Final Report of the FK 124147 NKFIH project

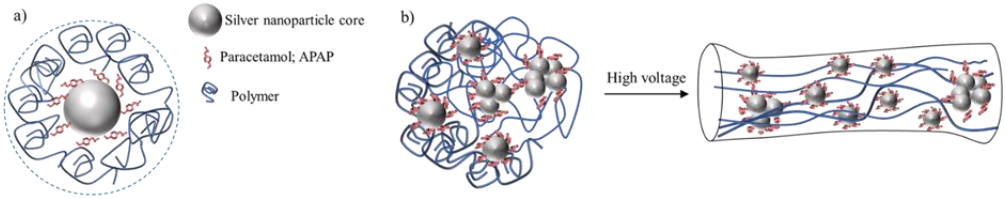
Poly(succinimide) has been recently gaining attention as its reactive nature makes it a versatile, potential component for functionalised systems. Its production is fairly simple, as it only requires heat (as it is produced via thermal polycondensation), a catalyst (e.g. phosphoric acid) and the monomer itself (L-aspartic acid). PSI has been utilized in different forms (powder, gel, particles) but the literature on nanofibrous membranes is very limited. Electrospinning is a fascinating method to fabricate meshes, membranes or scaffolds composed of nano- or micro-sized fibres. Briefly, the system's most basic setup, requires a high voltage power supply, a syringe pump, a syringe filled with a polymer solution and a grounded collector as a target. As the polymer solution is pushed through a needle attached to the syringe, due to the high voltage the electrostatic power is larger than the surface tension of the solution resulting in the spatter polymer fibres in the process.

PSI is hydrolysed in slightly alkaline media and water (albeit quite slowly in the latter) and recent studies of our group have already demonstrated its cyto- and potential biocompatibility therefore it could be considered as a candidate for environmental and biomedical applications [1].

1, The first task in my research frame of the grant was to prepare electrospun fibers loaded with AgNPs (silver nanoparticles), test the antibacterial effect of the gel fiber mesh in bacteria cultures and cell experiments.

Wound dressings have evolved to sophisticated materials with intricate structures and specialized components, designed specifically to enhance and promote faster wound healing rates. While simple gauze dressings are still widely used, wound dressings are now not exclusively composed of textiles. The research focus is now turning to advanced materials, for example, films, hydrogels, foams, and nanofibers with an emphasized objective on the prevention of bacterial infection, since such an event can seriously delay the healing process and poses as a major risk towards severe clinical conditions. While typically bacterial flora found on the skin poses no risk to a patient, as the balance of the skin is disrupted due to chronic wounds or immunocompromised conditions, even representatives of normal bacterial flora can lead to pathological conditions. To overcome the widespread antibiotic resistance of various pathogens, several strategies have been continuously proposed and examined. These tactics include antibacterial polymers, essential oils, and other natural antibiotics such as honey, antimicrobial peptides, phage therapy, and nanoparticles.

In our project, we were demonstrating how AgNPs as well a pharmaceutical agents can be electrospun after a one-pot synthesis method (Figure 1). The pharmaceutical agent of choice was paracetamol (APAP), ibuprophen and diclophenac. But in this final report I will show just the results with the APAP, because the rest of the experiments are ongoing ones and the resuts are under publication. Our aim, by pairing paracetamol with AgNPs is to assess the effect of a small-molecular drug on AgNP and how it could potentially enhance or decrease the antibacterial effect of PSI based membranes. Furthermore, PSI being a highly biocompatible polymer the objective is to produce a functionalized biocompatible and biodegradable wound dressing.



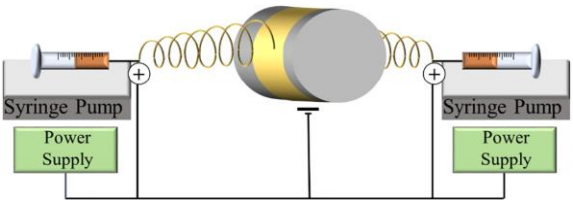


Figure 1. A theoretical NP coverage with polymer and APAP (a) and the fiber formation (b). In the lower schematic draw represent a two-needle electrospinning setup for the fabrication of nanofibrous meshes.

Antibacterial properties of silver nanoparticles are known to be strongly dependent on the size of the particles therefore size determination is a key aspect in designing effective silver nanoparticle-containing matrices. For a thorough characterization, the mean size of the silver nanoparticles was calculated not just based on intensity, but the size and volume distribution as well (Figure 2).

