Electrospinning as a novel fabrication technique for drug delivery and tissue engineering

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To my family

List of Original Publications Included in this Thesis

Parts of this thesis were published in the following articles:

- **Article I:**

  S. Seif, L. Franzen, M. Windbergs. Overcoming drug crystallization in electrospun fibers – Elucidating key parameters and developing strategies for drug delivery. *International Journal of Pharmaceutics*, 2015. 478(1): p. 390-397. DOI: 10.1016/j.ijpharm.2014.11.045.

- **Article II:**

  S. Seif, V. Planz, M. Windbergs. Controlling the release of proteins from therapeutic nanofibers: the effect of fabrication modalities on biocompatibility and antimicrobial activity of lysozyme.
  Planta Medica, 2017. 83(05): p. 445-452. DOI: 10.1055/s-0042-109715.

- **Article III:**

  S. Seif, V. Planz, M. Windbergs. Delivery of therapeutic proteins using electrospun fibers – recent developments and current challenges.
  Archiv der Pharmazie, 2017. 350. DOI: 10.1002/ardp.201700077.

- **Article IV:**

  S. Seif, F. Graef, S. Gordon, M. Windbergs. Monitoring drug release from electrospun fibers using an *in situ* fiber optics system.
  Dissolution Technologies, 2016. 23(2). doi.org/10.14227/DT230216P6.

- **Article V:**

  V. Planz*, **S. Seif***, J. Atchison, B. Vukosavljevic, L. Sparenberg, E. Kroner, M. Windbergs. Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers.
  Integrative Biology, 2016. 8: p. 775-784. DOI: 10.1039/C6IB00080K.

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i. Short summary

Electrospinning as an advanced fabrication technique for polymeric fibers has drawn a substantial interest in the last few decades. Recently, one focus was directed towards the biomedical applications of this technique to fabricate biocompatible fibers for drug delivery and tissue engineering. This is mainly attributed to the numerous advantages provided by electrospun fibers including high surface area to volume ratio, ability to encapsulate different types of drugs, structural flexibility, similarity to the extracellular matrix, and many others.

This work aimed at studying the factors affecting the biomedical applicability of electrospun fibers for drug delivery and tissue engineering. At first, factors leading to inappropriate drug encapsulation as a result of hydrophilic drug crystallization on the surface of hydrophobic polymer fibers were identified and successfully overcome. Further, prolonging the release of proteins from hydrophilic electrospun fibers by means of different post-modification approaches was studied, focusing on investigating the effect on proteins' activity and cytotoxicity. Moreover, novel approaches for *in situ* dissolution analysis of electrospun fibers were investigated, with the focus on developing strategies to prevent sample folding during dissolution testing. Further, novel electrospun fiber mats composed of a combination of natural and synthetic polymers were generated and investigated for their application as substrates for cultivation of human cells. Overall, the results obtained in this work enhance our understanding of the factors that affect the biomedical applications of electrospinning, and can serve as guidance for rational development of novel drug delivery systems and tissue engineering scaffolds based on electrospun fibers.

ii. Kurzzusammenfassung

Elektrospinnen als fortgeschrittene Herstellmethode für polymerbasierte Fasern hat in den letzten Jahren großes Interesse erweckt. Ein Schwerpunkt ist die Verwendung von Elektrospinnen um biokompatible Fasern für biomedizinische Anwendungen wie Arzneistoffträgersysteme und Gewebezüchtung herzustellen. Dies ist hauptsächlich zurückzuführen auf die zahlreiche Vorteile von elektrogesponnen Fasern wie einem hohen Oberfläche-Volumen-Verhältnis, die Möglichkeit, verschiedene Arten von Wirkstoffe zu verkapseln, strukturelle Flexibilität, Ähnlichkeit mit der extrazellulären Matrix, und vielen anderen.

Ziel dieser Arbeit war die Untersuchung der Faktoren, die die biomedizinische Anwendbarkeit von elektrogesponnenen Fasern für Arzneistoffträgersysteme und Gewebezüchtung beeinflussen. Zunächst wurden die Faktoren, die zu unzureichender Wirkstoffverkapselung in Form von Kristallisierung hydrophiler Wirkstoffe auf der Oberfläche hydrophober Polymerfasern führen identifiziert und überwunden. Außerdem wurde die Verlängerung der Proteinfreisetzung aus hydrophilen elektrogesponnenen Fasern mittels unterschiedlicher Nachbearbeitungsmethoden untersucht, mit Fokus auf der Aktivität der verkapselten Proteine sowie auf der Zytotoxizität. Darüber hinaus wurden neue Ansätze für die *in situ* Analyse der Wirkstofffreisetzung aus elektrogesponnenen Fasern untersucht, diesbezüglich wurde neue Strategien entwickelt, um die Faltung der Proben während der Freisetzungsversuche zu vermeiden. In dem letzten Teil dieser Arbeit wurde neuartige elektrogesponnen Vliese bestehend aus einer Kombination von natürlichen und synthetischen Polymeren entwickelt und auf ihre Einsetzbarkeit als Substrate für die Kultivierung von menschlichen Zellen untersucht. Insgesamt, die Ergebnisse dieser Arbeit sind wichtige Bausteine für unser Verständnis der biomedizinischen Anwendbarkeit von elektrogesponnenen Fasern für Arzneistoffträgersysteme und Gewebezüchtung.

iii. List of Abbreviations

CAF	caffeine
CRM	confocal Raman microscopy
DMF	dimethylformamide
DSC	differential scanning calorimetry
ECM	extracellular matrix
EtOH	ethanol
FFA	flufenamic acid
FT-IR	fourier-transform infrared spectroscopy
GTA	glutaraldehyde
LDH	lactate dehydrogenase
MeOH	methanol
MTT	methylthiazol tetrazolium
PBS	phosphate buffer saline
PCL	polycaprolactone
PEG	poly(ethylene glycol)
PVA	poly(vinyl alcohol)
SEM	scanning electron microscopy
TFE	2,2,2-trifluoroethanol
UV	ultraviolet

1. Introduction

1.1. Background and process of electrospinning

Electrospinning is a straightforward and versatile technique that allows the production of continuous polymeric fibers in the nano- and micrometer range utilizing electrostatic forces [1]. The basic principle of the technique was established based on the cumulative efforts of researchers from various interdisciplinary fields who paved the way for the current state-of-the-art of the technique [1, 2]. The earliest contribution is attributed to William Gilbert in the 16th century who was the first to describe how electrostatic forces can deform the shape of water droplets without any physical contact [2, 3]. Despite the few studies that followed the work of Gilbert, the main purpose of applying electrostatics on liquids was for entertainment with no real application being realized [2]. It was in the 20th century when Anton Formhals succeeded in preparing polymeric fibers using electrostatic forces, hence his crucial patent filed in 1934 motivated researchers to further investigate and contribute to this field [4]. At this point, the interest in electrospinning was drastically increasing, leading to more achievements being reached in the following few years. Researchers started to thoroughly investigate the process of fiber formation using electrostatic forces. In this context, one of the most important contributions is attributed to Sir Geoffrey I. Taylor who mathematically modeled the shape of the cone formed by a fluid droplet upon applying electric field to it. Hence, his work was the first to provide deeper understanding on the process of electrospinning revealing the underlying parameters responsible for fibers formation [1, 5]. These early discoveries form together the foundation stone that allowed for the current electrospinning developments.

