produce a 25 w/w% solution. The polymer solution was loaded into a 5 mL syringe fitted with a metal blunt-ended 18G needle and it was electrospun directly on an aluminum foil-covered standing collector or drum collector (10 rpm; diameter = 10 cm) of the electrospinning system with a 20 cm needle tip to collector distance. A syringe pump was used to deliver the polymer solution to the needle tip at a constant feed rate of 1.0 ml/h. Electrospinning was conducted below 40 % humidity level, at the temperature of 22-26°C for 4 hours using a high-voltage DC power supply at 12 kV. The resultant nanofibrous scaffold sheet

was cut into adequate forms (circular: diameter 6 mm or 16 mm; rectangular: 2 x 2,5 cm, the longer side of the rectangle is parallel with the axis of rotation (when drum collector was used).

## 3.2. Post-electrospinning processing

### 3.2.1. Compressing

In the first part of my work, standing collector was used and to make the samples mechanically durable and shelf-stable, nine layers were compressed together with a hydraulic press (1.2 MPa). In the second part, a rotating drum collector was used, and the samples were investigated without compression.

### 3.2.2. Chemical modifications

The polymer chains were chemically crosslinked after the electrospinning process (post-electrospinning) by wet chemistry. Crosslinking was performed by submerging each scaffold in 0.5 M DAB in anhydrous ethanol for 3 hours (resulting PSIDAB). After submersion, scaffolds were repeatedly washed with ultrapure water until neutral pH.

The hydrolysis of PSIDAB was conducted overnight in a mild alkali medium, in a water-based imidazole buffer at pH 8, where the ionic strength was 250 mM. After the hydrolysis, the resultant hydrogel samples (PASPDAB) were washed with ultrapure water to remove the remaining salts.

### 3.2.3. Ultrasonication

The PASPDAB samples were immersed into ultrapure water in a glass sample container at room temperature and ultrasonicated using a USC900THD ultrasonicator (VWR, Germany) with a power of 200 W. The duration of the treatment was in the range of 1 min to 120 min. For further

characterization, PASPDAB scaffolds subjected to 60 min ultrasonication treatment were chosen.

#### 3.2.4. Freeze-drying

Before freeze drying, the samples were washed thoroughly with ultrapure water, then were frozen at -20°C covered with ultrapure water. The freeze-drying was carried out using a Christ Alpha 1-4 LSC freeze dryer overnight (16 h).

### 3.3. Chemical and morphological analysis

Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) was carried out to characterize the chemical structure of the dry or freeze-dried samples using a JASCO 4700 (type A) FT/IR spectrophotometer equipped with a diamond ATR head.

Morphological investigations of the fibrous structure of the dry or freeze-dried samples were carried out using a JSM 6380LA scanning electron microscope (JEOL, Japan).

### 3.4. Mechanical analysis

The mechanical properties of the samples were measured using a uniaxial mechanical testing machine (4952, Instron, USA). For this purpose, rectangular samples (2 cm x 2,5 cm) were cut from each scaffold (n=5). PSI scaffolds and freeze-dried samples were measured in a dry state at room temperature, below 30% humidity level. PSIDAB and PASPDAB samples were measured under physiological saline solution (150 mM, 25°C) immediately after the modification, or after washing with ultrapure water, freeze-drying, and rehydrating in saline solution. The specimens were assessed until rupture at a crosshead speed of 1 mm/min.

