Formulation of nano and microfibrous drug delivery systems and *in vitro* and *in vivo* examination of topical therapeutic applicability of fibrous scaffolds

Ph.D. thesis

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INTRODUCTION

Among the current pharmaceutical technological challenges of generic and original drug development, the enhancement of the solubility of poorly soluble drugs, as well as the stable and designable industrial and clinical reproducibility of targeted and controlled drug delivery are of special impact. The development of combinatorial chemistry has greatly influenced the selection of therapeutically effective appropriate drugs at the level of active ingredients, but this has not yet relieved the formulation scientists from the solubility problem, which requires almost original research work during generic drug development. In the course of the development of innovative, non-conventional drug delivery systems both of the polymeric-based formulation techniques and the physical, physico-chemical and biological characteristics of the drug-excipients system should be investigated. The optimization and scale-up of the applied technology is required. The micro and macrostructural properties of the formulated systems should be examined in relation to the applied polymers, drugs and the formulation technique in order to obtain information about the therapeutic values, industrial implementation and novelty of the formulation. Further development and optimization, meeting the pharmaceutical, clinical and regulatory requirements, can be stated only after these preliminary investigations. Among the techniques suitable for formulation of innovative drug delivery systems, the pharmaceutical and biomedical application of nano- and microfiber structures produced by different methods is at the practical and industrial feasibility. The advantageous properties of fibrous systems provide a wide margin in the drug development. Based on these, it is possible to formulate compositions of enhanced solubility and potential bioavailability of poorly soluble active substances, reducing the dose or repositioning existing chemical entities.

It is feasible to apply the fibrous systems for wound healing in the form of absorbable topical formulations based on their tissue regeneration effects.

OBJECTIVES

- Optimization of the laboratory spinning technique with pharmacopoeial polymers for the preparation of morphologically uniform fibrous systems,
- Examination of the rotational spinning ability of polymeric gels based on their physicochemical characterization with instrumental monitoring and the micro and macrostructural characterization of the formulated fibrous structure,
- Incorporation and *in vitro* testing of antibacterial agent in microfibres,
- Implemenation of a novel spinning technology (contact spinning) for the formulation of microfibers and elaboration of the technical conditions,
- Formulation of drug-loaded polymeric microfibers into tablets and their in vitro dissolution study,
- *In vitro* and *in vivo* study of the antibacterial and tissue regeneration effect of electrospun APO (All Peptide Optimized) polypeptide-loaded nanofibers,
- Formulation of electrospun multilayer colistin sulfate-loaded nanofibrous scaffolds for wound dressing,
- Custom-made design of a drug release cell for the examination of the controlled and sustained drug release from topical nanofibrous delivery systems,
- Study of the antibacterial and wound healing effect as well as the therapeutic applicability of the multilayer nanofibrous topical formulation,
- Mathematical modelling of drug release from the multilayer nanofibrous topical formulation.

METHODS

Preparation of hydrogels

For the optimization of the high-speed rotary spinning, the fiber forming PVP polymer was dissolved in the mixture of water-alcohol (98 % ethanol and distilled water). As an antibacterial agent povidone iodine complex was used in the form of Braunol solution (7.5 w/w% povidone iodine complex) added to the system and also served as a solvent.
Tabletting of the microfibers was made in comparison with free films of the same composition. The stock solution of vitamin B_{12} model drug of 5 mg/ml concentration also served as a solvent for polymers.
For further experiments, nanofibers were prepared from 15 w/w% PVA (Mowiol® 18-88) gels. In one part of the empty gel, colistine sulfate was dissolved while in the other part A3-APO polypeptide was dissolved in 2 w/w% of total solutes.

Preparation of microfibrous mat with custom-made high-speed rotary spinning device

The fiber formation was carried out with a WSE 602M (AEG, Germany) motor based on high-speed rotary spinning technique. The rotating reservoir was a aluminium-polyamide spinneret with 30 ml internal volume. The various fibers made from the PVP 30 hydrogels at 411 RCF (relative centrifugal force) with about 1 g/min production rate. The rotating speed was controlled with a toroidal transformator and measured with laser revolution counter (DT-10L, Voltcraft, Germany). The internal diameter of the nozzles was 0.3 mm.

Micronization and tabletting of fibers

The first fine cutting of fibers was carried out with 2000 rpm for 10 minutes in Stefan UMC5 apparatus in order to obtain a sample capable for micronization. The micronization process was done in Retsch MM301 vibrating mill with balls of various diameters and 900 rpm revolution rate. The micronization parameters were the followings: Number and diameter of balls: 6 and 15 mm.
Number and diameter of balls in the second micronization step: 30 and 5 mm; Time of micronization was 15 minutes in each step. The physical mixture of micronized fibers and different tabletting excipients was homogenized in Turbula T2F mixer with 34 rpm

revolution number for 60 minutes. After the homogenization the obtained powder mixture was directly compressed with Diaf (Denmark) single punch press of 12 mm in diameter at constant compression force.

