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Summary of Ph.D. thesis

DEVELOPMENT AND COMPARISON OF SINGLE-NEEDLE AND NOZZLE-FREE ELECTROSPINNING OF POLYMERIC NANOFIBERS FOR DRUG DELIVERY APPLICATIONS

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1. INTRODUCTION

Nanotechnology has emerged as one of the most promising technologies of the twenty-first century, opening up new avenues in a variety of scientific fields. One small slice of this extensive area deals with longitudinally elongated structures with nano-sized diameters – the nanofibers. Nanofibers show improved properties such as large surface area, high porosity, diverse composition, and controllable morphology, which make them attractive in many different applications from textiles to electronics and biomedical uses. In the case of biomedicine, the medicines and medical devices of the future are being investigated worldwide. Namely, face mask and other protective clothing, biosensors, tissue engineering scaffolds, wound dressings, and drug delivery systems are in the pipeline. In recent years, as more and more research groups have been involved, the collective knowledge of this field has developed fast.

Electrospinning (ES) is the most common process for nanofiber production as it is a simple, cost-effective, but a versatile and practical method. The majority of nanofibers are produced by ES and the topic has an impressively growing literature. According to the main databases, a total number of 141 scientific articles about nanofibers for biomedical applications and 5505 articles about ES procedures were published last year.

Despite the intensive research in the field, very little attention is paid to modeling ES. Thus, a universally accepted simulation model for accurately predicting the nozzle-based or nozzle-free ES parameters has not been developed yet. Due to this, the majority of ES investigations rely on parametric analyses and empirical understanding of the process parameters. Therefore, further study and optimization of different ES techniques are necessary to gain more information about the advantages and limitations of each technique, paying particular attention to the developed ES parameters (including solution, process, and ambient conditions), reproducibility, and scaling-up. This approach will help to better understand and control the ES process, and develop optimized preparation protocols for each technology, which may later be employed for industrial reasons.

Abbreviations: ANOVA – Analysis of Variance; *API – Active Pharmaceutical Ingredient; CaCo-2 – Human Colorectal Adenocarcinoma Cell Line; CIP – Ciprofloxacin; DL – Drug Loading; DSC – Differential Scanning Calorimetry; EE – Entrapment Efficiency; ES – Electrospinning; FTIR – Fourier Transform Infrared Spectroscopy; HPLC – High Performance Liquid Chromatography; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NF – Electrospun Nanofibers Prepared by Nozzle-Free Electrospinning; PBS – Phosphate-Buffered Saline; PVP – Polyvinylpyrrolidone;* R^2 *– Regression Coefficient; SEM – Scanning Electron Microscopy; SN – Electrospun Nanofibers Prepared by Single-Needle Electrospinning; UV-Vis – Ultraviolet-Visible Spectroscopy; XRPD – X-ray Powder Diffractometry*

2. AIMS OF THE WORK

This Ph.D. work aimed to develop, investigate, and compare different polyvinyl-pyrrolidone (PVP) nanofibers loaded with ciprofloxacin (CIP) prepared by both nozzle-based and nozzle-free ES methods (Figure 2.). The final goal was to describe an optimized preparation protocol and thus extend the repertoire of drug-carrier nanofibers that could be used in the future as novel, innovative medications.

The research work was planned and carried out according to the following steps:

I) A literature review of the mechanism of ES, types of ES, and the preparation parameters affecting the properties of nanofibers was needed to set up an appropriate research plan for the experimental work.

II) As the first step of the experimental work, CIP-loaded nanofibers with proper morphology and physicochemical properties were fabricated using the conventional single-needle ES method. This step aimed to provide a starting point for the optimization, in addition to obtain nanofibrous formulations with improved physicochemical properties.

III) The next step was to optimize the single-needle ES by increasing the concentration of the active substance inside the nanofibers while maintaining all other improved physicochemical properties as before.

IV) In the third step of the experimental work, the goal was to increase the productivity of ES which required a shift to a newer and nozzle-free ES technology. CIP-loaded nanofibers were fabricated via a lab-built roller ES equipment. Additionally, the impact of the technology shift on nanofibers was investigated.

V) Finally, the single-needle and nozzle-free ES techniques were compared in terms of morphology, physicochemical properties, *in vitro* cytocompatibility, and stability of the prepared nanofibers. Highlighting the advantages and the drawbacks of the two techniques, a suggestion was made about which ES technique and which nanofiber formulation could be used as an innovative drug-delivery system.

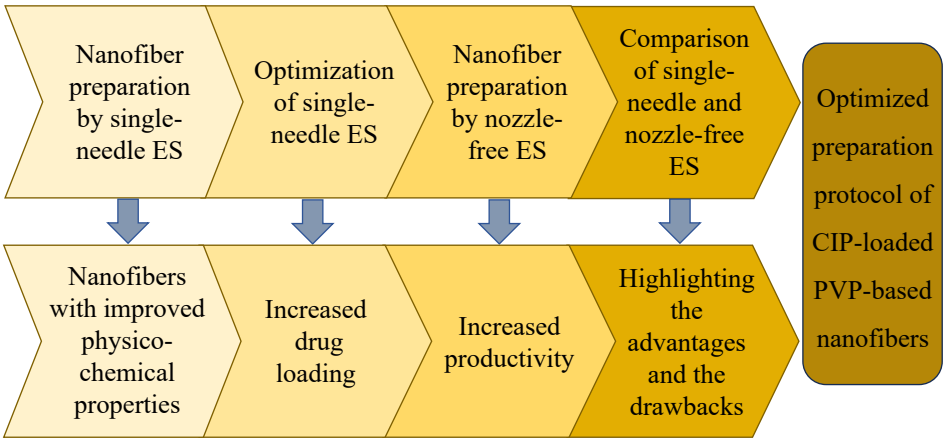

Figure 2. *The aims of the Ph.D. work*

3. MATERIALS AND METHODS

3.1. Materials

As mentioned above, in the case of drug-loaded nanofibers, the API is dispersed in the polymer matrix. In this Ph.D. work ciprofloxacin (CIP; Teva Pharmaceutical Works Ltd., Debrecen, Hungary) was incorporated into high molecular weight polyvinylpyrrolidone (PVP; Mw = 1,300,000; Alfa Aesar, Heysham, UK). The solvents for the ES solutions were chloroform (Fisher Scientific, Loughborough, UK), ethanol (Fisher Scientific, Loughborough, UK), and glacial acetic acid (Sigma-Aldrich, Hamburg, Germany).