A general electrospinning setup is composed of a syringe containing polymer solution, a syringe pump, a high voltage power supply and a metallic collector. The polymer solution is pumped at a defined flow rate through the syringe tip which is connected to the high voltage power supply and therefore acts as an electrode. The high voltage starts to deform the polymer solution droplet ejected from the tip of the syringe forming a cone shape known as the Taylor cone. Once the voltage is high enough to overcome the surface tension of the polymer solution, the solvent starts to rapidly evaporate allowing electrospun fibers to be formed. The fibers are directed to

the metallic collector which is located at the opposite of the syringe tip and connected to a power supply with an opposite polarity. The fibers move at a very high speed and deposit on each other forming one coherent fiber mat. A schematic of a typical electrospinning setup is illustrated in Figure 1.

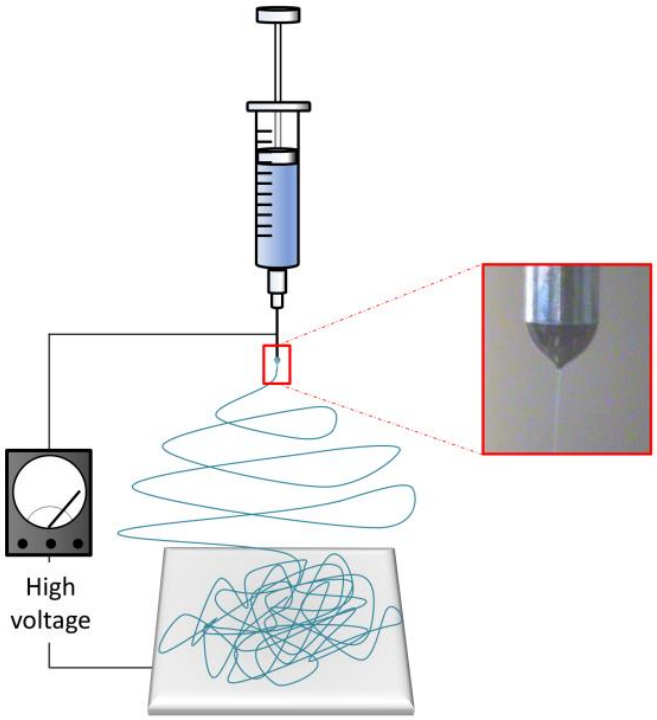


Figure 1: Typical electrospinning setup composed of a syringe containing polymeric solution, high voltage power supply and metallic collector. The Taylor cone formed upon increasing the voltage is depicted in the magnified image.

1.2. Applications of electrospun fibers

One major advantage of electrospinning is attributed to its versatility and flexibility, bearing the possibility to produce different types of fibers for a high variety of applications [1, 6]. In this context, full understanding of the electrospinning process is crucial for the fabrication of tailor-made electrospun fibers suiting the desired application [6, 7]. There are many parameters that affect the shape and nature of the fabricated electrospun fibers, and can mainly be divided into polymer-solution related parameters (such as polymer solubility, polymer molecular weight, solvent boiling point, solvent vapor pressure, pH value, conductivity, dielectric constant, surface tension, etc.) and process related parameters (including tip-to-collector distance, collector shape, flow rate, voltage intensity, temperature, relative humidity, etc.) [1, 6, 8]. The ability to control each parameter offers numerous options in terms of fibers morphology and functionality which led to a high variety of applications being suggested, some of which could successfully be introduced to the market. For instance, in 1936 Igor Petryanov-Sokolov, from the aerosol department at the Karpov Institute of Physical Chemistry, noticed that electrospun membranes made from thinner fibers would make very effective filters [9, 10]. Thereafter, his innovative work was employed for the production of industrial scale electrospun fibers that were popularized as “Petryanov filters” and were applied for military gas masks in the former Soviet Union [9, 10]. Despite this early breakthrough, filters based on electrospun fibers were not commercialized until the 1980s by the Donaldson company in the United States of America [2]. Another example of pioneer approach for applying electrospun fibers was in the textile fabrication. In this regard, researchers suggested combining electrospun fibers with conventional textiles (e.g. as an interlining) in order to provide additional features such as regulating the water-gas permeability, improving the thermal insulation, providing protection against chemical or biological hazards [1, 11]. Further interest led to innovations in many areas including biomedical field (e.g.: drug delivery systems and tissue engineering), energy applications (e.g.: solar cells, capacitors, batteries, etc.), catalysis, agriculture, self-cleaning surfaces (super hydrophobic surfaces) and many others [1, 12-14]. However, as the main focus of this thesis is on the biomedical applications, the following sections will highlight the major advantages, application possibilities and state-of-the-art of utilizing electrospun fibers in this interdisciplinary field.

1.3. Biomedical applications of electrospun fibers

Electrospun fibers offer a very attractive platform for various biomedical applications due to the versatility of electrospinning and the unique ultrafine fibrous structure of electrospun fibers [7, 15, 16]. In more details, the mechanical flexibility, high surface area to volume ratio, tunable surface porosity, ability to incorporate different types of drugs and most important the similarity to the extracellular matrix are prominent examples of advantages provided by electrospun fibers. A substantial interest in applying electrospun fibers for biomedical purposes evolved in the middle 1990s and kept rapidly increasing leading to an impressive trend of articles and patents being published [7, 17]. Furthermore, the current advances in the field of polymer chemistry and the development of polymers with special functionality (such as polymers with antibacterial activity, shape memory polymers, etc.) provided more options in terms of materials selection. Novel drug delivery systems, tailor-made wound dressings, heart valves and vascular prostheses are only few examples of what can be achieved using electrospinning [7, 18]. The various biomedical applications of electrospun fibers can mainly be categorized in two main groups: 1) drug delivery applications, and 2) tissue engineering applications.

1.3.1. Electrospun fibers for drug delivery applications

For the application of electrospun fibers for drug delivery, one of the most important features of electrospinning is the flexibility in incorporating drug molecules in electrospun fibers. So far, different types of drugs could successfully be encapsulated where various potential applications were evaluated [7, 19]. Drug molecules ranging from low molecular weight drugs, macromolecules (e.g. proteins and peptides) and nucleic acids could successfully be encapsulated in electrospun fibers [7, 18]. Further advantages of electrospun fibers as novel drug carriers include the high drug loading capacity and the high encapsulation efficiency in comparison to other drug carriers [20]. In addition, the flexibility in choosing the desired drug/polymer combination provides additional control over the mechanism of drug release and can further enhance the therapeutic performance. Drug encapsulation in electrospun fibers can mainly be achieved by one of the following methods:

1) Post-spinning modification
2) Direct drug incorporation
3) Co-axial electrospinning

These methods are discussed with further details in the following sections.

Post-spinning modification

Post-spinning modification is a straightforward method for incorporating sensitive drug molecules [18, 21]. The general idea of this method is quite simple, electrospun fibers are first produced, and then drug molecules are added to the surface of the fabricated electrospun fibers. For instance, a study by Bolgen et al. reported the incorporation of ornidazole as a model drug on the surface of electrospun polycaprolactone (PCL) fibers, this was achieved by evenly adding defined amount of the drug solution to pieces of electrospun PCL [21]. Major drawback of this method was that the drug only attached to the fibers surface via physical adsorption, which led to a very rapid burst release reaching 80 % in the first 3 hours [21]. In order to overcome this issue of rapid drug release, researchers suggested improving drug binding to fibers via chemical conjugation in order to control the release of the

incorporated drug. For instance, matrix metalloproteinases (MMPs) were introduced on the surface of polycaprolactone-poly(ethylene glycol) (PCL-PEG) copolymer fibers as cleavable linker in order to allow for efficient binding of poly(ethyleneimine) (a linear cationic polymer), which in turns can bind to DNA allowing for efficient incorporation for gene therapy [22]. DNA release could be realized by the cleavage of the MMPs linker occurring over time, thus providing an efficient controlled gene delivery system [22]. Despite the advantages provided by post-spinning modification especially for encapsulating sensitive drugs, only limited studies investigating this approach are available [18].