From the recorded data, the extension and the regarding load were measured. The specific load capacity (maximal

sustained load [N] divided by the area density [g/m2], and the elongation were calculated.

$$Specific\ Load\ Capacity\left[\frac{Nm^2}{g}\right] = \frac{Maximal\ Sustained\ Load[N]}{Area\ Density\left[\frac{g}{m^2}\right]}$$

$$\boldsymbol{Elongation}\ [\%] = \frac{\boldsymbol{Deformation}\ [\boldsymbol{mm}]}{\boldsymbol{Initial\ length}\ [\boldsymbol{mm}]} \cdot \mathbf{100}\ \%$$

### 3.5. Cell viability assay on the surface of scaffolds (2D samples)

Disks of an average diameter of 6 mm were cut from the compressed electrospun scaffolds (PSIDAB and PASPDAB). Before introducing the disks to the cells, the scaffold samples were sterilized in a 300 ppm chlorine-dioxide solution (in PBS) for 10 min and they were incubated in the completed medium for 1 hour. First, the gel disks were placed into the wells of low cell binding 96 well microplates (flat bottom, Nunc, Denmark). The surface of the low binding plates is not suitable for cells to attach to, and it was used to ensure, that the cells can only adhere on the scaffolds. After that, the MG-63 cells (adherent cell line) were seeded onto the gel disks at a concentration of 20 000 cells/well in 200 µl medium/well and incubated for 24 or 72 hours at 37˚C. Cell viability was evaluated by a colorimetric assay using a cell proliferation reagent (WST-1).

### 3.6. Indirect cytotoxicity assay (3D samples)

Circular samples of PASPDAB and PASPDAB US with a diameter of 6 mm (m = 0.81 ± 0.07 mg) were cut from each scaffold. The sterility of the test samples was ensured by 60 minutes of UV light exposition. The in vitro cytotoxicity test was performed applying the extracts of the test samples with completed cell culture media medium as an extraction vehicle.

In the case of each sample, the extraction was carried out in a 48-well-plate in 500 µl medium at 37°C for 24 hours.
Cells were seeded into 96-well plates at a concentration of 3200 cells/cm2 in 100 µL (n=5 for each sample) and maintained at 37°C for 24 h.

The culture medium was aspirated from the cells after 24 h incubation. The medium was replaced with 200 µl of extract (without dilution), while in the case of control, the replacing solution was fresh culture medium (=treatment). The blank wells did not contain cells. Cell viability was evaluated by a colorimetric assay using a cell proliferation reagent (WST-1) before treatment and after 24 or 72 h treatment.

### 3.7. Multiphoton microscopy

To visualize the MG-63 cells growing on the surface or the 155BR cells infiltrating the ultrasonicated fibrous scaffolds, they were labelled with a fluorescent vital dye (Vybrant DiD) before seeding. For the MG-63 cells, scaffolds of 6 mm in diameter (compressed PSIDAB and PASPDAB) were placed into Lab-tek 8 chamber slides (Nunc, USA) with tissue culture surface treatment. For 155BR cells, PASPDAB and PASPDAB US scaffolds of 16 mm in diameter ($m = 4.75 \pm 0.86$ mg) were prepared and freeze-dried.

After the sterilization, the samples were immersed in the completed cell culture medium for 2 hours, then placed into 6-well-plates, and 40 000 cells were seeded onto each disk. The same amount of control cells was seeded on a cover glass. After 24 and 72 hours, the samples were fixed by soaking in 4% paraformaldehyde solution (in PBS) for 2 hours at room temperature with subsequent washing with PBS. The samples were investigated under a multiphoton microscope (Femto2D, Femtonics, Hungary) applying a 10x objective and 800 nm wavelength to excite the photoactive dye. The low red channel (600-700 nm) was used to detect the red fluorescence of the cells due to the Vybrant DiD vital staining while the low green

channel (490-560 nm) was used to detect the autofluorescence of the PASPDAB scaffolds.

## 4. Results

T1

Electrospinning was used to create two-dimensional polysuccinimide (PSI) fibers and with post-electrospinning methods, poly(aspartic acid)-based fibrous hydrogel scaffolds (PSI crosslinked with DAB (PSIDAB) and its hydrolysed form, PASPDAB) were produced. The cell study on PSIDAB revealed that hydrolyzing PSIDAB in cell culture media caused a pH shift toward the acidic region, resulting in cell death in the MG-63 osteosarcoma cell line. PASPDAB scaffolds showed no signs of cytotoxicity after 24 and 72 hours on the same cell line (Figure 1a). While visualizing the cellular attachment of pre-labelled MG-63 cells with multiphoton microscopy 24 hours after seeding, on PSIDAB scaffolds no attached cells were observable, nevertheless on the surface of PASPDAB scaffolds plenty of attached cells could be seen (Figure 1b). [RP1]