For the in vitro drug release studies, pH 7.4 phosphate buffer solution (PBS) was prepared and used as dissolution media, and a commercially available, 250 mg CIP containing, filmcoated *per os* tablet was used as reference.

3.2. Methods

3.2.1. Different Electrospinning Methods During the Research Work

Initially, the aim was to prepare defect-free, fast-dissolving nanofibers by single-needle ES setup. To accomplish this goal, the composition (SN-0 – SN-1.5) and the flow rate (SN-F0.5 – SN F4) of the ES solution were altered to obtain different formulations. ES solutions were made by mixing the previously prepared CIP-in-chloroform and PVP-in-ethanol solutions in appropriate ratios to earn the desired drug-loading w/w%. After the viscosity had been measured (Haake Rheostress 1 Rheometer; Karlsruhe, Germany), the prepared ES solutions were filled in 2 mL syringes fitted with stainless-steel 20 G needles. The applied potential difference was 24 kV, and the needle-collector distance was maintained at 10 cm. The temperature in the chamber was 23 °C and the relative humidity was 36–42%. For the single-needle ES process, a commercially available ES device (IME Medical Electrospinning, Waalre, The Netherlands) was used.

In the second optimization step, the solvent of the CIP was changed for two reasons. Firstly, ***the objective was to increase the drug loading***, so a better solvent of CIP was needed to prepare a more concentrated solution. Secondly, chloroform is not considered a green solvent, so it was desirable to replace it with acetic acid. The setup was equipped with a stainless-steel 20 G needle, as the tip of a 2 mL syringe connected to a syringe pump (IME Medical Electrospinning; Waalre, the Netherlands). The applied potential difference was 24 kV, and the needle-collector distance was set at 15 cm. The flow rate was kept at 1 mL/h. The ES chamber was at room temperature, with a relative humidity of 31–36%. In this step, formulation NF-5 and NF-10 were prepared (Table 1.).

Table 1. *List of the prepared nanofibrous formulations with the process parameters and viscosity data.*

Sample name		CIP solution (concentration + solvent)	CIP-loading (w/w%)	ES setup	Flow rate (mL/h)	Viscosity of the ES solution (mPa s)
SN-0		-	0	single-needle ES	2	103 ± 6
SN-1	**SN-F0.5**	1 mg/mL in chloroform	1	single-needle ES (20 G)	0.5	148 ± 32
	SN-F1		1		1	148 ± 32
	SN-F2		1		2	148 ± 32
	SN-F3		1		3	148 ± 32
	SN-F4		1		4	148 ± 32
SN-1.3			1.3		2	201 ± 2
SN-1.5			1.5		2	283 ± 45
SN-5		20 mg/mL in acetic acid	5	single-needle ES (20 G)	1	336 ± 19
SN-10			10		1	79 ± 2
NF-5		20 mg/mL in acetic acid	5	roller ES	-	336 ± 19
NF-10			10		-	79 ± 2

In the next step, the aim was to form CIP-loaded nanofibers by a novel, nozzle-free ES technique. The roller ES setup contained a rotating mandrel electrode which provided the free surface for the Taylor-cone creation and jet ejection from the ES solution. The mandrel was partially immersed in the ES solution bath and +30 kV voltage was given to it. Placed opposite, the collector was 15 cm above the bath, covered by aluminum foil, and supplied with -15 kV. So, the applied voltage was +45 kV, nearly twice of the single-needle ES method. The apparatus was temperature-controlled, and the experiments were performed at 21.9 °C and 33% relative humidity. This roller ES equipment was built by our cooperation partner, Norbert Radacsi at the University of Edinburgh.

3.2.2. Preparation of the Physical Mixture

In the case of some *in vitro* studies, physical mixtures were used as reference samples. Therefore, PVP and CIP powder were mixed under controlled conditions (50 rpm, 10 min) and in the appropriate ratio using a shaker mixer (Turbula System Schatz; Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland).

3.2.3. Characterization of the Nanofibers

3.2.3.1. Micrometric Investigation

The morphology of the different nanofibrous mats was observed by ***Scanning Electron Microscopy*** (SEM; Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan) at 10 kV.

The diameter and the diameter distribution were measured by ImageJ 1.44p software (Bethesda, MD, USA) using the SEM images. The mean fiber diameter was calculated from the size of 50-100 nanofibers in the case of each formulation.

3.2.3.1. Structural Investigation

Fourier transform infrared spectroscopy (FTIR; Thermo Nicolet AVATAR 330, Thermo Fisher Scientific, Madison, USA) was performed to investigate the nanofiber structure in the molecular aspect. The KBr-sample discs were scanned 128 times over the range 4000–400 cm^{-1} and with a resolution of 4 cm^{-1}.

For the ***differential scanning calorimetry*** (DSC; Mettler Toledo 821e DSC; Mettler Inc., Schwerzenbach, Switzerland) measurements, approx. 3–5 mg samples were measured between 30–300 °C at a heating rate of 5 °C/min. Every measurement was normalized to the sample size.

X-ray powder diffraction (XRPD) was measured by the BRUKER D8 Advance Diffractometer (BRUKER AXS GmbH, Karlsruhe, Germany). All manipulations (Kα2-stripping, background removal, and smoothing) were performed with DIFFRAC plus EVA software (Karlsruhe, Germany). XRPD was also used to evaluate the stability of the formulations.

Raman spectroscopy (Thermo Fisher DXR Dispersive Raman microscope, Waltham, Thermo Fisher Scientific, Waltham, USA) was performed to determine the homogeneity of the nanofibrous mats. Raman maps of specimens from the collector's edge and the center were collected and compared.

3.2.3.2. In Vitro Studies

The ***drug loading*** and the ***drug entrapment efficiency*** were quantified by UV spectrophotometry (ABL&E-Jasco UV/VIS Spectrophotometer V-730, Budapest, Hungary). The UV absorbance was measured at λ_{max} = 277 nm after suitable dilution and the CIP concentration was calculated by a calibration curve ($y = 0.1391x$, $R^2 = 1$). In addition, the UV spectrophotometry was used to investigate the homogeneity of the electrospun mats.

In vitro solubility studies were carried out both in distilled water (pH 6.3) and pH 7.4 PBS. CIP powder, physical mixture, and CIP-loaded nanofibers were stirred for 24 h at room temperature in 3 mL of solvent. Then the samples were measured by UV spectrophotometry (ABL&E-Jasco UV/VIS Spectrophotometer V-730, Budapest, Hungary). The measurements were carried out three times.