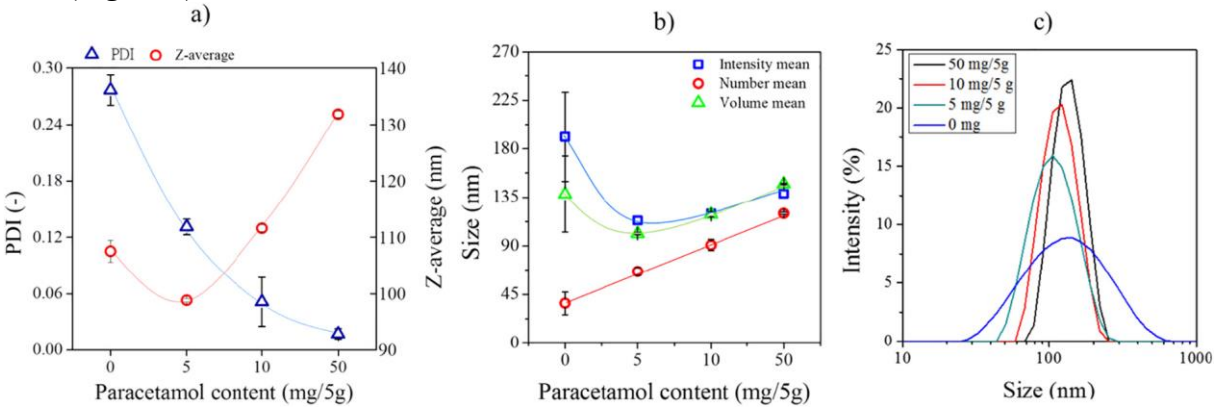


Figure 2. Effect of paracetamol on properties of silver nanoparticles a) polydispersity index (PDI) and z-average b) particle size and c) intensity-based size distribution.

As it was indicated by DLS results, AgNP synthesis was successfully performed and PSI, together with paracetamol, facilitates the formation of a highly monodispersed silver colloidal system of which properties (size, aggregative stability, PDI) could be tuned by adjusting paracetamol content.

The prepared fibers from the solutions containing polymer and AgNP also presented a homogenous fiber diameter between 210-325 nm up to the paracetamol content (with increasing drug concentration the fiber diameter increased). Although the mechanical evaluation indicated that silver nanoparticles can reinforce pure PSI membranes, and interestingly, the addition of paracetamol can further enhance the mechanical properties of the nanocomposite meshes, but no difference was found between the drug amounts.

For the antibacterial evaluation we used 4 clinically relevant bacterial suspensions both Gram positive and Gram-negative bacteria strains (Figure 3). The results suggested that antibacterial efficacy is a function of the paracetamol content highlighting that the addition of a small molecule can change the antibacterial properties of nanoparticles which is in accordance with previous findings.

Release kinetics showed a four-stage drug release. The released amount of drug (%) was a function of exact composition as paracetamol seems to alter the hydrolytic stability of the meshes as well [2].

Thus all the aim was mention in this frame of the project was successfully done and demonstrated in a manuscript. Some ongoing experiments are still under research with ZnNPs in the presence of diclofenac, but these results are not published yet.