Direct drug incorporation

Unlike post-spinning modification, direct incorporation of drugs in electrospun fibers is performed by adding the drug to the polymeric solution before electrospinning. Direct drug incorporation is the most used method for fabricating drug loaded electrospun fibers. This is mainly attributed to the simplicity of this approach allowing the drug to be efficiently incorporated within the fibers, and therefore a better drug encapsulation efficiency and improved controlled release kinetics can be achieved. Kenawy et al. investigated in an early study the incorporation of tetracycline HCl in electrospun fibers so that the drug and the polymer were dissolved together in the same solvent system and electrospun directly [23]. In their study, the controlled release of tetracycline HCl was investigated and compared to the release from Actisite® (a commercially available tetrycline HCl containing polymeric dosage form) [23]. Different types of drugs were successfully incorporated within electrospun fibers using this method including antibiotics, antioxidants and non-steroidal anti-inflammatory drugs [7, 18]. For effective drug incorporation, the physicochemical properties of both, the drug and the polymer have to be taken into consideration. In this context, drug solubility within the polymeric solution is a crucial factor determining whether the drug can be molecularly dispersed within the polymeric solution or an emulsion has to be formed. For instance, stable polymers that can resist rapid degradation and therefore can be used for controlled drug delivery are mainly soluble in organic solvents. So if the drug of interest is only soluble in water such as proteins and peptides, the best way to encapsulate such drugs within stable polymers is by forming water in oil (W/O) emulsion where the drug is in the inner phase and the

polymer is in the outer phase. As an example, Maretschek et al. prepared a W/O emulsion containing cytochrome C as a model protein in aqueous solution and poly(l-lactide) (PLLA) in chloroform [24]. Based on this formulation, they were able to produce cytochrome C loaded PLLA nanofibers while reserving the activity of the encapsulated protein. In addition, by adding PEG as a hydrophilic polymer to the cytochrome C/PLLA emulsion, they were able to improve the release kinetics and control the duration of drug release [24]. While the study of Maretschek et al. showed no significant loss in the activity of encapsulated cytochrome C, other reports indicated that the activity of another types of proteins could partially be damaged as a result of the electrospinning process [25]. In such cases, the use of stabilizers to preserve the protein activity was essential. For instance, Chew et al. encapsulated nerve growth factor (NGF) in electrospun poly(caprolactone-co-ethyl ethylene phosphate) fibers [25]. In their study, bovine serum albumin (BSA) was added as a filler to help stabilizing NGF during the electrospinning process. They showed by cellular assay that the bioactivity of NGF could -at least partially- be maintained for up to 3 months [25].

Co-axial electrospinning

Co-axial electrospinning (also known as core-shell electrospinning) is an advanced method that allows combining two polymeric solutions to form one concentric jet with core-shell structure [26]. This method provides even more options compared to the other conventional drug incorporation techniques, as it bears the potential to utilize two polymer solutions with different physicochemical properties allowing for more flexibility in terms of choosing desired drug-polymers combinations [26]. Generally, core-shell electrospinning is suitable to encapsulate sensitive drug molecules (e.g. proteins) in the core using a hydrophilic water soluble polymer, while the shell composed of stable hydrophobic polymer provide the necessary protection against degradation and allows for controlled drug release to be achieved. For instance, Jiang et al. fabricated core-shell electrospun fibers containing lysozyme as a model protein added to PEG forming the core, and PCL as a hydrophobic polymer forming the shell [27]. Using this approach, direct contact between the protein and the organic solvent used for PCL could be avoided. As a result, controlled release kinetics could be reached while successfully maintaining the structure and bioactivity of lysozyme

[27]. Although the general idea of core-shell electrospinning is to protect the components of the core using the shell, other approaches were suggested. For instance, a co-axial electrospinning setup was used to fabricate core-shell fibers where the core was composed of PCL and cationized gelatin (prepared by derivation with N,N-dimethylethylenediamine) forming the shell [28]. While PCL provided the necessary support, the cationized gelatin allowed for efficient incorporation of bovine serum albumin (BSA) or heparin onto the fibers' surface. Furthermore, the addition of vascular endothelial growth factor (VEGF) as a second step to the heparin loaded fibers resulted in efficient immobilizing of VEGF to the fibers due to the specific binding of VEGF to heparin where VEGF was slowly released over 15 days [28]. Core-shell electrospinning was not only used to encapsulate drugs such as low molecular weight molecules, proteins and DNA [28-31], recent studies suggested the encapsulation of living cells for advanced tissue engineering applications. For instance, Townsend-Nicholson et al. reported using co-axial electrospinning setup to encapsulate cells in electrospun fibers where a biosuspension of living cells was forming the core and medical grade poly(dimethylsiloxane) medium was forming the shell [32]. After fabrication, the cells were cultured and the results were quite promising as the cells viability was not affected upon fabrication showing no sign of cellular damage during the electrospinning process [32].

While each of these methods has its own up- and down-sides, choosing the suitable method depends mainly on the type of drug and the intended application [18]. Table 1 provides an overview of different studies for applying electrospun fibers for drug delivery.

Table 1: Examples of electrospun fibers for drug delivery applications.

Drug	polymer	Method of drug incorporation	Intended application	Ref.
Ornidazole	PCL	Post-modification (adsorption)	Prevention of post-surgery abdominal adhesion	[21]
epidermal growth factor (EGF)	PCL and PEG	Post-modification (Chemical conjugation)	Protein drug delivery	[33]
fibroblast growth factor (FGF-2)	Collagen or gelatin	Post-modification (Chemical conjugation)	Tissue engineering	[34]
Heparin or bovine serum albumin (BSA)	PCL and gelatin	Post-modification (Chemical conjugation)	Protein drug delivery	[28]
DNA	PCL-PEG copolymer	Post-modification (Chemical conjugation)	Gene therapy	[22]
Tetracycline HCl	poly(lactic acid) and poly(ethylene-co-vinyl acetate)	Direct drug incorporation (blend)	Drug delivery	[23]
Cytochrome C	poly(l-lactide)	Direct drug incorporation (Emulsion)	Protein drug delivery	[24]
Bone morphogenetic protein-2 (BMP2)	poly(D,L-lactide-co-glycolide)/hydroxy-lapatite (PLGA/HAp)	Direct drug incorporation (Emulsion)	Protein drug delivery	[35]
Bovine serum albumin (BSA)	PCL	Core-shell electrospinning	Protein drug delivery	[30]
Plasmid DNA	PCL and PEG	Core-shell electrospinning	Gene therapy	[31]