The ***in vitro CIP release*** (Hanson Research SR8-Plus Release Device; Hanson Research, Chatsworth, USA) from the electrospun nanofibers was studied and compared with raw CIP powder and the physical mixtures or 250 mg tablets. 50 mL of pH 7.4 PBS medium was used at 37 °C and the paddle was rotated at 100 rpm. Samples of 0.5 mL volume

were taken manually from the buffer solution after 5, 10, 15, 30, 60, and 90 min. After sampling, the volume was replaced with fresh PBS.

The concentration of the drug present in the aliquots was determined in two different ways. In the beginning of the work, ***UV spectrophotometry*** was used without any separation but after ***high-performance liquid chromatography*** (HPLC) separation was executed in order to obtain better quality data.

The ***release kinetics of CIP*** from the nanofibers were determined and compared with the reference samples. Five different mathematical models (zero order, first order, Hixson–Crowell, Higuchi, and Korsmeyer–Peppas model) were fitted to confirm the release kinetics.

As ***in vitro cytotoxicity test***, MTT assay was carried out by our collaborative partners at the Department of Medical Microbiology and Immunobiology, University of Szeged. MTT assay was used to quantify mitochondrial activity using Caco-2 (Human Colorectal Adenocarcinoma) cells. The assay was repeated four times for each concentration.

3.2.3.3. Long-Term Storage Stability Tests

The nanofibrous samples were kept in a desiccator at room temperature (22 °C) and shielded from light. After 3, 5, 8, 16, and 26 months of storage, the stability was examined using SEM images and XRPD. The crucial samples were then subjected to *in vitro* drug release experiments.

3.2.3.4. Statistical Evaluation of the Results

Statistical analysis was performed to assess if there was a significant difference between the measured data. At the beginning, the data of the solubility and drug release were statistically compared by a two-sample t-test. After, one-way ANOVA with post hoc Tukey HSD test was executed on the fiber diameter and drug release data. The experimental results with p values <0.05 and <0.01 were assumed to be statistically significant.

4. RESULTS AND DISCUSSION

4.1. Preparation and Characterization of the Single-Needle Electrospun Nanofibers

At the start of this Ph.D. work, the goal was the production and investigation of CIP-loaded PVP-based nanofibers. Different compositions and flow rates were used to alter the production parameters (Table 1. Row 1-8.). Afterward, the morphology, structure, and *in vitro* properties of the samples were investigated.

In the initial literature review, it was found that the flow rate ranged between 0.2 and 2 mL/h in the case of drug loaded PVP nanofibers and the average fiber diameter of the different formulations varied largely. Therefore, in this study, the flow rate was set to 0.5, 1, 2, 3, and 4 mL/h while keeping the other preparation parameters constant (SN-F0.5 – SN-F4).

Additionally, it is known that the composition and the viscosity of the ES solution affect the properties of the nanofiber. This impact was studied by varying the PVP : CIP volume ratios during the ES solution mixing (SN-0 – SN-1.5).

4.1.1. Effect of Composition and Flow Rate on Micrometric Properties

The morphology of nanofibers was observed using SEM images, and then the average fiber diameter was measured by ImageJ software (Figure 1.). During the optimization process, the narrowest nanofiber formulation with the finest morphology was targeted.

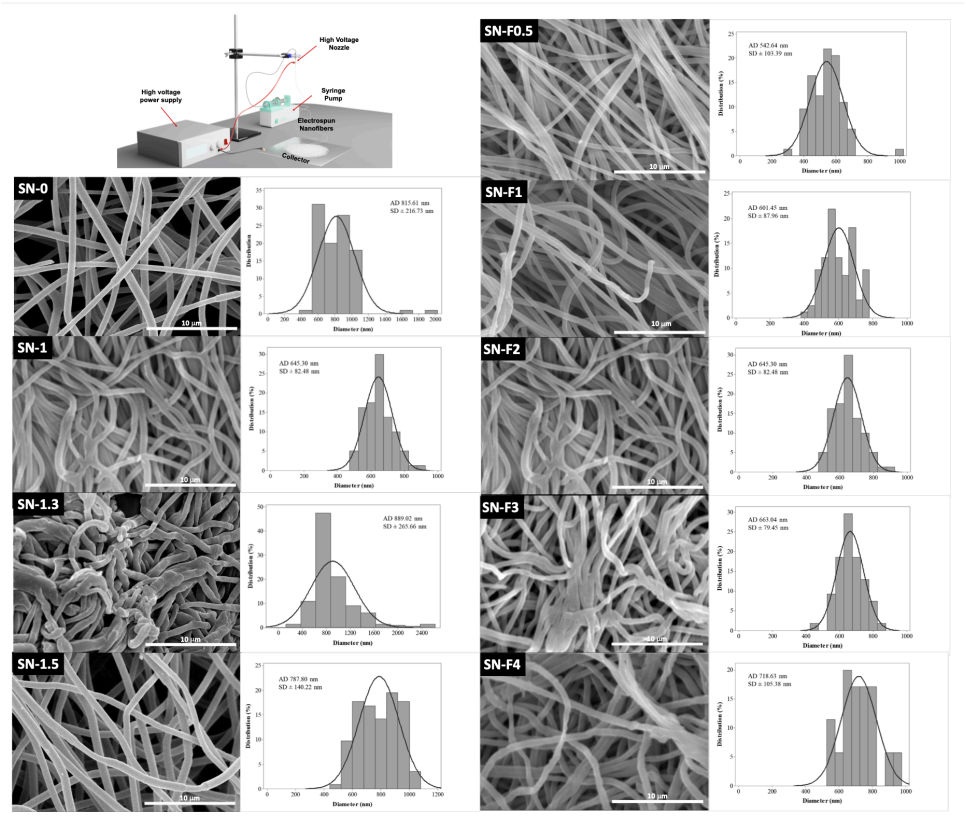

Figure 1. *SEM images and diameter distribution of the nanofibers prepared with single-needle ES*

Overall, continuous, smooth-surfaced nanofibers were successfully prepared from all the solutions studied except PVP:CIP 1:2 volume ratio (SN-1.3). SEM images of SN-1.3 formulation showed discontinuous, worm-like nanofibers with largely varying diameters (889 ± 265 nm). The average diameter of the pure PVP fibers (SN-0) was 815 ± 216 nm, and it had some narrowing that slightly resembled the formation of beads (Figure 1.). This was probably due to the low viscosity. In the case of the continuous fibers, the addition of the CIP decreased the fiber diameter. Higher solution conductivity could facilitate the elongation of the jet and the formation of thinner fibers.