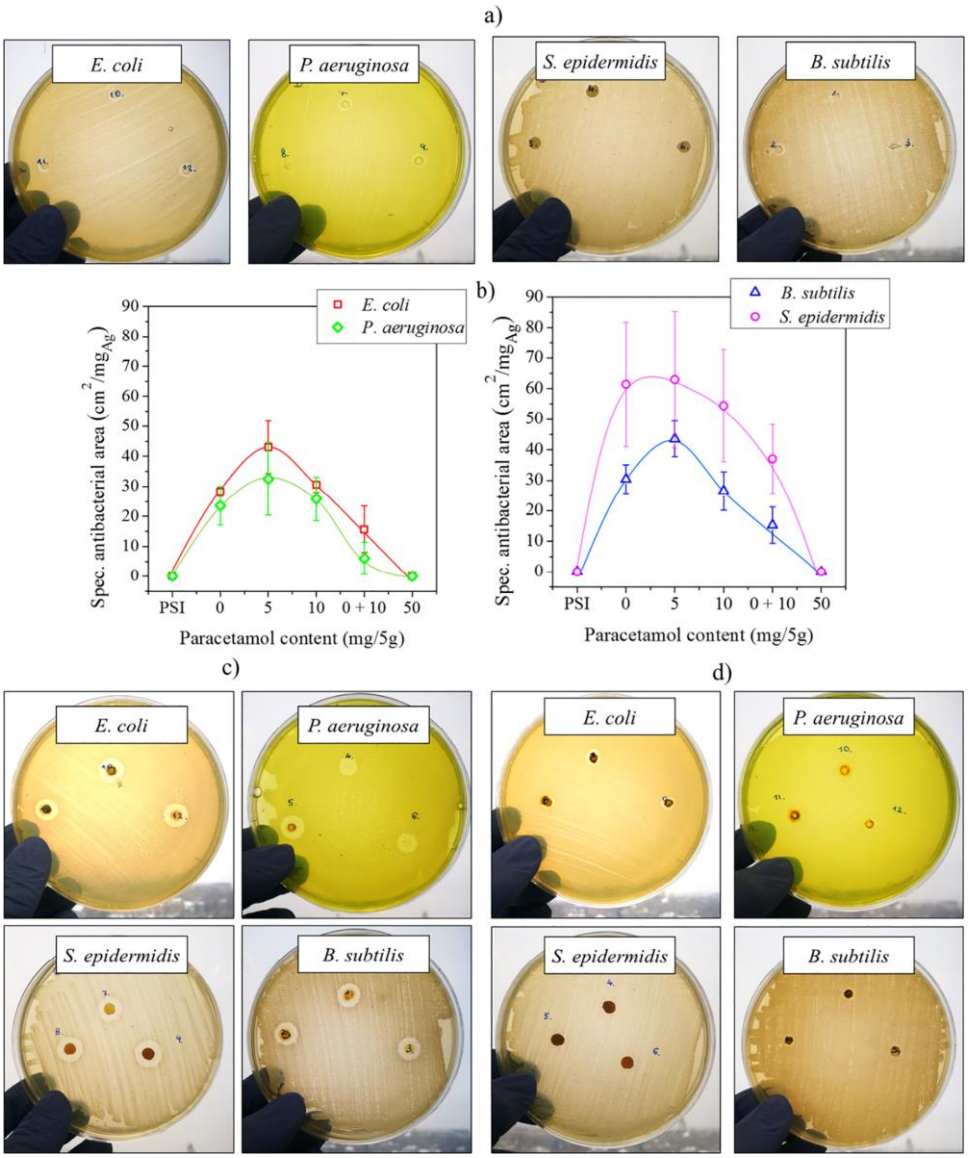


Figure 3. a) Kirby-Bauer or Disc diffusion method using PSI membranes b) Specific antibacterial area of silver nanoparticles within electrospun meshes and diffusion zone of c) the most effective sample: 5 mg/5 g APAP meshes d) the least effective sample: 50 mg/5 g APAP meshes with all tested bacteria strains.

2, The second task in my research frame of the grant was to prepare electrospun fibers loaded with magnetic NPs, test the creation of scaffold structure, test the cell growth and viability on the scaffold, and measure the hyperthermic effect of the meshes.

Magnetite nanoparticles could be used for theranostics, as they can act as contrast agents in MRI (T2 relaxation) and can also induce magnetic hyperthermia. The combination of magnetite nanoparticles with electrospun PSI scaffolds could effectively entrap and localise these particles, therefore allowing multiple treatments to be done with the same scaffold.

The aim of our research was to create an artificial poly(succinimide) mesh loaded with magnetite nanoparticles, that is capable of killing cancer cells due to its magnetic hyperthermic effect and is a good contrast agent in MRI. Therefore, poly(succinimide) membranes were created with the electrospinning technique, the same way as it was mentioned above. To avoid the short biodegradation of the membrane, DAB crosslinker was to use between the polymer chains [3]. Magnetite nanoparticles were synthesized inside the uniformly cut meshes (Figure 4).

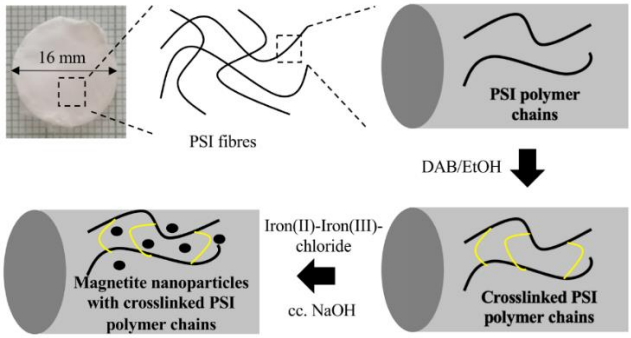


Figure 4. Schematic representation of the chemical treatment of the fibrous scaffold-magnetite synthesis.