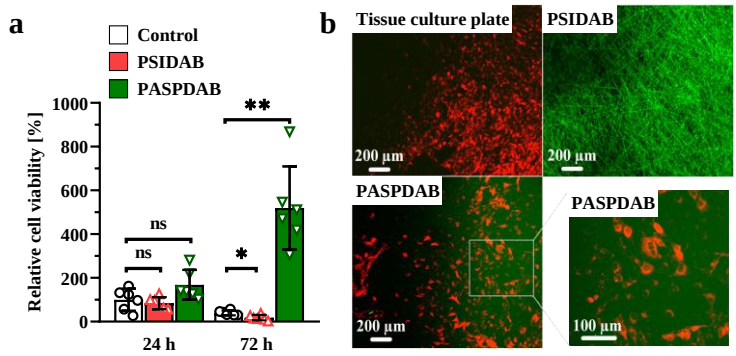


*Figure 1 Viability of MG-63 cells after 24 h and 72 h cultivation on the surfaces of low cell binding plastic plates (control) and PSIDAB or PASPDAB scaffolds (a). Multiphoton microscopic images of the Vybrant DiD-labelled MG-63 cells after 24-hour-long cultivation on the surfaces of tissue culture plastic plates, or PSIDAB,*

*or PASPDAB based fibrous scaffolds (b). The cells show red fluorescence due to the Vybrant DiD staining while the green color indicates the autofluorescence of the PSI or PASP based scaffolds.*

T2

Ultrasonication can be used to increase scaffold porosity and the cross-sectional thickness by 400% after 1 hour of treatment (200 W) of the two-dimensional PASPDAB scaffolds. This can enable cellular penetration (Figure 2).

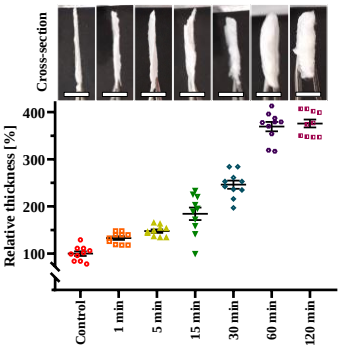


*Figure 2 The cross-sectional thickness of freeze-dried PASPDAB scaffolds as a function of ultrasonication time. The scale bars are 5 mm.*

The average fiber diameter (PASPDAB: 1106 ± 113 nm; PASPDAB US: 1155 ±151 nm) and chemical composition of the scaffolds did not change as a result of ultrasonication, as evidenced by SEM micrographs and ATR-FTIR analyses (Figure 3). [RP2]

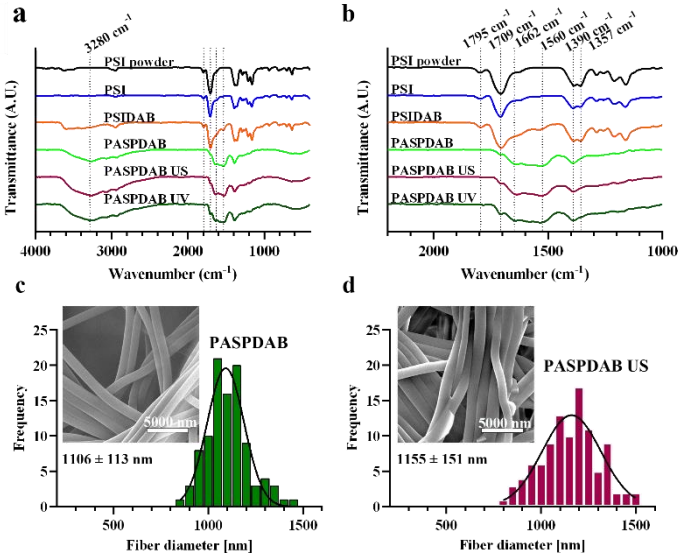


*Figure 3 ATR-FTIR spectra of PSI powder, PSI electrospun fiber, and its derivatives. (a, b) SEM micrographs and fiber size distribution of PASPDAB (c), and ultrasonicated PASPDAB (d). The fiber diameters were presented as the means ± standard deviations. The figure was adapted from [RP2] with minor modifications.*