1.3.2. Electrospun fibers for tissue engineering and cellular cultivation

The extracellular matrix (ECM) forms the basic framework for all tissues in the human body, providing an outstanding environment for the cells and controlling their growth and behaviors *in vivo* [36]. ECM is mainly composed of nanofibrous proteins forming a three-dimensional network that holds the cells together and helps in determining their shape and activities [37]. Many attempts were taken by researchers to mimic the ECM for providing functional scaffolds for tissue engineering applications. However, only limited success could be achieved. This was related to the fact that conventional fibers production methods either results in fibers much thicker than ECM fibers (>10 µm in diameter), or lack the ability to produce continuous fibers [36]. It was only in the recent years when electrospinning emerged as a versatile technique for producing continuous ultrafine fibers based on high variety of synthetic and natural polymers [16, 36]. Since then, very rapid progress could be achieved thus offering new solutions for cell cultivation and tissue engineering covering different types of aspects. In this context, many applications were evolved in the recent years including: cell cultivation [36], vascular tissue engineering (artificial prosthesis, heart valves) [38, 39], nerve tissue engineering [40, 41], bone regeneration [42, 43], skin reconstruction [44] and many others [7, 16, 45]. In order to achieve the desired mechanical properties while facilitating cellular attachments and growth, it is very important to choose the suitable materials after considering their characteristics. For instance, combining synthetic hydrophobic polymer (providing mechanical stability) with natural hydrophilic polymer (improving cellular attachment, growth, etc.) in the same scaffold is a successful strategy for fabricating electrospun fibers for tissue engineering. In this regard, deep understanding of the human ECM is crucial for the design of tailor-made biomimetic electrospun fiber scaffold for tissue engineering and cell cultivation.

2. Aims of the Thesis

The aim of this work was to study of the factors affecting the biomedical applicability of electrospun fibers. Thorough investigation of the parameters determining drug encapsulation in electrospun fibers in terms of drug, distribution within the fibers, drug stability, and drug release has to be conducted while the suitability of electrospun fibers for applications on human cells has to be confirmed. In this context, the experimental studies should establish the basis for tailor-made electrospun fibers for drug delivery and tissue engineering.

More specifically, the aims of this thesis were:

- To investigate the factors influencing the encapsulation of hydrophilic drugs in hydrophobic polymers, focusing on overcoming drug crystallization upon fabrication.
- To study the effects of different post-modification treatments of hydrophilic electrospun fibers to prolong drug release for protein delivery.
- To establish novel approaches for *in situ* dissolution analysis of electrospun fibers and to develop strategies to prevent sample folding during dissolution testing.
- To generate novel electrospun fibers with tunable biomechanical properties as substrates for cultivation of human cells.

3. Scientific Outcome

The following sections encompass the scientific outcome of this work published in articles (I – V). A more detailed description of the experimental and results can be found in the respective publications.

3.1. Fabrication and characterization of drug-loaded electrospun fiber mats: evaluating drug stability upon fabrication

3.1.1. Introduction

Even though electrospinning has been proved as a versatile technique for fabricating fiber-based drug delivery systems [7], encapsulating hydrophilic drugs in hydrophobic polymers for controlled drug release bears several challenges [46]. One important issue is represented by uncontrolled drug recrystallization in the dosage form after/during fabrication which is a common challenge for thin polymeric dosage forms, and can potentially cause inhomogeneous drug distribution, affecting uniformity of the drug delivery system and consequently affecting drug release kinetics as well as physical stability during storage [47]. In this section, a comprehensive study aimed at understanding the underlying parameters responsible for crystal formation of caffeine (CAF) as a model drug in electrospun fibers comparing different polymers. After elucidating the role of the solvent system in controlling drug crystal formation, a successful fabrication of crystal-free electrospun fibers could be achieved, providing an improved drug distribution and therefore improved dissolution behavior could be realized.

Corresponding article: I.

3.1.2. Overcoming drug crystallization in electrospun fibers – elucidating key parameters and developing strategies for drug delivery

For fabricating drug-loaded electrospun fibers and for investigating the effect of uncontrolled drug crystallization, CAF as a model drug was combined with two different biocompatible and biodegradable polymers possessing different physicochemical characteristics. Poly(vinyl alcohol) (PVA) was chosen as a hydrophilic polymer while polycaprolactone (PCL) was selected as hydrophobic polymer. PVA is normally utilized to form immediate release matrices, while PCL is rather used for controlled release systems. CAF was chosen as a hydrophilic model drug due to its high crystallization tendency [47].

In general, the high evaporation rate of the solvents in electrospinning is thought to facilitate the formation of solid solutions of the drug in the fibers [48]. However, few studies reported the presence and growth of drug crystals on electrospun fibers affecting the morphology and the physicochemical characteristics of the fibers which can mainly be observed when encapsulating hydrophilic drugs in hydrophobic polymer fibers [46, 49]. In this study, electrospinning of the CAF-PVA solution resulted in a homogeneous fiber mat with fiber diameters in the lower micrometer range. Scanning electron microscopy (SEM) images revealed continuous fibers with a smooth outer surface (Figure 2A) and confocal Raman microscopy (CRM) visualized a homogeneous distribution of the drug in the polymer fibers without any detectable crystals (Figure 2B). In comparison, while electrospinning of the CAF-PCL solution resulted in homogenous fibers in the lower micrometer range, the SEM images revealed crystal formation on the fiber surface (Figure 2C). CRM allowed identifying the observed crystals as pure CAF (Figure 2D, CAF is depicted in red).