Preparation of multilayer nanofibrous mat for wound healing

Multilayer mat was prepared by electrospinning from polyvinyl alcohol (PVA) gels.The multilayer mat comprised various PVA fibrous layers prepared with or without the active ingredient. One of the electrospun layers contained water-soluble colistin sulfate (CEL) and the other was prepared from the same polymer type and composition without the active drug and was finally heat-treated. The heat treatment modified the supramolecular structure and conferred the polymer nanofibre with the rate-controlling function (MEL).

Preparation of microfibrous mat with custom-made contact spinning device

A novel custom-made contact spinning method was developed for the preparation of microfibers which was published under the Patent submission P1400283, issued on 28.08.2015.

Optical digital microscopic images

Optical microscopic images of the fibers and fibrous samples were taken using a 20-200x magnification digital microscope (Digimicro 2.0 Scale, DNT®, Germany).

Scanning Electron Microscopic images

Multiple magnifications (1000-10000-fold) of the samples were made by scanning electron microscopy (SEM; JEOL 6380LVa, Tokyo, Japan). Based on the photos, the average fiber thickness of the individual fibers and the average distance between the fibers were determined.

Rheological-based optimization of fiber forming hydrogels

Optimization of polymer-solvent ratio of gels containing PVP 30 for high-speed rotary spinning was followed by the determination of dynamic viscosity. Measurements were made with Kinexus Pro (Malvern Instruments Ltd, United Kingdom) at 25 °C (± 0.1° C).

Measurement of the tensile strength and the elastic properties of nanofibers

The measurements were carried out with Zwick Z005 (Zwick Roell GmbH, Germany) universal testing instrument at 5 mm/min test speed and 25 mm clamping length using 15 N upper force limits at room temperature. The tensile strength and Young's moduli of randomly oriented fiber webs were calculated using the following equation:

$$\sigma\left(Pa;\frac{N}{m^2}\right)=\frac{F_{max}(N)\cdot\rho\left(\frac{kg}{m^3}\right)}{TEX(\frac{kg}{m})} \qquad \textbf{(E1)}$$

Fmax is the maximum force, ρ is the average density of the fibers, TEX is the linear density which equals to W/L where W is the mass of the fibers and L is the length of fiber. Elongation was determined by maximum force function of displacement curve.

Positron Annihilation Lifetime Specroscopy (PALS)

For the microstructural characterization of the fibres, PALS was applied as a unique method since it is exceptionally sensitive to free volume. The measurement is based on the interaction between the free-volume holes and the so-called ortho-positronium (o-P) atom. For positron lifetime measurements, a positron source made of carrier-free ^{22}NaCl was used. Three parallel spectra were measured at each concentration to increase reliability. After summarizing the parallels, the spectra were evaluated by the RESOLUTION computer code; the indicated errors were the deviations in the obtained lifetime parameters. Three lifetime components were found in all samples. The MELT code was used to extract lifetime distributions from the spectra. These latter evaluations were used to characterize the size distribution of the free-volume holes in the samples throughout the o-P lifetime.

Drug release study of microfiber-based tablets

Dissolution and release of B_{12} vitamin from the fiber-based solid tablet was determined by Hanson SR8PLUS machine with rotating shovel apparatus at 37 °C. The rotating speed was 50 rpm and the dissolution medium was 250 ml of aqueous phosphate buffer (pH=6.8) with 2 ml sampling volumes were taken in the predetermined time intervals. The measuring time was 1 hour.

Quantitative determination of B_{12}-vitamin by HPLC-UV technique

LC separation was performed on an Agilent 1050 HPLC equipped with a degasser, a quaternary pump, an autosampler, a column thermostat and a variable wavelength detector. Gradient elution was carried out at a flow rate of 1 mL/min using 0.05% V/V trifluoroacetic acid acid in MilliQ as "A" and acetonitrile as "B" eluents. The gradient separation was started at 10% B then this composition was held for a minute. A linear gradient was increased to 90% B within 4 minutes. After it the eluent composition was lowered to 10% B immediately. 3 minutes equilibration time was needed before the next injection. Before the injection samples were centrifuged for 5 minutes at 3300 RCF in a Z230 Hermle centrifuge. Sample aliquots of 200 µl were injected onto a 100 mm x 4.6 mm x 2.6 µm Kinetex XB-C18 (Kinetex, Phenomenex, Hungary) core-shell column. The column temperature was kept at 40 °C during the analysis. The detection wavelength was 362 nm and the peak width was 0.053 minutes. The HPLC method was validated between 50 ng/mL and 50 µg/mL.