As the flow rate increased, the average fiber diameter increased. The morphology of the fibers produced at higher flow rates was not appropriate. 3 mL/h flow rate (SN-F3) caused incomplete solvent evaporation and resulted in merged fibers, while the sample

produced by 4 mL/h flow rate (SN F4) showed some bead-like structures on top of the merged fibers. So, the nanofibers with proper morphology and the fastest ES, namely the sample SN-1/SN-F2 was selected as optimal formulation and was used for further studies.

4.1.2. Physicochemical Properties

XRPD and ***DSC*** studies were executed to investigate the crystallinity of the untreated CIP and PVP powders, the physical mixture, and the CIP-loaded nanofibers. While CIP powder and physical mixtures showed the characteristic peaks, the nanofiber diffractogram and thermogram both indicated the amorphous form of CIP created by the fast evaporation of the solvent during ES.

The ***FTIR*** spectra proved the main drug-polymer interactions. The shifts and widenings demonstrated that the CIP was successfully incorporated into the nanofibers.

4.1.3. Increased In Vitro Solubility and Drug Release

In the first phase of the research, high CIP content was not yet objective, so nanofibers with 1% theoretical CIP loading were prepared and tested. The percentages were calculated from the absorbance values of UV spectroscopy. In the case of the sample SN-F2, the ***calculated drug loading*** was 0.92 ± 0.08 w/w%.

CIP has a pH-dependent, U-shaped ***solubility*** profile since its isoelectric point is 7.42. The drug has high solubility if the pH < 5 or pH > 10, but CIP is poorly soluble around neutral pH. For this reason, pH 6.3 distilled water and pH 7.4 PBS were used as solvents to investigate the effect of the polymer nanocarrier on the solubility.

As the results showed, in the case of the physical mixture, the solubility was higher than the pure CIP powder, but not significantly (Table 2.). Probably the presence of PVP could enhance a little the solubility by increasing wettability. On the other hand, comparing the electrospun samples to the untreated materials, a significant increase ($p < 0.01$ in the case of water and $p < 0.05$ in the case of PBS) was observed. Nanofibers, as solid molecular dispersions, can guarantee increased solubility by not only improving wettability but stabilizing the amorph drug incorporated into the polymer matrix. Thus, this finding correlates with the results of the structural characterization.

Table 2. *Solubility data of the samples. The dissolution of the ciprofloxacin (CIP) shifted the pH from 6.3 to 7.1 in distilled water. Statistical analysis: Two-sample t-test (** $p < 0.01$; * $p < 0.05$).*

Sample	Solubility [mg/mL] in Water (pH 6.3 → pH 7.1)		Solubility [mg/mL] in PBS (pH 7.4)	
CIP	0.071 ± 0.001	**	0.099 ± 0.001	*
Physical mixture	0.182 ± 0.035	**	0.123 ± 0.001	*
Nanofiber	0.862 ± 0.074		0.629 ± 0.186	

The ***in vitro release*** of the CIP powder was not complete within 90 min, only 41 ± 3% (Figure 2.). Most probably its reason was the poor solubility of the drug at this pH. Similarly, at the end of the 90-minute measurement, the drug release from the physical mixture was 67 ± 12%. However, the electrospun sample showed a significantly higher dissolution rate than CIP powder ($p < 0.001$) at every measured point. Furthermore, while all the samples showed fast dissolution, the nanofibers released the CIP the most quickly. The faster dissolution rate resulted in 94 ± 6% dissolved CIP in only 5 minutes. The large surface-to-volume ratio, high wettability, and amorphous drug may explain the distinct difference between the CIP powder and nanofibers.

The ***drug release kinetics*** of the samples were also tested. Korsmeyer–Peppas model almost perfectly described the CIP release from the nanofibers (R^2 = 0.9993). This is reasonable since the model describes drug release from a polymeric system. It considers various mechanisms at the same time, such as water transport into the polymer matrix, swelling, and polymer dissolution.

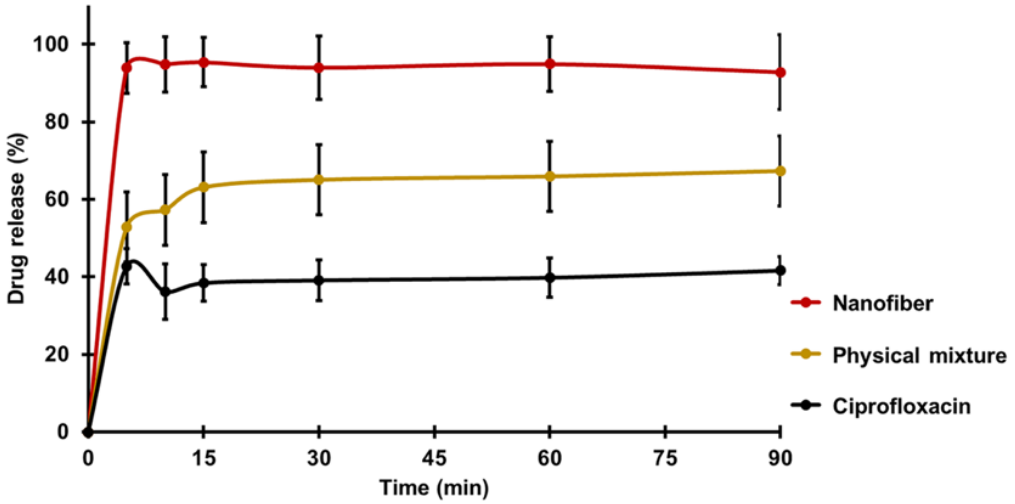

Figure 2. *In vitro dissolution of CIP from the nanofiber, physical mixture, and CIP powder*

4.2. Preparation and Characterization of the Single-Needle Electrospun Nanofibers with Increased CIP-content

In the second step of the research work, PVP-based nanofibers with 5 and 10 w/w% CIP content were fabricated by the same ES device (Table 1. Row 9-10.). To achieve higher drug loading, it was necessary to change the solvent of CIP from chloroform to acetic acid.

4.2.1. Effect of Viscosity on Micrometric Properties

Continuous fibers with smooth surfaces were formed from both compositions. However, the SN-10 sample had many large beads and sack-shaped formations indicating the inappropriate viscosity of the ES solution. With the increase of the viscosity, the average fiber diameter increased: SN-$5_{visc.}$= 336±19 mPa s, SN-$5_{diam.}$= 735±91 nm, and SN-$10_{visc.}$= 79 ± 2 mPa s, SN-$10_{diam.}$= 323 ± 51 nm. This phenomenon is well described in the literature.