T3

Ultrasonication process affected the mechanical properties of the PASPDAB scaffolds. After ultrasonication, the specific load capacity of the dry PASPDAB samples decreased significantly (from $0.23 \pm 0.03$ $Nm^2/g$ to $0.11 \pm 0.01$ $Nm^2/g$) (Figure 4a). The specific maximum load of wet state scaffolds was lower by an order of magnitude, and ultrasonication had no discernible effect on mechanical strength (Figure 4b). The elongation at breakpoint of dry state (freeze-dried) PASPDAB scaffolds significantly increased after ultrasonication (PASPDAB: $35 \pm 5\%$; PASPDAB US: $146 \pm 8\%$) (Figure 4c). On wet scaffolds, due to ultrasonication elongation at breakpoint increased from $73 \pm 8\%$ to $177 \pm 18\%$ in freshly prepared samples (Figure 4d). [RP2]

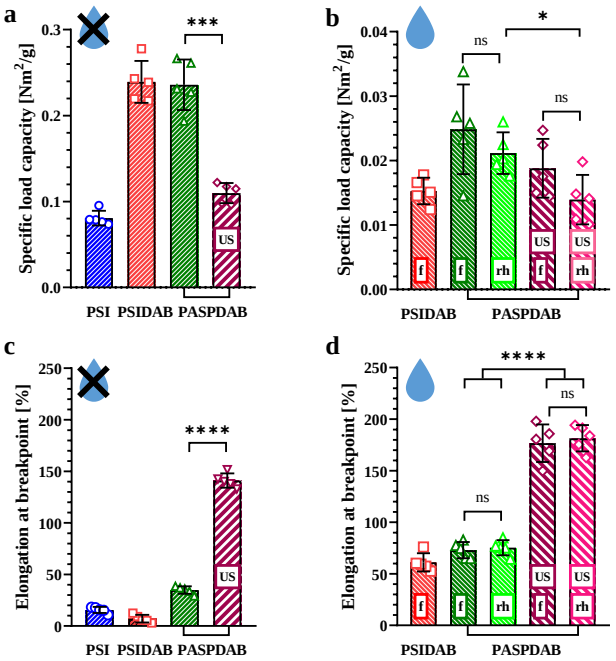


*Figure 41 Specific load capacity (a, b) and elongation at breakpoint values (c, d) of dry (a, c) and wet (b, d) samples measured by a tensile test. The presented data show the average of 5 independent measurements. The error bars correspond to the standard deviation of the mean. The "*" indicates statistical significance exists between two groups (one-way ANOVA, *p<0.05; **p<0.01; ****p<0.0001).*

T4

To prevent PASPDAB and PASPDAB US hydrogel scaffolds from degradation, structural damage and microbiological spoilage caused by wet state storage, freeze-drying as an alternative condition was used to increase the shelf-life of the samples (Figure 5).

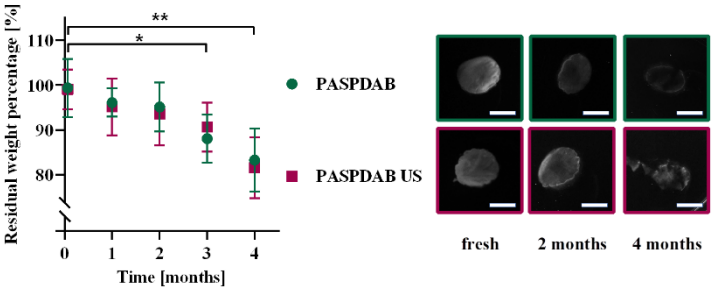


*Figure 52 Degradation of PASPDAB and PASPDAB US samples after storing in PBS at RT for 4 months. The presented data show the average of 5 independent measurements. The error bars correspond to the standard deviation of the mean. The "*" indicates if statistical significance exists between two groups (one-way ANOVA, *p<0.05; **p<0.01). The scale bars indicate 1 cm.*