The fibrous crosslinked structure can swell and shrink in different solvents or ionic strengths. This phenomenon was investigated by measuring the diameter of the cylinders during the merging steps. It is obvious that if the crosslinking reaction happened and the polymer fibres turned to gel-fibres, then after putting them in different solutions the diameter changes, showing the swelling and the shrinking. This is an indirect proof for the crosslinking reaction and the gel-fibre formation of the mesh.

SEM studies (not shown here) proved that fibres were not damaged and kept their initial structure during the treatments. It is important to note, that fibres did not merge, which is usually the biggest issue when it comes to treating fibrous structures with solutions. The average diameter of fibres was 0.87 ± 0.21 μm. However, cells like this type of fibre arrangement more, as it is closer to the structure of the extracellular matrix. The composition of crystals on the surface of fibres was investigated by EDS spectrometry, which indicated high proportions of iron and oxygen. Therefore, the presence of magnetite was not only visually proven but due to the EDS detector it is clear that the magnetite did not just attach on their surface but was also present inside the polymer fibres themselves.

The SQUID, ESR and magnetic hyperthermic studies proved the magnetic behaviour of the particles inside the fibrous structure (Figure 5). SQUID studies of scaffolds showed no hysteresis in the full investigation range (Figure 5 (a)), which is consistent with data found in the literature. The maximum magnetization of the PSI-DAB-Magn sample was 44 emu/g, which is enough for the hyperthermic application. The ESR signal of the sample (Figure 5 (b)) followed the reference signal well, which confirms that the nanoparticles are indeed magnetite.

The magnetic hyperthermic effect of the PSI-DAB-Magn sample was investigated at different frequencies and magnetic fields (here just an example is shown). After a lot of optimisation (results not shown) here the results at 109.4 kHz and 20.56 mT are shown (Figure 5 (c) (d)). The typical criteria in the literature for an effective hyperthermic treatment is that samples should induce an increase at least 5°C in temperature under 5 minutes. Since we start from the 37°C of our bodies, an increase of 5°C leads to 42°C, which is enough to denature proteins in cells and therefore kill them. Our sample heated up more than the minimum 5 °C (around 10 °C for each sample), which means they could be used for hyperthermic treatments effectively. The SAR value of the sample is 10.8 ± 1.6 W/g.

PSI-DAB-Magn samples were able to heat up at least 8 °C under 300 seconds in every cycle (Figure 5 (c)) during the heating-cooling measurement. This means, after implanting the sample, multiple treatments could be done with it with the same efficiency.

However, cancer cells become resistant to heat shocks after a while, so it is also important to investigate if samples are able to induce hyperthermic effect on a daily basis. Therefore, the hyperthermic effect of PSI-DAB-Magn sample was investigated every day for 5 days. During the 5-day repetitive measurement, samples consistently heated up more than 12

°C every day (Figure 5 (d)). Thus, PSI-DAB-Magn samples could be used for hyperthermic treatment after even days after implantation.