Topical modelling of drug-release in a custom-made dissolution device

A dynamic individually designed glass release cell was used for the drug-release study of the active ingredient. The effective volume of the dissolution container was 70.41 ml and the volume of the taken samples was 1.0 ml. The dissolution medium was a phosphate buffer. The sampling was performed with a Hamilton syringe. The volume of the taken sample was replenished from the equalization tank with the same volume at the moment of sampling. The test was performed at room temperature (25°C ± 2°C) under magnetic stirring at 50 rpm for 240 min at the following predetermined times: 1, 3, 5, 7, 10, 13, 16,

20, 25, 30, 45, 60, 90, 120, 180, 240 min. The sample was fixed and sealed in order to contact the buffer solution only on one side.

Quantitative determination of colistin with ultra-performance liquid chromatography–mass spectrometry (UPLC–MS)

Liquid chromatographic separation was performed on a Waters Acquity UPLC H-Class system. A Waters Acquity QDa Detector equipped with an ESI ion source was used for detection. Data acquisition and evaluation were performed using Empower 3 software. Hermle Z230A was used to perform centrifugation. HPLC grade water was provided by a Direct-Q 5 (Millipore) system. Gradient separation was performed on a Thermo Hypersil Gold C18 column (30 mm × 2.0 mm × 1.9 μm) at a flow rate of 0.5 ml/min. 0.5% (v/v) formic acid in Direct-Q5 was used as eluent 'A' and 0.5% (v/v) formic acid in acetonitrile was used as eluent 'B'. The separation started with a 0.5 min isocratic part at 10% 'B'. Then, the composition of the eluent was changed with a linear gradient to 95% 'B' within 1.5 min. This composition was maintained for 0.2 min and then immediately decreased to 10% 'B', i.e. the starting composition. 1.2 min was necessary to equilibrate the system before the next injection. The injection volumes were 0.5 μl and 50 μl. Seven seconds post-inject wash with isopropanol/water 1/1 containing 0.5% (V/V) formic acid was used as a needle wash. The column thermostat was set to 40°C. Ions were generated with the ESI ion source in the positive ionisation mode. For detection, the single ion recording mode was applied with monitoring 381.1 for quantification and 578.5 and 1156 for qualification. The sampling rate was set at 10 points/s. The probe temperature was 600°C and the capillary voltage was 1.0 kV. To protect the ion source against salts, the eluent from the column was diverted into the waste at the beginning of the separation. After 1 min, the valves were switched and the effluent containing colistin flowed into the ion source. At 1.75 min – after the elution of colistin – the effluent was directed into the waste again to avoid the contamination of the ion source with PVA. Every sample was centrifuged at 5500 rpm for 10 min before injection.

Antimicrobial susceptibility testing

For the antimicrobial susceptibility tests the following bacterial strains were used: Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 29923, Streptococcus

pyogenes ATCC 30013, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853. Long-term storage of bacterial strains was performed at -70 ˚C in a brain–heart infusion (BHI) broth with 20% glycerol. The antimicrobial susceptibility tests were performed using confluent growth of each bacteria (0.5 McFarland) on Mueller-Hinton (MH) agar or MH agar supplemented with 5% sheep blood (Becton Dickinson, Sparks, MD) (for S. aureus and S. pyogenes), and incubated at 37°C overnight (CLSI2005). For antimicrobial testing the following fiber discs were used: (i) discs with polymers S-I, S-II, S-III, S-IV, S-V alone; (ii) discs with polymers containing iodine S-IB, S-IIB, S-IIIB, S-IVB, S-VB. For antimicrobial susceptibility testing the following hydrogels were also used: (iii) hydrogels with polymers S-I, S-II, S-III, S-IV, S-V alone; (iv) hydrogels with polymers containing iodine S-IB, S-IIB, S-IIIB, S-IVB, S-VB. Discs (diameter =13 mm, average width = 1 mm, weight = 50 and 100 mg) were prepared from the forcespun fibers of amorphous glassy polymers with Specac type pneumatic press at 1000 N compression force. Glassy-to-rubbery transition can be observed due to the swelling of discs in contact with the agar gel.