The mechanical properties of fibrous poly(aspartic-based) hydrogels are unaffected by freeze-drying and hydrating. The specific load capacity of freshly prepared PASPDAB wet samples was 0.025 ± 0.007 $Nm^2/g$, while that of freeze-dried and rehydrated samples was 0.021 ± 0.003 $Nm^2/g$ (Figure 4b). The mechanical strength of PASPDAB US was 0.019 ± 0.004 $Nm^2/g$ in fresh and 0.014 0.004 Nm2/g in rehydrated form (Figure 4b). The elongation at breakpoint was 73 ± 8% for freshly prepared wet PASPDAB samples and 75 ± 7% for rehydrated ones (Figure 4d). During the investigation of PASPDAB US samples, the elongation of fresh samples was 177 ± 18%, while rehydrated samples had 181 ± 13% (Figure 4d). Based on these results, fibrous hydrogel scaffolds in freeze-dried form can be stored, transported, and rehydrated before use without changing mechanical properties. [RP2]

T5

PASPDAB and PASPDAB US scaffolds were found to be noncytotoxic to 155BR fibroblast cells after 24 and 72 h of an indirect cytotoxicity test (Figure 6).

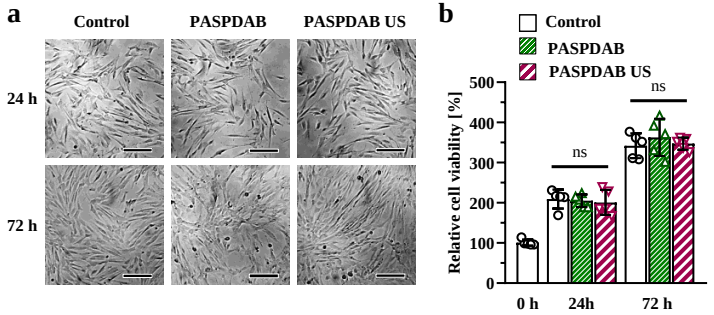


*Figure 3 Phase-contrast microscopical images of 155BR cells (a) without treatment (control), treated with PASPDAB or PASPDAB US scaffolds after 24 and 72 hours. The scale bars indicate 100 μm. The cytotoxicity results (b) of PASPDAB and PASPDAB US scaffolds after 24 and 72 hours (n=5). The "ns" indicates the lack of statistical significance between the two groups (one-way ANOVA, p>0.05).*

Ultrasonication process created a porous structured PASPDAB US and enabled the penetration of 155BR cells inside the poly(aspartic acid) based scaffolds based on the multiphoton microscopy results, on the other hand, the cells seeded on the PASPDAB scaffolds, were attached on the surface (Figure 7). [RP2]

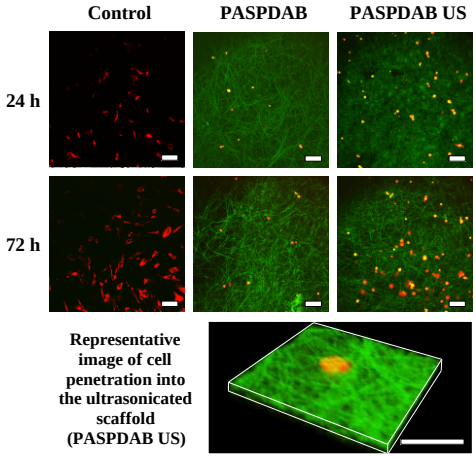


*Figure 4 Multiphoton images of Vybrant DiD-labelled 155BR cells seeded on the surface of a glass coverslip (control) or PASPDAB or PASPDAB US fibrous scaffolds after 24 and 72 hours. The cells show red fluorescence due to the Vybrant DiD staining, while the green color indicates the autofluorescence of the PASPDAB scaffolds. Scale bars indicate 200 μm.*