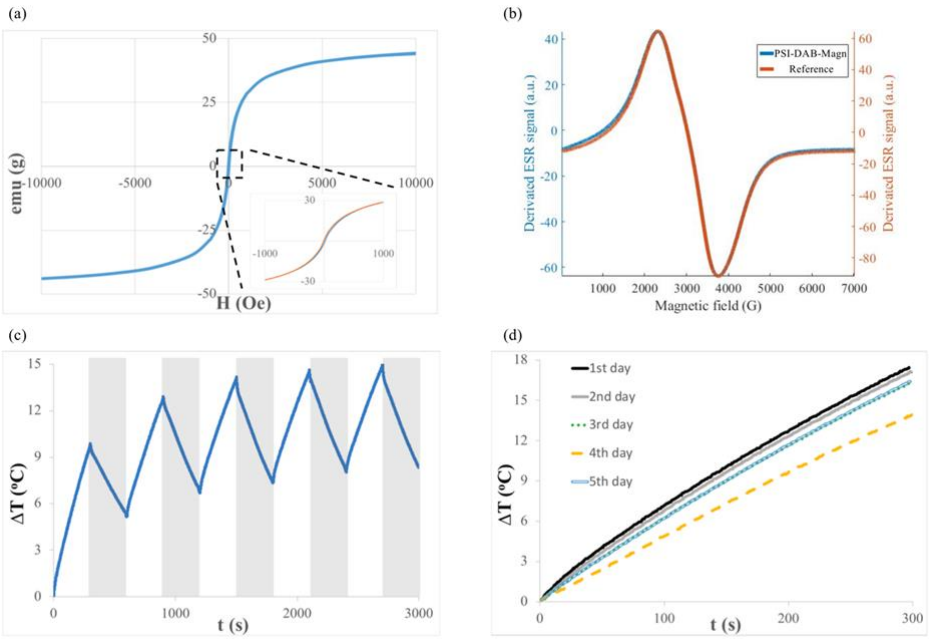


Figure 5. a) SQUID study of the crosslinked magnetite loaded fibrous sample. (b) The ESR signal of magnetite matches the reference signal. (c) The change in temperature of the sample during five 5 minutes heating-5 minutes cooling hyperthermic measurement cycle. (d) The change in temperature of the sample during the 5-day repeated hyperthermic measurement.

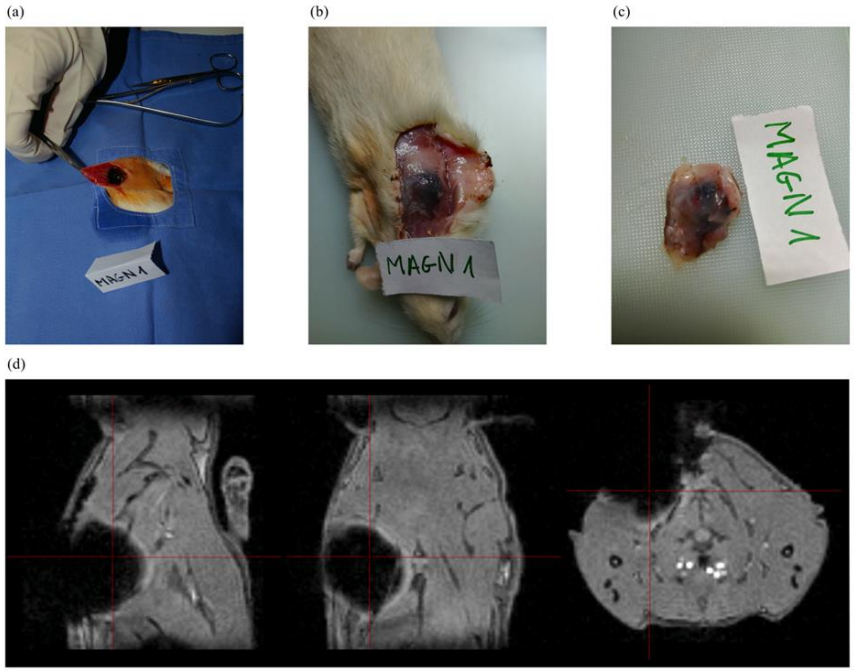


Figure 6. (a) (b) (c) Samples were placed at the back of the Wistar rat. (d) MRI scan on the 1st day following implantation.

To investigate the biocompatibility of the membranes as well as the their potential use as MRI contrast agents in vivo experiments were performed on Wister Rats (Figure 6).

During the surgical procedure no issues or difficulties were documented. The meshes can be easily handed even with traumatic surgical instruments and they are robust enough for suturing and surgical fixation.

Our goal was to determine whether superparamagnetic magnetite nanoparticles entrapped within an artificial membrane can enhance contrast in MRI for a longer time period. In images taken on the 1st post-operative day (Figure 6 (d)), samples exhibited their excellent contrast features. The samples can clear be seen, their dimensions and borders are clear while no inflammation is observed. MRI images were taken on the 8th day following implantation also to prove, that samples have been losing magnetite slowly, even within a living organism, since they gave excellent contrast in MRI even after 8 days. The sample dimensions did not change during the 8-day period, and no lesions were seen in surrounding tissue.

After 8 days, the animals were terminated (Figure 6 (b) (c)) and histopathological evaluation was done. No inflammatory signs or other complications were macroscopically observed on the meshes themselves or the surrounding tissue. Histopathology speciments were prepared with haematoxylin-eosin to observed and assess the inflammatory reaction and berlin blue stains to examine the release of the iron magnetic nanoparticles. No serious complications can be observed on the slides. The granulation tissue is rather thin while cells have already started to infiltrate the mesh. Therefore, PSI-DAB-Magn samples could be used in the future as a supplementary treatment of tumours, and as a support for cells during tissue regeneration [4].

3, The third task in the research frame of the grant was to investigate the special 3D structure of the scaffolds.

The aim in this frame was to test the target shape effect, using in-house designed targets of appropriate shapes, and also the possible nanoparticle effect (using both charged and uncharged nanoparticles) on the creation of the 3D structured mesh.