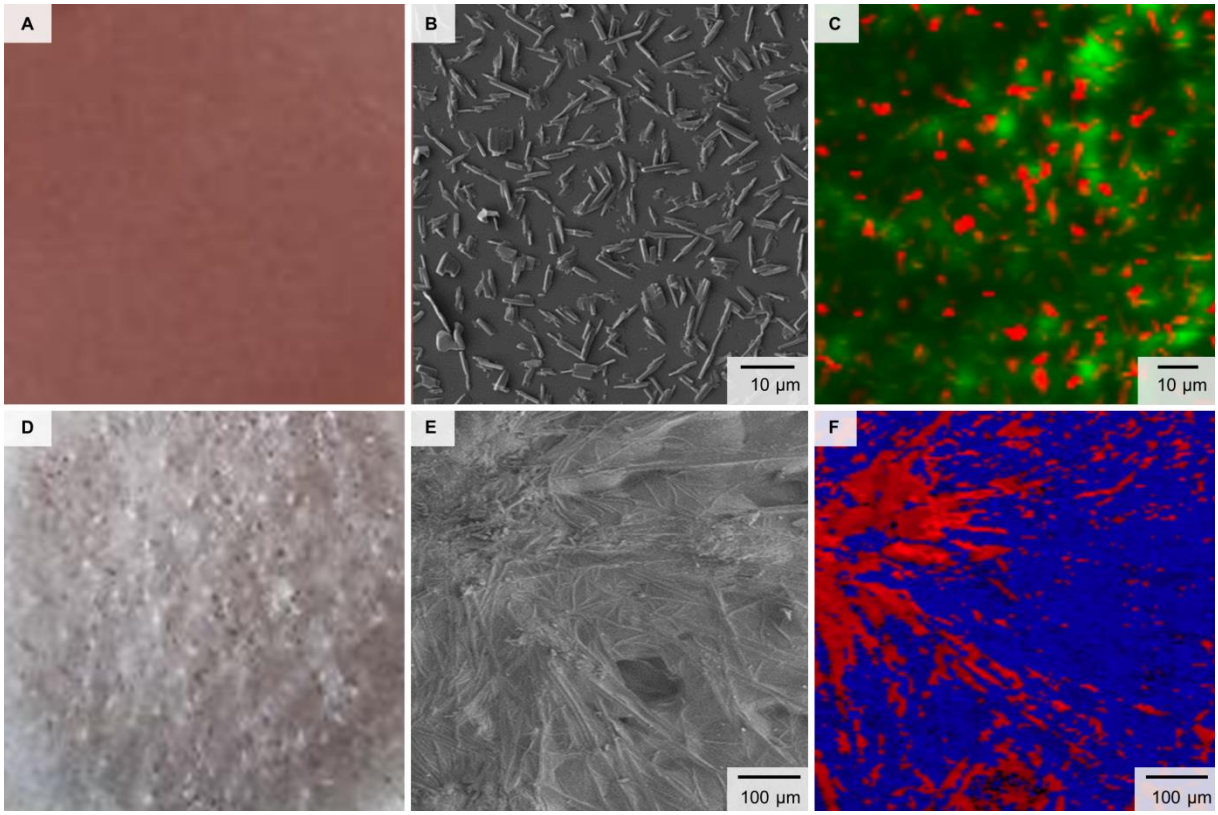


Figure 2: A) SEM image of electrospun CAF-PVA fibers, B) CRM false color image showing CAF-PVA fibers. Both compounds could be detected in each pixel (green), C) SEM image of electrospun CAF-PCL fibers, D) CRM false color image of electrospun CAF-PCL showing crystalline CAF in red and PCL containing pixels in blue. Reproduced with permission from [50].

While electrospinning is thought to overcome crystallization of incorporated drugs during fabrication due to the high evaporation rate of the solvent, CAF crystallization on the outer surface of PCL surface raised the question whether the rate of fiber formation is the only affecting key factor. In order to understand the crystal formation of CAF on the outer surface of PCL fibers, comprehensive studies aimed at investigating the process of fibers formation focusing on two main aspects: 1) process parameters and 2) properties of the polymeric solutions.

As electrospinning process parameters are known to affect the final fibers characteristics [6], the following experiments focused on testing different set of parameters for electrospinning using the same CAF-PCL solution, and the fabricated fibers were investigated with respect to drug crystal formation on the fiber surface. Pumping flow rate of the polymer solution, nozzle-collector distance, and voltage were the main parameters tested (Table 2). After fabrication, the prepared fibers using these parameters were investigated with SEM and the corresponding images are depicted in Figure 3. Generally, only a minor influence of process parameter variation on fiber diameter as well as on the crystal formation was observed. Therefore, the following part of this study focused on the CAF-PCL solution and the physicochemical properties of its components.

Table 2: Summary of the investigated electrospinning process parameters.

	Parameter set 1	Parameter set 2	Parameter set 3	Parameter set 4	Parameter set 5	Parameter set 6
Flow rate (ml/h)	0.3	0.3	0.3	2	3	3
Distance (cm)	13	13	16	18	13	16
Voltage (kV)	6.48	11.51	6.48	8.26	12.28	12.30
SEM image	3-A	3-B	3-C	3-D	3-E	3-F

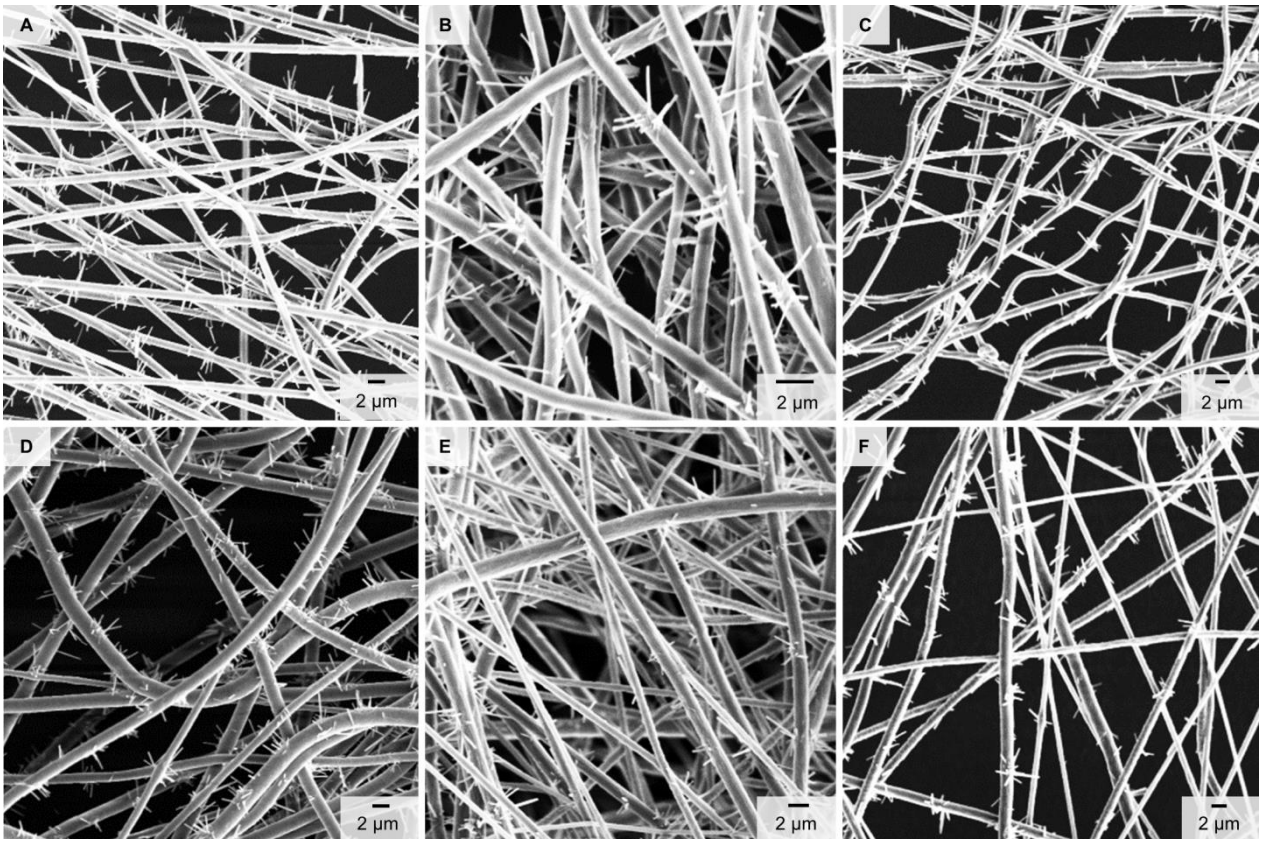


Figure 3: SEM images of CAF-PCL fibers prepared with different electrospinning process parameters according to table 2. Reproduced with permission from [50].