The size distribution of the nanofibers was homogeneous, as the standard deviations were small, and the distribution diagrams were monodispersed, and bell-shaped.

4.2.2. Structural Characterization

Similar to the previous step of the whole work, ***DSC*** and ***XRPD*** were performed to prove that the ES method produced amorphous solid dispersions.

4.2.3. Increased *In Vitro* Drug Release

Drug loading and ***entrapment efficiency*** of both formulations were sufficiently high. Moreover, the nanofibrous formulations demonstrated rapid and 100% release. Comparing the nanofibers and the 250 mg tablets, the dissolved drug amount was significantly higher ($p < 0.01$) at every measured point.

4.2.4. Testing for Cytocompatibility

An MTT assay was used to assess ***in vitro cytotoxicity***. The untreated CIP powder solution was shown to be cytotoxic at concentrations of 163 μg/mL, whereas the solutions containing different nanofibers were not lethal to CaCo-2 cells at any of the observed values.

4.3. Preparation and Characterization of the Nozzle-Free Electrospun Nanofibers

In the third step of the research work, the focus moved toward productivity improvements. Roller-type free-surface ES device was used to produce nanofibers from 5% and 10% CIP-containing ES solutions (Table 1. Row 11-12.). It was observed that a higher voltage was needed to spin the 5% solution than previously. Additionally, the viscosity of the 10% solution was too low, so fiber preparation was impossible, even by varying the collector-bath distance over a range of 10–20 cm, or by varying the applied voltage between +20-30 and −30-−15 kV on the rotating mandrel and the collector, respectively. The fibers stuck together and fused, resulting in an incorrect product, as Figure 3. shows. Thus, fiber diameter measurement and further tests with the NF-10 sample were disregarded.

4.3.1. Habit and Micrometric Properties

In the case of the NF-5 sample, the free-surface ES was successful, and continuous, smooth-surfaced nanofibers were produced. The average fiber diameter was 1167 ± 415 nm, which was 3.6 times larger than that of the SN-5 formulation. The fiber diameter varied widely, and the diameter distribution was polydisperse and strongly skewed left. The histogram shows that 42% of the measured nanofibers were between 600 and 1000 nm, and 34% were between 700 and 900 nm. So, the modus was near the average value of the SN-10 sample (735 ± 91 nm).

The non-uniform fiber diameter distribution may be explained by the principle of the nozzle-free ES. Taylor cones form in several spots of the thin liquid layer on the surface of the rotating electrode, so the spinning happens in several jets at the same time.

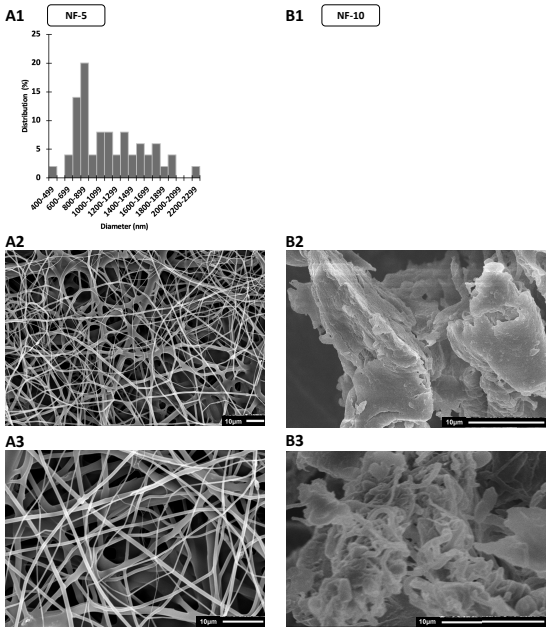

Figure 3. *SEM images and diameter distributions of samples produced by nozzle-free ES. NF-10 sample was not fibrosus, so the fiber diameter was not measurable, and the distribution was not interpretable.*

4.3.2. Structural Characterization

In the previous sections, it was seen that the CIP amorphized during the single-needle ES. The purpose of the structural characterization was to see if the same was also true in the case of the roller ES technique. Both ***DSC*** and ***XRPD*** showed the same results as in the previous steps. Based on these results, the ES method did not affect the behavior of the drug and facilitated amorphization.

4.3.3. Increased In Vitro Drug Release

The ***drug loading*** calculated from the NF-5 sample was 4.55 ± 0.36 w/w%, and the ***entrapment efficiency*** was 90.1 ± 0.7%. Thus, sufficiently high entrapment efficiency was achieved by the nozzle-free ES method.

Due to the water-soluble polymer, the amorphous drug, and the previous experience, fast CIP release was expected from the nanofibers. The ***release study*** proved the concept, as 75 ± 7% CIP was released in the first 5 minutes and the dissolution was complete in 10 minutes. Moreover, the CIP ***solubility*** increased in the nanofibrous formulation, two and a half times in the case of the commercially available tablets, and five times in the case of the untreated CIP powder. The ***release kinetics*** of the NF-5 sample could be best described using the Korsmeyer–Peppas model and first-order kinetics.

4.3.4. Testing for Cytocompatibility

Cytotoxicity was tested on CaCo-2 cells in the case of the nozzle-free electrospun fibers, similar to the previous step. The nanofiber formulation was found to be cell compatible, which was expected, as PVP is a biocompatible polymer.

4.4. Comparison of the Single-Needle and the Nozzle-Free Electrospun Nanofibers

The electrospinnability of the same 5% and 10% ES solutions on single-needle and nozzle-free ES devices were investigated. The morphology, crystallinity, drug loading and entrapment efficiency, *in vitro* solubility and dissolution, and *in vitro* cytotoxicity of the prepared nanofibers were presented in the previous sections. The results of these tests were similar regardless of the ES type. However, the drug distribution and the long-term stability were different in the two cases.

4.4.1. Homogeneity of the Nanofiber Mats

Two approaches (Raman and UV spectroscopy) were used to investigate the homogeneity of the CIP distribution in the nanofiber mats. In both cases, sample specimens taken from the center and the edge of the collector were tested.