## 5. Conclusion

Electrospinning is commonly used in the biomedical field to create tissue engineering scaffolds. In ideal case, the cells colonize the scaffold, synthesize new extracellular matrix as the scaffold degrades. Finding the appropriate material for each type of tissue with tuneable properties is currently a challenge for scientists. In this work, poly(aspartic acid) (PASP) and its anhydride, polysuccinimide (PSI) was used as a base polymer to create hydrogel scaffolds with electrospinning setup.

First, I produced electrospun fibrous scaffolds of PSI and after crosslinking with 1,4-Diaminobutane (DAB), hydrogels with varying chemical compositions (PSIDAB and PASPDAB) were formed. While investigating their interactions with cells, PSIDAB made the culturing media acidic in vitro, decreasing the cell viability. However, PASPDAB was found

noncytotoxic for MG-63 cells and the two-photon microscopy confirmed that after 24 hours, cells were able to attach to the surface of the PASPDAB scaffold. Based on these results, PASPDAB is a possible material for scaffolds, but based on the micrographs there was no evidence of cellular penetration into the scaffolds.

The reason for the poor infiltration is the densely deposited structure of randomly arrayed electrospun fibers resulting in low porosity. Thus, I attempted to overcome this limitation by using ultrasonication treatment on PASPDAB, to loosen the structure, and enhance the cellular infiltration. Ultrasonication has increased the cross-sectional thickness of the scaffolds to 400%. The ultrasonication affected the mechanical properties of the scaffold. The specific load capacity of the dry samples has halved; however, the elongation at breakpoint has significantly increased by both dry (4-fold) and wet state (2,5-fold) scaffolds. As an alternative for wet state storage, freeze-drying was used. Based on my findings, drying and rehydrating of the fibrous poly(aspartic based) hydrogels do not affect their mechanical properties, thus the freeze-dried form of scaffolds can be stored, transported, and be prevented from degradation. While investigating their interactions with cells, ultrasonicated PASPDAB scaffolds were found to be noncytotoxic for 155BR fibroblast cells, and ultrasonication process created a porous structure and enabled the cellular penetration inside the poly(aspartic acid) based scaffolds.

Due to the increased cellular infiltration of the PASPDAB scaffolds and their alternative dry state storage, this kind of scaffold could be a huge step towards an off the shelf and effective product for tissue regeneration.

## 6. Bibliography of the candidate's publications

### Publications relevant to the Thesis

[RP1] Molnar, K., Voniatis, C., Feher, D., Szabo, G., Varga, R., Reiniger, L., Juriga, D., Kiss, Z., Krisch, E., Weber, G., Ferencz, A., Varga, G., Zrinyi, M., Nagy, K. S., Jedlovszky-Hajdu, A. (2021). Poly(amino acid) based fibrous membranes with tuneable in vivo biodegradation. Plos One, 16(8), e0254843. (2021). https://doi.org/10.1371/journal.pone.0254843

[RP2] R. Pázmány, K.S. Nagy, Á. Zsembery, A. Jedlovszky, Ultrasound induced, easy-to-store porous poly ( amino acid ) based electrospun scaffolds, J. Mol. Liq. 359 (2022). https://doi.org/10.1016/j.molliq.2022.119243.

### Publications not relevant to the Thesis

K. Molnar, R. Varga, B. Jozsa, D. Barczikai, E. Krisch, K.S. Nagy, G. Varga, A. Jedlovszky-Hajdu, J.E. Puskas, Investigation of the cytotoxicity of electrospun polysuccinimide-based fiber mats, Polymers (Basel). 12 (2020) 1–11. https://doi.org/10.3390/polym12102324.