Electrospun fibre meshes are often considered 3D objects (just like natural ECM) as compared to the lower than a few micron fibre diameters, the thickness of these meshes is very big. However, the fibres are tightly packed creating very small pore sizes inevitably hindering cells from entering the inner layers of the matrix. Although cell culturing is still feasible and often give better results than standard cell culturing plates, cells still cannot obtain their natural 3D morphology, unless they enter the matrix. An effort has been made for raising the pore size between the fibres in the mesh, thus loosening it up to provide cells with a platform, in which free migration is possible. We actually added inorganic salt to the polymer solution to create real 3D structure with a certain pore size (Figure 7).

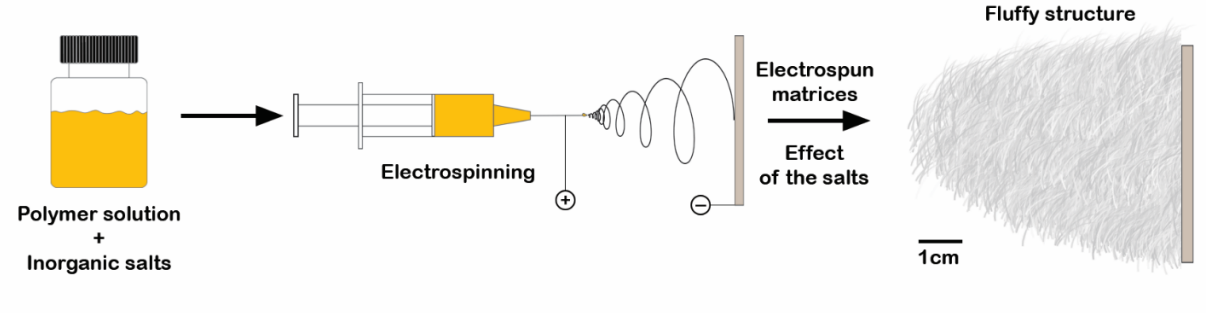


Figure 7. Schematic representation of the 3D scaffold preparation using electrospinning technique.

From the biological point of view, chloride is a more suitable co-ion in the living system, thus we prepared our experiments just with LiCl, $MgCl_2$ and $CaCl_2$. To have information at the molecular level on the ion-ion and ion-DMF solvent interactions, we recorded the IR spectra of these mixtures (Figure 8). The full characterization of the spectra was published here: [5].