The physicochemical properties of the polymer solution are known to have an important impact on the final morphology and characteristics of the formed electrospun fibers [51]. Especially, compatibility of drug and polymer were already shown to directly affect the drug encapsulation and its release kinetics [46]. In this context, the hydrophilicity of CAF in contrast to the hydrophobic PCL can be expected to influence the crystallization process of the drug [52]. In order to verify this hypothesis, CAF was exchanged against the hydrophobic drug flufenamic acid (FFA) and electrospun fibers were prepared from the same polymeric solution as for the CAF experiments. FFA was efficiently encapsulated within PCL resulted in smooth crystal-free fibers as shown in (figure 4A). These results indicate that the hydrophilic-hydrophobic relationship of drug and polymer highly affects CAF crystallization on the PCL fiber surface. Based on our results, we focused in the following studies on modifying the solvent regarding the solubility of the drug and the polymer and its impact on the electrospinning process. It is known that fiber formation can be affected by the properties of the applied solvents such as boiling point, surface tension and dielectric constant [6]. Among them, the solvent dielectric constant (as an indicator of solvent polarity) was found to be a strong influential parameter. Solutions with high

dielectric constant were shown to facilitate and improve the electrospinning process as well as the morphology of the final fibers [53, 54]. Therefore, we investigated the effect of solvent polarity on our polymer solutions. At first, we prepared solutions with 2,2,2-trifluoroethanol (TFE), a solvent with high polarity capable to dissolve CAF as well as PCL. Interestingly, electrospun fibers based on these solutions showed significantly decreased crystal formation (Figure 4B). Apparently, the higher polarity of TFE facilitated CAF encapsulation within PCL. In order to corroborate these results, we investigated dimethylformamide (DMF) as a solvent with higher polarity than TFE. However, as pure DMF cannot efficiently dissolve PCL, a mixture of TFE:DMF (75:25 v/v) was used for the next experiments. Crystallization of CAF on the fiber surface was successfully prevented as depicted in Figure 4C. However, the reduction in the diameter of the individual fibers as a result of the high dielectric properties and high conductivity of the polymer solution led to rapid release kinetics for the embedded CAF (data not shown). Therefore, we reduced the amount of DMF and repeated the experiment with a TFE:DMF ratio of 90:10 v/v. The resulting fibers were smooth and homogeneous with no CAF crystals on the surface as depicted in the corresponding SEM images in Figure 4D. The diameters of the fibers were in the lower micrometer range and thus comparable to those produced by CAF-PVA (Figure 2A). These results prove the importance of solvent selection in preventing CAF crystallization on the fiber surface. The mixture of TFE:DMF (90:10 v/v) assured an efficient encapsulation of CAF compared to the previously used chloroform:ethanol (50:50 v/v) mixture. Considering the physicochemical properties of both solvent systems, we accredit the effectiveness of the TFE:DMF (90:10 v/v) mixture to its high polarity which influenced the electrospinning leading to the homogeneous CAF distribution in the fibers, and thus efficiently overcoming drug crystallization on the fiber surface.

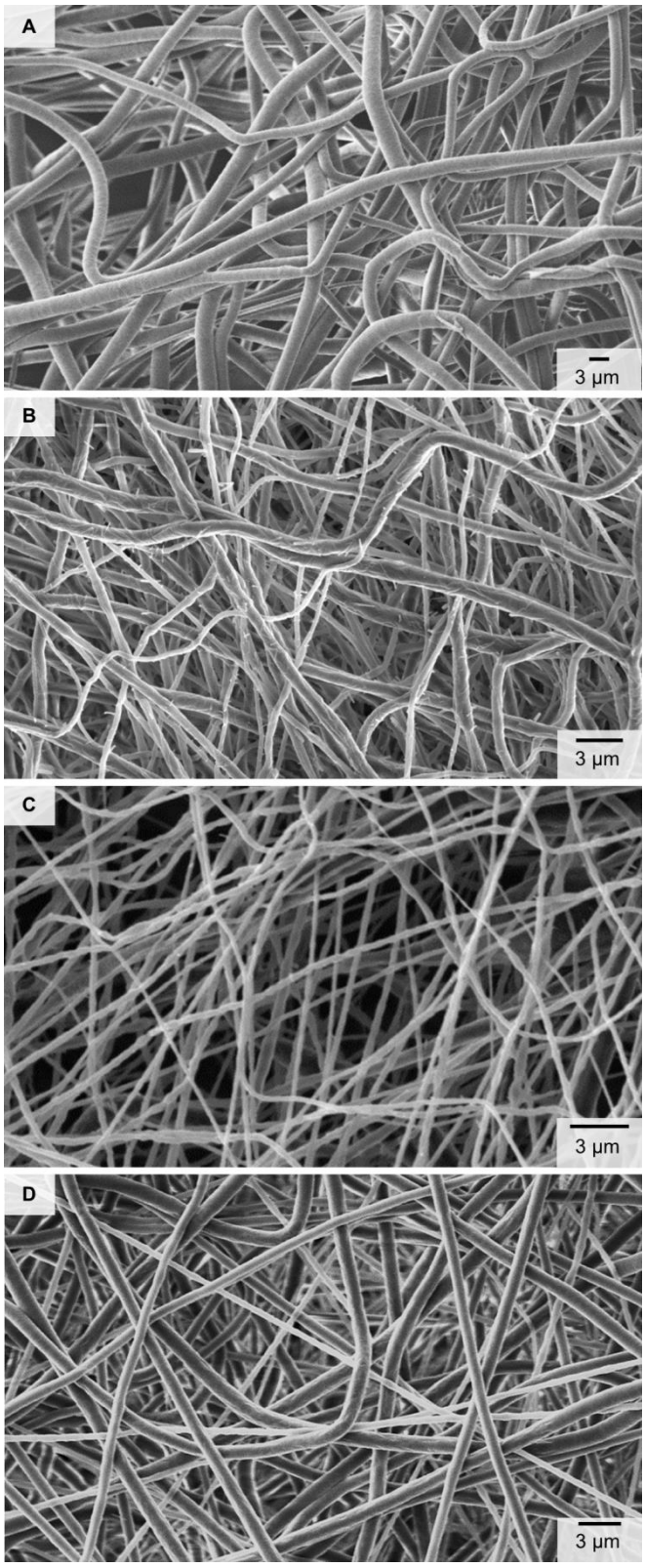


Figure 4: SEM images representing: A) FFA-PCL fibers, B) CAF-PCL fibers prepared in TFE, C) CAF-PCL fibers prepared in TFE:DMF 75:25 v/v, D) CAF-PCL fibers prepared in TFE:DMF 90:10v /v. Reproduced with permission from [50].

Subsequent analysis concentrated on the physicochemical characterization of the final CAF-PCL fibers prepared using TFE:DMF (90:10 v/v). CRM analysis confirmed

that for the final CAF-PCL fiber formulation, no CAF crystals were detected on the surface of the fibers, instead a homogeneous distribution of both compounds could be visualized in each pixel (Figure 5A and B). Differential scanning calorimetry (DSC) analysis was performed to determine the physical form of CAF within the electrospun PCL fibers. In contrast to the initial CAF-PCL fibers (with CAF crystals); the thermogram of the final crystal-free CAF-PCL showed no melting peaks representing crystalline CAF, hence, indicating the formation of a solid solution (Figure 5C).