Kinetic assay

The in vitro bactericidal activity of the fiber discs – S-IB and S-VB - was determined for S. pyogenes ATCC 30013 using the time–kill methods (CLSI, 2005). The initial concentrations of S. pyogenes ATCC 30013 was 7.602 log10 colony-forming units (CFU)/ml. The concentrations of the polymers were the following S-IB: 21.38 mg/ml
S-VB: 21.5mg/ml. The viable bacterial counts were determined after 1, 3, 6, 12 and 24 hours following the incubation with polymers IB and Vb. Dilutions were used to minimize the carryover effect of antimicrobial agent. The amount of 0.1 ml was subcultured on agar plates and incubated at 37 ˚C for 24 h for the CFU determination.

Skin abrasion and infection

Thirty-six C57/BL/6 mice, 17–20 g in weight, were kept under normal housing conditions for 2 weeks. On the day of surgery they were anesthetized with ketamine, shaved and subjected to a full thickness dorsal skin biopsy using an 8 mm diameter punch surgical tool. Twenty mice received 109 cfu A. baumannii BAA-1805 in 10 μL phosphate buffered saline suspension.

Tracking of the tissue regeneration by body thermal analysis with thermal camera

The animals were photographed by a heat-sensitive camera (type: FLIR A325sc), and the wound sizes were measured in two directions with a caliper. The evaluation was carried out with FLIR Research IR Max software.

Simplified histology study

For histological examination the wounds of the animal involved in the in vivo experiments were removed by excision after the treatment. Microscopic examination of samples with hematoxylin and eosin was performed using a Zeiss microscope equipped with a camera.

Extinction from tissue homogenisation

To determine the bacterial contamination of wounds from mice the wound homogenate was extinguished at Mueller-Hinton media with a 24-hour incubation time.

Mathematical modelling of drug release

The time-dependent change of the drug concentration in the dissolution vessel released from the multilayer scaffolds consisting of multilayer membranes (MEL, Membrane Electrospun Layer) and colistin sulfate-loaded jelly layers (CEL, Colistin sulfate-loaded Electrospun Layer) was determined.

Comparison of colistin release from different fibrous systems by Weibull distribution

A general empirical equation described by Weibull was successfully applied for the characterization of the dissolution of colistin from the different fibrous scaffolds into the diffusion media:

$$M_t = M_\infty\left(1 - e^{\frac{(t-t_0)^\beta}{\tau_d}}\right) \qquad \textbf{(E2)}$$

where

M_t = the dissolution (%) at time ‘t’ (min),

M_∞ = the dissolution (%) at infinite time,

t_0 = the lag-time (min) of the dissolution,

β = shape parameter of the curve,

τ_d = time (min) when 63.2% of M∞ has been dissolved.

The Microsoft Excel 2010 solver function was used in the nonlinear parameter estimation.

Finite Element Method (FEM)

The numerical analysis of the diffusion of the active substance in the alternating arrangement of the layers of CEL and MEL of the multilayer wound dressing system was performed by the *Finite Element Method* (FEM) developed for the technical calculations. The FEM is a numerical technique for finding approximate solutions to boundary value problems for partial differential equations.

RESULTS

Novel statements of the thesis

- We have developed a laboratory-size, easy-to-operate technical version of the centrifugal spinning device for the appropriate quality and reproducibility of the microfiber preparation as a potential drug delivery system [1, 2].

- In the course of the optimization of the production of PVP polymer-based microfibers using the centrifugal spinning device according to the technical design, I examined the effect of water-ethanol solvent mixture and polymer concentration on the fiber formation [1]. By examining the dynamic viscosity of the initial fiber forming solutions, I determined the specifications for the production of the corresponding fibers of required morphology, which I confirmed by scanning electron microscopic (SEM) images. I have mapped the supramolecular relationships of the polymer structure and predictive free volumes for drug storage capacity with Positron Annihilation Lifetime Spectroscopy (PALS). Using the results of PALS, I determined the relationship between the moisture content and the mechanical tolerance of the fibers. The obtained results of PALS measurements are the basis for designing the use of PVP based microfibers.

- In order to find novel and advantageous solutions to the fiber-forming of the solution phase, I developed the functional technical design of the contact drawing machine, which produces fibers of micrometer diameters by periodic, contact methodic movement of the spinning comb. Parallel and perpendicularly oriented fibers can be separated from the electrostatic collector by a square form of wire rope. The operation of the device is described in detail in patent specification P1400283.