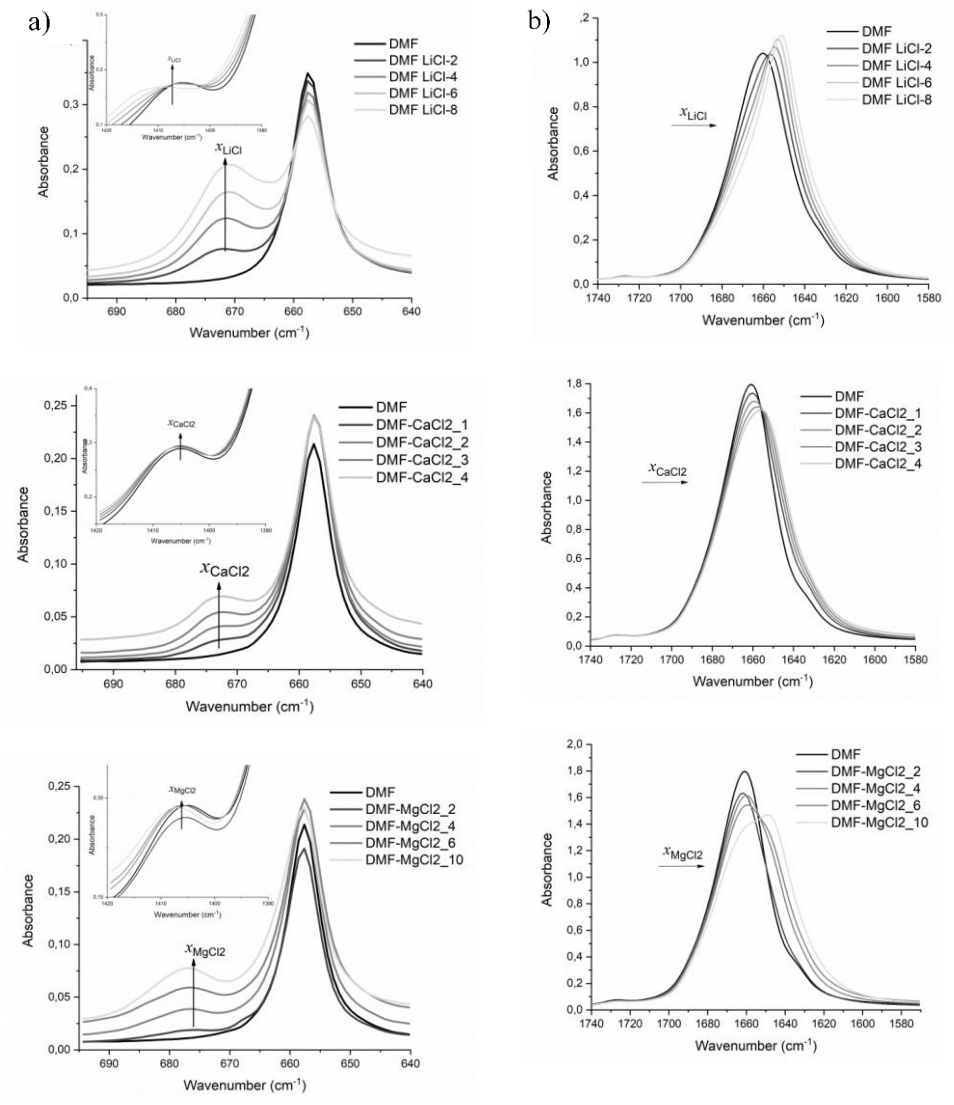


Figure 8 IR spectra of the NCH=O a) and the C=O b) vibration modes in mixtures of LiCl (2, 4, 6 and 8 w/w%) CaCl2 (1, 2, 3 and 4 w/w%) and MgCl2 (2, 4, 6 and 10 w/w%) with DMF.

After the careful characterization of the salt-solvent solutions, fibrous structure preparation was attempted. As it can be seen on Figure 9 the macroscopic structure looks roughly the same in all 3 cases. SEM showed that fibres had smooth surfaces and homogenous thicknesses (around 570 nm).

In the case of $MgCl_2$ we experienced gelation during the electrospinning. This phenomenon is well-known in polymer science and medical science for both Mg^{2+} and Ca^{2+} ions, both of them create physical crosslinks between polymer chains resulting in a gel-like structure [68]. In our system, the Mg^{2+} created an inhomogeneous structure during the fibre formation. Although the fluffy structure formation was reproducible, the fibre formation changed in time parallel with fibre diameter.

Based on these findings, fluffy 3D structured meshes of polysuccinimide were prepared reproducibly with $CaCl_2$, $MgCl_2$ and LiCl. We also did DFT simulation (results are showed in the relevant publication) and the FTIR analysis proved that there is interaction between the solvent (DMF) and the ions used, that strongly depends on their quality. This indicates that the reason why the presence of salts causes 3D fluffy structure during electrospinning, might not be just because of charge accumulation (as described in the literature), but also because of solvent-ion interactions.

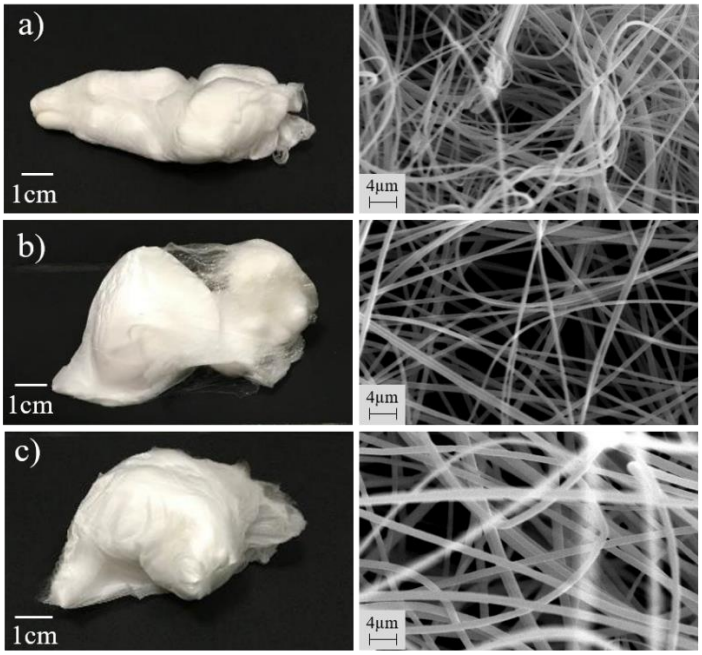


Figure 9. Macroscopic picture of the 3D effect of different salts used and their SEM pictures on the right a) LiCl 1 w/w% b) $MgCl_2$ 1 w/w% c) $CaCl_2$ 2 w/w%. The scale on the SEM images correspond to 2µm.