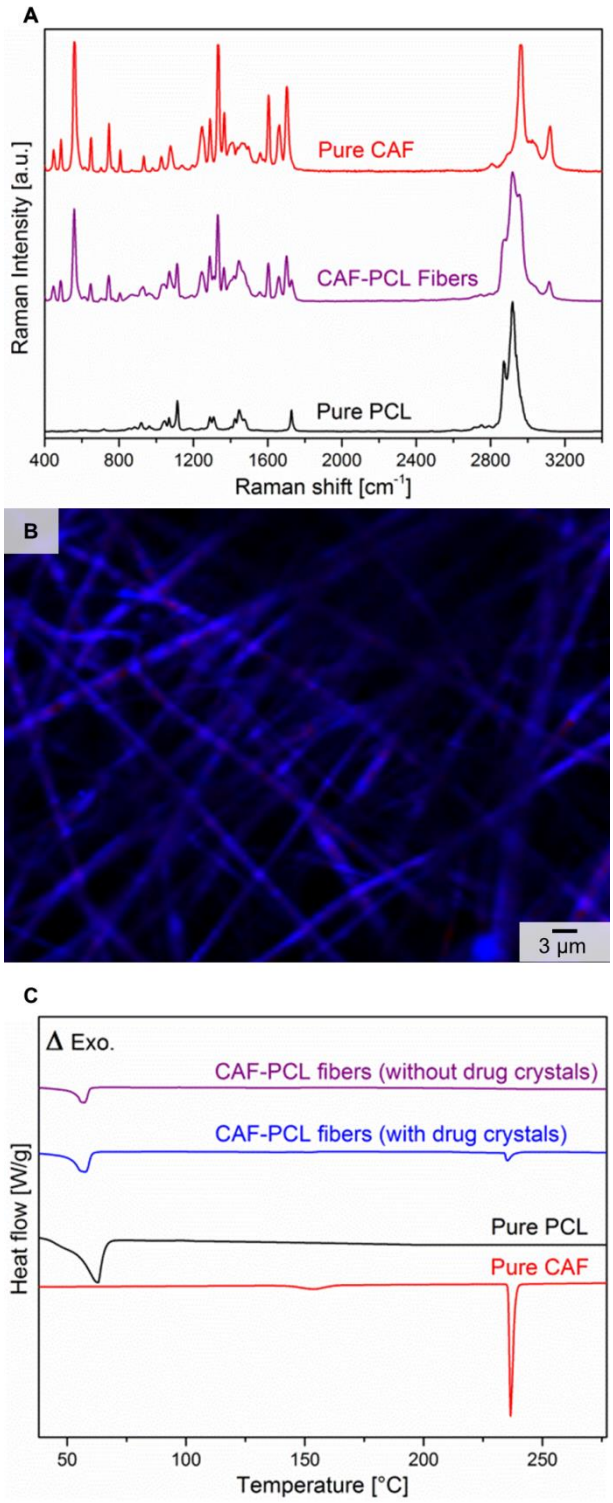


Figure 5: A) Raman spectra of pure CAF, CAF-PCL fibers and pure PCL, B) CRM image of CAF-PCL fiber mat prepared with TFE:DMF (90:10 v/v), crystalline CAF is depicted in red and pixels containing CAF and PCL are assigned in purple, C) DSC thermograms of the electrospun fibers and pure substances. Reproduced with permission from [50].

In the final step of this study, we investigated drug release from the fabricated PCL fiber mats. For comparison, we determined the CAF release from hydrophilic PVA

fibers, the initial CAF-PCL fiber mats (with CAF crystals) and the final crystal free CAF-PCL fiber mats (prepared with TFE:DMF (90:10 v/v)). The hydrophilic PVA fibers showed an immediate release of CAF (Figure 6). Almost 100% of the encapsulated CAF was detected in the release medium after only 15 min. This correlates to the expected burst release due to fast degradation of the hydrophilic polymer PVA and the good aqueous solubility of CAF as a hydrophilic drug. The release of CAF from the initially prepared PCL mats with visible CAF crystals on the fiber surface follow immediate release kinetics similar to CAF-PVA fiber mats (Figure 6). Since CAF is mainly located at the outer surface of the fibers, its solubility in the release medium determines the release kinetics, rather than the diffusion from the PCL fibers. In contrast, the crystal free CAF-PCL fibers prepared in TFE:DMF (90:10 v/v) show a sustained drug release. As 100% release was not reached until four hours, an encapsulation of the CAF in the PCL and release by passive diffusion can be assumed.

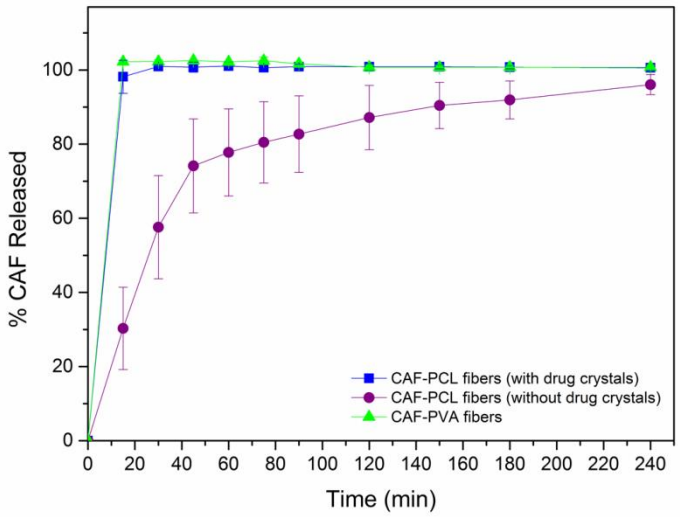


Figure 6: *In vitro* release of CAF from the prepared PVA and PCL based fiber. (mean ± SD, n≥3). Reproduced with permission from [50].

In conclusion, crystallization behavior of hydrophilic drugs in hydrophobic electrospun fibers is affected mainly by the composition of the polymer solution as well as - to a minor extent - by the electrospinning process parameters. Based on a systematic investigation of caffeine crystallization in electrospun PCL fibers, the solvent polarity was found to have a major impact on drug crystallization. Successful prevention of uncontrolled drug crystallization led to homogeneous drug distribution, accordingly controlled drug delivery could effectively be achieved.

3.2. Controlling drug release from hydrophilic electrospun fibers for protein drug delivery by means of post-modification treatments

3.2.1. Introduction

Electrospun nanofibers gained considerable attention as novel drug carriers for the delivery of natural proteins [24, 55]. However, even though immediate drug release from such fibers can easily be realized, the fabrication of fiber mats providing controlled protein release over longer time periods still bears challenges [24]. In this section, a systematic investigation on the effect of different post-modification treatments of hydrophilic electrospun nanofibers to prolong protein release was performed. Analysis of the fibers focused on the effect of post-modification on fiber morphology, chemical composition, release of embedded proteins, protein activity as well as cytotoxicity.

Corresponding articles: II and III.

3.2.2. Controlling the release of proteins from therapeutic nanofibers: the effect of fabrication modalities on biocompatibility and antimicrobial activity of lysozyme

Lysozyme, a natural protein was encapsulated in PVA fibers followed by postmodification with methanol (MeOH), ultraviolet (UV) irradiation, or glutaraldehyde (GTA) vapor. After modification, the fibers were stored in a desiccator for 24 hours at 4 °C prior to analysis. Untreated lysozyme-PVA fibers (without any post-modification) displayed a rather homogeneous smooth surface and a uniform fiber diameter in the nanometer range (mean around 500 nm) as depicted in Figure 7A. This also holds for fibers treated with MeOH (Figure 7B). However, it can be noticed that the fibers treated with MeOH appear closer packed, thus resulting in a slightly denser fiber network. As a second postmodification approach in our study, GTA was applied as a broadly known chemical cross-linking agent for the stabilization of polymers [56]. While earlier studies showed that treating electrospun fibers with GTA vapor for long time (5 hours) can cause the fibers to collapse forming a coherent film-like structure [56], in our study we exposed our fibers to GTA vapor for only 1 hour in order to avoid

such effect. As a result, the GTA treated fibers maintained their individual shape and morphology and only a slightly more stacked fiber mat structure can be visualized (Figure 7C) as already found for the MeOH treated samples (Figure 7B). For the third approach, controlled application of UV irradiation on the fiber mats was executed. In contrast to previous studies, where UV light in combination with chemical sensitizers to electrospun fibers were applied [57, 58], we solely exposed each side of the drug loaded fiber mats for 3 hours to UV light to avoid potential interactions of lysozyme and sensitizer molecules. The resulted fibers after UV treatment do not show any morphological changes (Figure 7D) compared to the untreated fibers (Figure 7A). In summary, no significant change on the fiber morphology upon treatment with any of the approaches could be observed, only for the cases of MeOH and GTA treatment, where the fiber mats appeared slightly denser packed.