- One of the possible ways of using drug-loaded fibers that is relevant to industrial drug technology is the formulation of a tablet or capsule dosage form with further processing of nano and microfibers. I made PVP microfibers containing vitamin

B12 model material and micronized them, and then, following the simplified manufacturing process, I made tablets by adding additional excipients. It was the first microfiber-based tablet formulation published in the literature [2]. I investigated the dissolution of tablets produced by direct compression of end homogenate. By the use of PALS measurements of the micronized microfibers, I analyzed the effect of micronization on the fibrous structure and its supramolecular relations with the aim of demonstrating the non-destructive effect of the technological operation on the material system and its ability to fabricate tablet dosage form.

- Another potential field of the use of polymeric fibers is the direct pharmaceutical or biomedical application of the developed systems, where the primary aim is the examination of the efficacy of the released drug in the desired therapeutic indication. In these cases the composition of the fiber-forming solution, the way of production and the physicochemical properties of the emerging material system are the main characteristics, which determine the drug release kinetics and the other end-product properties. In order to investigate the direct application for antibacterial treatment, povidone-iodine complex-loaded microfiber mats were prepared and in vitro microbiological measurements were carried out to determine the antibacterial capacity of the system and the corresponding microstructural properties of fibers [3].

- By further exploring the possibilities of direct use of fibrous drug delivery systems, I have produced PVA-based nanofibrous monolayers by electrospinning. The fabricated system comprised various polyvinyl alcohol fibrous layers prepared with or without the active ingredient. One of the electrospun layers contained water-soluble colistin sulfate and the other was prepared from the same polymer type and composition without the active drug and was finally heat-treated. The heat treatment modified the supramolecular structure and conferred the polymer nanofibre with the rate-controlling function. In vitro, in silico, and in vivo testing methods were carried out to design a multilayered wound dressing system of optimized drug release kinetics and antimicrobial efficacy [4, 5]. The

topical formulation of the locally applicable colistin sulphate active agent against multiresistant bacteria and the APO monomer molecule which assisted tissue regeneration were incorporated and tested for the efficacy of the formulations obtained. The construction and operation of the composition are also described in patent application P1600505 / 19.

- For the drug release study of the multilayer wound dressing, I made a custom-made dissolution cell for simulating the biorelevant application conditions [5].

CONCLUSIONS

Practical relevance and applicability of the results

The use of nano and microfibers can provide many proven benefits as they can improve unfavorable physico-chemical properties of certain drugs by forming amorphous solid dispersions and solid solutions. By the application of fibrous drug delivery systems biomedically relevant and effective preparations can be developed.
It is of utmost importance to elaborate the fiber-forming techniques precisely, even at the laboratory level, so that the characteristics of the materials produced in reproducible quality can be examined from the point of the real drug-technology benefits. As a result of multiple redesigns, I have designed a centrifugal spinning device for optimal laboratory performance.
In the field of research and even in the course of industrial level preformulation studies, PALS technique is of utmost importance, as it can be used to examine the supramolecular environment of polymeric matrices and by early tracking of changes in it. Valuable information can be obtained about the dissolution, stability, compatibility and aging of drug delivery systems. With this, time and development costs can be saved in the industry and help to choose the right direction of further development.

The topical application of drug-loaded nanofibrous web is a very promising alternative for the treatment of wounds in the course of healing of wound, in case of infections with resistant bacteria or systemic loading with narrow therapeutic window antibiotics. The

long duration of the active ingredient can be assured in the area to be treated, while accelerating the healing of the wound. To test the *in vitro* drug release of such compositions, a unique custom-made release cell was designed and described in the thesis, which can be considered as a good tool for gaining predictive dissolution data.

LIST OF ORIGINAL PUBLICATION RELATED TO THE PH.D. THESIS

Publications connected to the Ph.D. thesis

1. Sebe I, Ostorhazi E, Bodai Zs, Eke Zs, Szakacs J, Kovacs NK, Zelkó R. (2017) In vitro and in silico characterization of fibrous scaffolds comprising alternate colistin sulfate-loaded and heat-treated polyvinyl alcohol nanofibrous sheets. Int J Pharm, 523: 151-158.

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3. Sebe I, Bodai Zs, Eke Zs, Kállai-Szabó B, Szabó P, Zelkó R. (2015) Comparison of directly compressed vitamin B12 tablets prepared from micronized rotary-spun microfibers and cast films. Drug Dev Ind Pharm, 41: 1438-1442.

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2. Sebe I, Kállai-Szabó B, Zelkó R, Szabó D. (2014) Polymers and Formulation Strategies of Nanofibrous Systems for Drug Delivery Application and Tissue Engineering. Curr Med Chem, 22: 604-617.

3. Sebe I, Szabó B, Zelkó R. (2012) A pozitron annihilációs élettartam spektroszkópia és gyógyszerészeti alkalmazása. Acta Pharm Hung, 82: 23-32.

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