<u>To summarize the research plan,</u> the proposed work was focused on creating special fibre structures with electrospinning techniques based on poly(amino acids) in the size range between a few micrometers and hundred nanometers for biomedical applications. Actually based on these research findings a lot of other publications came out during the 4 years' project period. All of them connected to the electrospinning setup, but we enlarged the scope of the polymer (PVA or PCL or polyisobuthylene was also used for different applications) or used bulk gels instead of the gel fibers for tissue engineering reasons. Thus, the rest of the results are presented in the following papers [6-14]. The sum impact factor for the publications are 51.032 (+3.125).

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Published papers:

[1] Voniatis, Constantinos; Gottschall, Ramóna; Barczikai, Dóra; Szabó, György; Jedlovszky Hajdu, Angela: Enhancing critical features of poly(amino acid) based meshes, Journal of Applied Polymer Science, accepted for publication 14th September 2021 (IF_{2020}: 3.125)

[2] Barczikai, Dóra ; Kacsari, Viktória ; Domokos, Judit ; Szabó, Dóra ; Jedlovszky-Hajdu, Angela: Interaction of silver nanoparticle and commonly used anti-inflammatory drug within a poly(amino acid) derivative fibrous mesh, JOURNAL OF MOLECULAR LIQUIDS 322 Paper: 114575 , 13 p. (2021) **(IF_{2020}: 6.165)**

[3] Molnar, Kristof ; Voniatis, Constantinos ; Feher, Daniella ; Szabo, Gyorgyi ; Varga, Rita ; Reiniger, Lilla ; Juriga, David ; Kiss, Zoltan ; Krisch, Eniko ; Weber, Gyorgy, Ferencz Andrea, Varga Gabor, Zrinyi Miklos, Nagy Krisztina S., Jedlovszky-Hajdu Angela: Poly(amino acid) based fibrous membranes with tuneable in vivo biodegradation, PLOS ONE 16 : 8 Paper: e0254843 , 21 p. (2021) **(IF_{2020}: 3.240)**

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[5] Juhász, Ákos György; Molnar, Kristof; Idrissi, Abdenacer; Jedlovszky-Hajdu, Angela: Salt induced fluffy structured electrospun fibrous matrix, JOURNAL OF MOLECULAR LIQUIDS 312 Paper: 113478 , 10 p. (2020) **(IF$_{2020}$: 6.165)**
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[7] Voniatis, Constantinos; Barczikai, Dóra; Gyulai, Gergő; Jedlovszky-Hajdu, Angela: Fabrication and characterisation of electrospun Polycaprolactone/Polysuccinimide composite meshes, JOURNAL OF MOLECULAR LIQUIDS 323 Paper: 115094 , 8 p. (2021) **(IF$_{2020}$: 6.165)**
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[9] Molnar, Kristof; Varga, Rita; Jozsa, Benjamin; Barczikai, Dora; Krisch, Eniko; Nagy, Krisztina S.; Varga, Gabor; Jedlovszky-Hajdu, Angela; Puskas, Judit E.: Investigation of the Cytotoxicity of Electrospun Polysuccinimide-Based Fiber Mats, POLYMERS 12 : 10 Paper: 2324 , 11 p. (2020) **(IF$_{2020}$: 4.329)**
[10] Molnar, Kristof; Jozsa, Benjamin; Barczikai, Dora; Krisch, Eniko; Puskas, Judit E.; Jedlovszky-Hajdu, Angela: Plasma treatment as an effective tool for crosslinking of electrospun fibers, JOURNAL OF MOLECULAR LIQUIDS 303 Paper: 112628 , 8 p. (2020) **(IF$_{2020}$: 6.165)**
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[12] Hegedűs, Orsolya; Juriga, Dávid; Sipos, Evelin; Voniatis, Constantinos; Juhász, Ákos; Idrissi, Abdenaccer; Zrínyi, Miklós; Varga, Gábor; Jedlovszky-Hajdú, Angéla; S. Nagy, Krisztina: Free thiol groups on poly(aspartamide) based hydrogels facilitate tooth-derived progenitor cell proliferation and differentiation, PLOS ONE 14 : 12 Paper: e0226363 , 20 p. (2019) **(IF$_{2019}$: 2,74)**
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