After selecting the spectral region, the electrospun specimens were subjected to ***Raman chemical mapping***. The center specimens prepared by single-needle ES and the SN-5 edge specimen showed medium-high CIP contents with noticeable variations within each map. Furthermore, the drug concentration of the SN-10 edge specimen was minimal. Thus, nanofiber mats prepared by single-needle ES contained a higher amount of the drug in the center than at the edges. So, the CIP distribution in the whole mat was not homogeneous. On the other hand, the nozzle-free specimens both had high levels of CIP. Thus, not only the two Raman maps themselves, but the whole nanofiber mat can be considered homogenous.

The drug loading and entrapment efficiency were calculated from absorbance data of ***UV spectroscopy***. The results corroborated the findings of the Raman mapping.

In summary, nozzle-free production results in a more homogeneous nanofiber mat gathering on the collector. The explanation for this might be that the jets' origin is not concentrated at a single spot, as with needle-based ES, but is released from several sites in the bath simultaneously.

4.4.2. Long-Term Storage Stability Tests

The long-term storage stability was investigated using three different analytical tests over a period of 26 months. The changes in the fiber morphology were visualized by SEM images, the amorphous-crystalline transformation was checked by XRPD, and the *in vitro* dissolution was compared. The stability of nanofibers was also investigated by other researchers, but in most cases, it only involved crystallinity testing and a maximum storage time of one year.

After examining the ***microscopic images*** and the fiber diameter distribution, it was found that the morphological stabilities of SN-10, NF-5, and SN-5 were 8 months, 16 months, and more than 26 months, respectively.

The amorphous-crystalline transformation of the CIP was examined using ***XRPD***. In the case of the SN-10 nanofibers, a minor acetate peak was found at 5 months, and high peaks at 16 and 26 months. At 8, 16, and 26 months, further characteristic CIP peaks could be seen in the patterns. Likewise, signs of re-crystallization appeared on the XRPD pattern of the 5% single-needle sample after 8 months, 3 months later than in the 10% sample.

In the case of the nozzle-free electrospun fibers, every XRPD pattern was flat, without any sharp peaks, and two of them showed the broad peak of PVP. Thus, nozzle-free ES provides better stability since CIP was amorphous until the end of the stability study.

Although SN-10 showed stability problems based on SEM and XRPD measurements, the ***drug release*** was not affected as the total amount of CIP dissolved in 5 minutes (Figure 4.). Similar behavior was observed with SN-5 after 8 months. The re-crystallization of CIP was already visible in the XRPD pattern, but the drug release was unchanged. However, in the case of the 16-month stored sample, reaching the 90% drug release took approximately three times longer than the fresh or the 8-month sample. The slower release might be explained by the formation of additional H-bonds between the CIP and the PVP molecules. On the other hand, the CIP remained amorphous after 26 months in the NF-5 sample, and there was no change in the *in vitro* drug release.

Overall, the nozzle-free electrospun fibers proved to be more stable in terms of drug release.

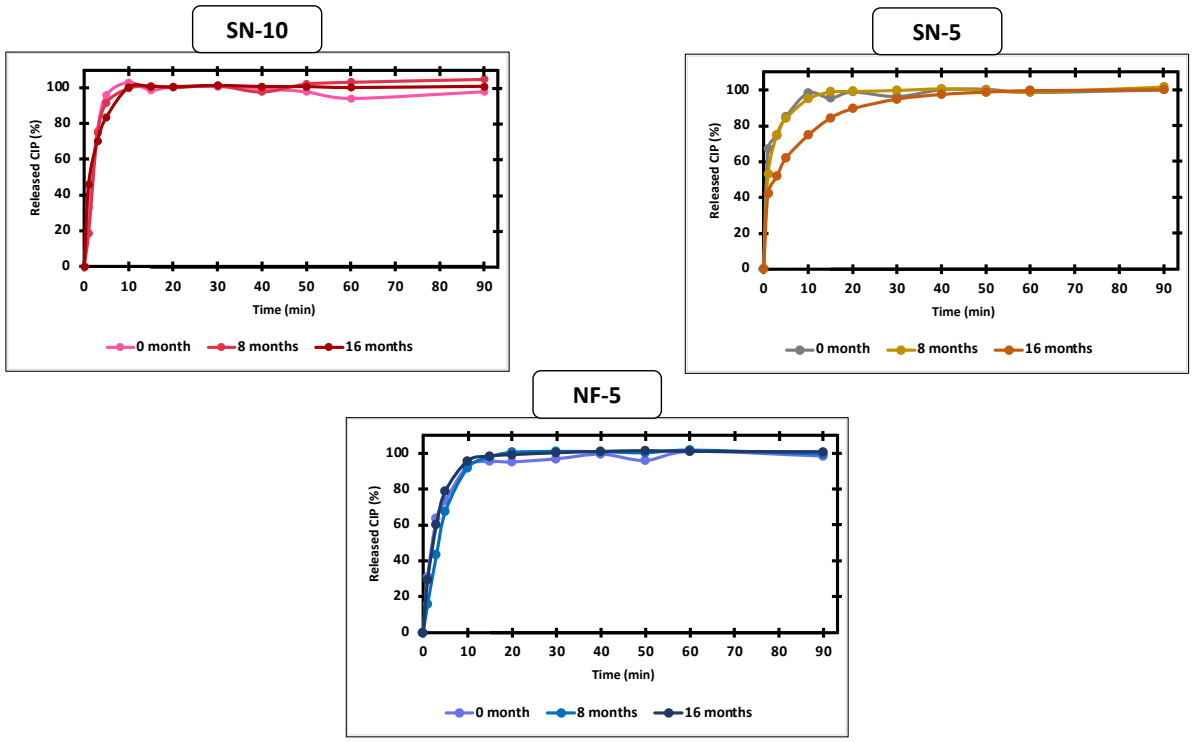

Figure 4. *Comparison of in vitro ciprofloxacin (CIP) release of nanofibers produced using single-needle (SN-10 and SN-5) and nozzle-free (NF-5) electrospinning after 0, 8, and 16 months of storage.*

4.5. Comparison tables

By single-needle and nozzle-free ES devices, the electrospinnability of the same 5% and 10% ES solutions was examined. The general and the specific advantages and disadvantages of the two methods are presented in Table 3.

Table 3. *The advantages and disadvantages of single-needle and nozzle-free electrospinning techniques.*

Technique	Advantages	Disadvantages
Single-needle ES	*General:* 1. simple setup, easy to operate 2. well-studied method 3. possibility of coaxial or Janus ES 4. possibility of multi-axial ES	*General:* 1. low productivity 2. clogging of the needle 3. neighboring needle jet repulsion and deviation
	Specific to this work: 1. fiber preparation from a low viscosity solution (~80 mPa s) 2. lower working voltage (24 kV) 3. higher entrapment efficiency (~90-95%)	*Specific to this work:* 1. time-consuming method (1-2 mL/h) 2. fixed plate collector (smaller nanofiber mats) 3. nanofiber formation concentrated in the center of the collector (inhomogeneity)
Nozzle-free ES	*General:* 1. multiple Taylor cones 2. possibility of controlling the number of Taylor cones (ES solution conductivity) 3. high production rates 4. no nozzle (no clogging or neighboring needle effect)	*General:* 1. more complex optimization (low controllability) 2. only one ES solution (simple fiber configuration, no core-shell or Janus) 3. solvent evaporation from the bath (changing ES solution concentration) 4. higher ES solution wastage
	Specific to this work: 1. faster production 2. rotating collector (bigger mat) 3. homogenous nanofiber mat	*Specific to this work:* 1. requires higher viscosity (no fibers from ~80 mPa s ES solution) 2. requires higher voltage (45 kV)

The morphology, physicochemical properties and in vitro properties of the prepared nanofiber samples were also investigated. A summary of these results is shown in Table 4.

Table 4. *Properties of the single-needle and the nozzle-free electrospun nanofibers loaded with ciprofloxacin.*

Sample	Technique	Morphology	Fiber diameter	Fiber diameter distribution	Stability of the morphology
SN-5	Single-needle ES	continuous, smooth, proper	735 ± 91 nm	monodisperse	26 months
SN-10	Single-needle ES	continuous, smooth, beads	323 ± 51 nm	monodisperse	8 months
NF-5	Nozzle-free ES	continuous, smooth, fiber net	1167 ± 415 nm	polydisperse	16 months
Sample	**Drug loading**	**Entrapment efficiency**	**Mat homogeneity**	**CIP crystallinity**	**Stability of the crystallinity**
SN-5	4.55 ± 0.93 w/w%	90.9 ± 1.9%	inhomogeneous	amorphous	5 months
SN-10	9.56 ± 0.79 w/w%	95.6 ± 0.8%	inhomogeneous	amorphous	3 months
NF-5	4.55 ± 0.36 w/w%	90.1 ± 0.7%	homogenous	amorphous	26 months
Sample	**CIP solubility**	**CIP release**	**Stability of the CIP release**	**CIP release kinetics**	**Cytotoxicity**
SN-5	improved	fast (5 min), complete	8 months	Korsmeyer–Peppas	non-toxic
SN-10	improved	fast (5 min), complete	16 months	Hixon-Crowell cube root	non-toxic
NF-5	improved	fast (10 min), complete	16 months	First order	non-toxic

In summary, the solubility and release of CIP in nanofibers prepared by both ES techniques increased significantly. The entrapment efficiency was sufficiently high, and the drug was amorphized in all samples. Also, all nanofibers were cytocompatible and nontoxic to CaCo-2 cells.

However, differences were also found. The single-needle ES could produce more uniform fibers, with significantly smaller fiber diameters and a monodispersed diameter distribution. Also, the identity of the fibers was preserved, while the free-surface ES resulted in a web-like nanofiber mat with greatly varying fiber diameter and polydisperse distribution. On the other hand, the CIP distribution of the whole nanofiber mat was more homogeneous in the case of the nozzle-free ES. Additionally, the storage stability was tested in 26 months, and differences were also seen between the samples both produced by the single-needle ES method. The results showed that the amorphous drug was better stabilized in the nanofibers produced nozzle-free. However, this did not affect the *in vitro* release, as both SN-10 and NF-5 samples showed the same release after 16 months as the fresh samples.

5. CONCLUSIONS

Nanofibers have unique physical characteristics due to their nanoscale size, making them suitable for applications in various fields. There are many different nanofiber production techniques, but ES is the most used. Although the process is quite simple and has been known for more than a century, it is still challenging due to the interaction of several connected elements. Thus, it is important to continue researching the mechanism of different ES methods since new discoveries will lead to more predictable process results and products that will perform new functionalities.

The aim of my Ph.D. work was to investigate CIP-loaded and PVP-based nanofibers prepared by both single-needle and nozzle-free ES. Also, the optimization of the two different types of ES methods was aimed. Additionally, the goal was to compare the ES methods by highlighting their advantages and drawbacks.

The five aims collected at the beginning of the thesis were fulfilled as follows.

I.) After a review of the literature on nanofibers, a four-step research plan was set up. Additionally, a review article on the potential applications and their regulation was also published.

II.) As first step of the experimental work, the conventional single-needle ES was used to prepare nanofibers with improved physicochemical properties. The effect of the composition and the flow rate were tested, and the initial process was optimized. The sample prepared from 1:1 volume ratio PVP solution : CIP solution (viscosity 148 ± 32 mPa s) pumped with 2 mL/h flow rate was found to be the best among the 8 formulations. A significant increase in solubility in both water and pH 7.4 PBS was achieved with this sample. The *in vitro* CIP release was fast and complete. The EE% was sufficiently high (92 ± 8%) but the DL% needed to be increased.

III.) As the second step, the amount of the incorporated CIP was increased to 5% and 10%. To achieve higher drug loading, it was necessary to change the solvent of CIP from chloroform to glacial acetic acid. Acetate peaks showed up on the X-ray diffractogram, but it did not affect the CIP release, as it was fast and complete. Besides the physicochemical properties, the cytocompatibility and the stability of the nanofibers were investigated. Overall, the change in the composition did not negatively affect the results previously achieved. Moreover, the formulations were not harmful for CaCo-2 cells and were stable in terms of morphology and drug release for at least 8 months.

IV.) In the third step of the research, a novel, nozzle-free ES setup was tested. To obtain easily comparable nanofibers, the same compositions of ES solutions (5% and 10% CIP) were used by the roller ES method. In the case of the 5% ES solution, it was possible

to produce nanofibers that meet all the requirements and improve the *in vitro* solubility and release of CIP. The storage stability was 16 months, twice longer than the single-needle sample. The formulation was not cytotoxic *in vitro*.

V.) Comparing the two ES methods, the following findings were concluded. The main advantage of the nozzle-free method were the faster preparation and the higher productivity which are obvious consequences of the production process itself. Moreover, the CIP distribution was more homogenous in terms of the whole nanofiber mat. However, the nozzle-free method required higher viscosity, since fiber formation was not achieved from ~80 mPa s ES solution, and it also required higher working voltage (45 kV) compared to the single-needle ES (24 kV). Finally, another difference is worth highlighting. The different ES methods produced nanofibers with very different fiber diameter distributions and mat structures. In the case of the single-needle ES, the fiber diameter was more uniform, and the individual fibers were randomly arranged. During the roller ES process, generally wider nanofibers with various diameters were prepared from the several jets forming simultaneously. The structure of the fiber mat was more net-like. However, it cannot be clearly stated that this is a disadvantage. This attribute might be beneficial in tissue engineering.

Overall, the results and findings fit into this scientific research area, extend the knowledge, and give a detailed overview of the different ES methods. Hopefully, the presented comparison helps to establish and perform studies on nanofibers with less effort and more success, and the observations can provide a valuable base for further developments.

The main new findings of the work:

- Because the production of electrospun nanofibers is empirically planned and predicted, there is a need to broaden the collective knowledge about ES. As a result, as more research findings are published, more information is available to develop electrospun nanofibers for medical applications. This thesis may also contribute since it provides up-to-date and summarized knowledge about single-needle and nozzle-free roller ES.
- In this work, I have investigated, optimized, and compared the preparation of CIP-loaded PVP nanofibers by conventional single-needle ES and novel-type roller ES. The effect of process parameters has been investigated for the first time in the presented compositions via both nozzle-based and nozzle-free ES methods.

- In research, the negative outcome can also provide important information. Unsuccessful nanofiber formation with roller ES due to too low viscosity of the polymer solution was published for the first time.
- It was proved, that the nanofibrous formulations showed improved physicochemical properties, e.g., the *in vitro* CIP solubility and release significantly increased. Also, the formulations were cytocompatible and stable for 8 and 16 months.
- The storage stability was tested in three different aspects (morphology, crystallinity, and drug release) over 26 months. This was the first such comprehensive stability study for drug carrier nanofibers.
- At the end of the thesis, all the results have been summarized in a table to facilitate the comparison of the two ES methods and the nanofibers produced. Additionally, observations and recommendations on the different ES processes were presented to provide a base that can be successfully adapted to other nanofiber formulations and preparation methods.

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. **Uhljar L. É.,** Ambrus R.: A sebkezelés jövője – avagy antibiotikum-tartalmú nanoszálak fejlesztése; GYÓGYSZERÉSZET 65 (2021) 29-35

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IV. **L. É. Uhljar,** R. Ambrus: Electrospinning of Potential Medical Devices (Wound Dressings, Tissue Engineering Scaffolds, Face Masks) and Their Regulatory Approach; PHARMACEUTICS 15 (2023) 417; **Q1, IF: 5.4** https://doi.org/10.3390/pharmaceutics15020417

PUBLICATIONS NOT RELATED TO THE SUBJECT OF THE THESIS

I. **L. É. Uhljar,** B. Kürtösi, R. Ambrus: Optimization of nanocapsule preparation protocols using solvent displacement method; Tavaszi Szél 2022 / Spring Wind 2022 Tanulmánykötet III. (2022) 66-74

PRESENTATIONS RELATED TO THE SUBJECT OF THE THESIS

Oral presentations

I. **L. É. Uhljar,** R. Ambrus: Investigation of bottom-up prepared nanostructures; *II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 23-24. January 2020. Szeged, Hungary*

II. **L. É. Uhljar,** A. Alshweiat, N. Radacsi, R. Ambrus: Investigation of polymer-based nanofibers to modify the physico-chemical properties of ciprofloxacin; *Medical Conference for PhD Students and Experts of Clinical Science 2020, 17. October 2020. online*

III. **L. É. Uhljar,** S. Y. Kan, N. Radacsi, R. Ambrus: Preformulation studies of ciprofloxacin loaded PVP nanofibers; *III. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 20-22. January 2021. online*

IV. **L. É. Uhljar:** Development of antibiotic-loaded electrospun nanofibers; *DOSZ OEO Science and Innovation Conference, 29-30. January 2021. online*

V. **L. É. Uhljar,** R. Ambrus: Különböző szálképzési módszerekkel előállított polimeralapú nanoszálak vizsgálata*; Kárpát-medencei fiatal magyar kutatók konferenciája, 30-31. March 2021. online*

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VII. **L. É. Uhljar,** R. Ambrus: Process optimalization of the preparation of PVP-based nanofibrous drug carrier loaded with ciprofloxacin; *V. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 18-20. January 2023. Szeged, Hungary*

VIII. **Uhljar L. É.,** Ambrus R.: Hatóanyag-hordozó nanoszálak fejlesztése eltérő elektrosztatikus berendezéssel; *MKE Kristályosítási és Gyógyszerformulálási Szakosztály Kerekasztal konferencia, 5-6. Juny 2023. Balatonszemes, Hungary*

Poster presentations

I. **L. É. Uhljar,** A. Alshweiat, S.Y. Kan, A. Esmail, N. Radacsi, R. Ambrus: Preformulation studies of ciprofloxacin-loaded polymer-based electrospun nanofibers; *Congressus Pharmaceuticus Hungaricus XVI., 10-12. September 2020. online*

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III. **L. É. Uhljar,** N. Radacsi, R. Ambrus: Comparison of single-needle and nozzle-free electrospinning methods; *IV. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 19-21. January 2022. Szeged, Hungary*

PRESENTATIONS NOT RELATED TO THE SUBJECT OF THE THESIS

Oral presentations

I. **Uhljar L. É.,** Ambrus R.: Nanokapszula előállítási módszer kiterjesztése különböző hatóanyagokra faktoriális kísérlettervezéssel; *XXVI. Spring Wind Conference, 5-7. May 2023. Miskolc, Hungary*

Poster presentations

I. **L. É. Uhljar,** P. Gieszinger, P. Szabó-Révész, R. Ambrus: Preformulation Studies of Polymeric Nanocapsules; *International Conference on Advances in Pharmaceutical Drug Development, Quality Control and Regulatory Sciences (DDRS 2021), 15-17. November 2021. Budapest, Hungary*

II. **L. É. Uhljar,** B. Kürtösi, R. Ambrus: Optimization of nanocapsule preparation protocols using solvent displacement method; *XXV. Spring Wind Conference, 6-8. May 2022. Pécs, Hungary*

III. **L. É. Uhljar,** B. Kürtösi, R. Ambrus: Development of a preparation method for nanocapsules using factorial design; *9th BBBB International Conference on Pharmaceutical Sciences - Pharma Sciences of Tomorrow, 15-17. September 2022. Ljubljana, Slovenia*

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