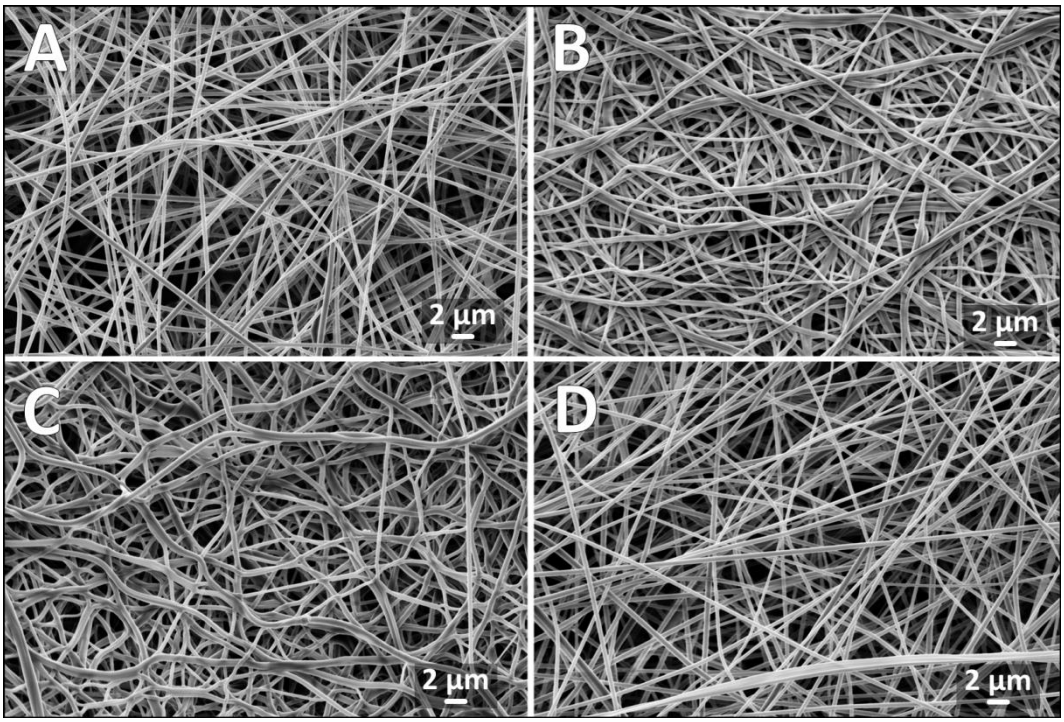


Figure 7: SEM images of the electrospun lysozyme -loaded PVA fibers: A) untreated fibers, B) MeOH treated fibers, C) GTA treated fibers and D) UV treated fibers. Reproduced with permission from [59].

In vitro drug release testing in phosphate buffered saline (PBS) (pH 7.4) was performed with all fiber mats. For lysozyme quantification, bicinchoninic acid assays (BCA assays) were used, as such assays provide the advantage of detecting proteins even in their denatured state, and therefore assure accurate calculation of the encapsulated protein content and its release kinetics [60]. As expected, the drug release from the untreated fibers was very rapid due to the fast disintegration rate of

the PVA fibers (Figure 8). For the first post-modification approach, lysozyme release from the MeOH treated fibers did not show significant difference compared to the untreated fibers (Figure 8). In an earlier study, Kenawy et al. reported that MeOH treatment of PVA fibers loaded with ketoprofen can prolong the release of the encapsulated drug reaching less than 40 % in two weeks [57], the differences of their data to the release kinetics for lysozyme represented in Figure 8 are most likely due to the hydrophilicity of lysozyme (in comparison to ketoprofen), rather than due to differences in the prepared fibers. Slower release kinetics could be achieved using GTA vapor where around 60-70 % of the encapsulated lysozyme was released in 18 days (Figure 8). The third postmodification approach using UV was shown to be more efficient in terms of prolonging the release, for which the released lysozyme did not exceed 40 % during 18 days.

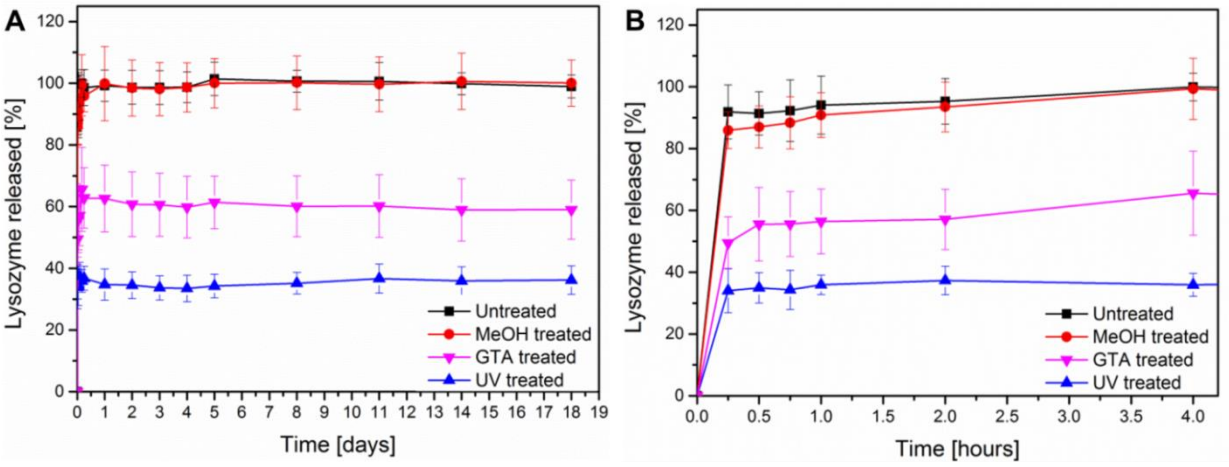


Figure 8: Lysozyme release from PVA electrospun fibers during a period of 18 days (A), and a close-up view on the release for the first 4 hours (B). Results are expressed as mean ± SD, n≥3. Reproduced with permission from [59].

For a better understanding of the effect of the release experiments on the fibers morphology, samples were immersed in PBS (pH 7.4) at 37 °C for 18 days, gently rinsed with water to remove any residual amount of the buffer and visualized with SEM. Interestingly, SEM images revealed that changes of the fibers surface structure correlate to the release kinetics for the encapsulated lysozyme. The surface of the untreated fibers changed into a homogeneous coherent film-like sheet with a slightly structured surface and individual fibers no longer visible (Figure 9A). In comparison, the initial fiber structure of MeOH and with GTA treated fibers is still generally visible, only in some smaller areas coherent film-like structures are also noticeable (Figure 9B and C). The UV treated fibers maintain their fiber morphology to a great extent,

which also corresponds to the slowest release kinetics for the embedded protein (Figure 9D).

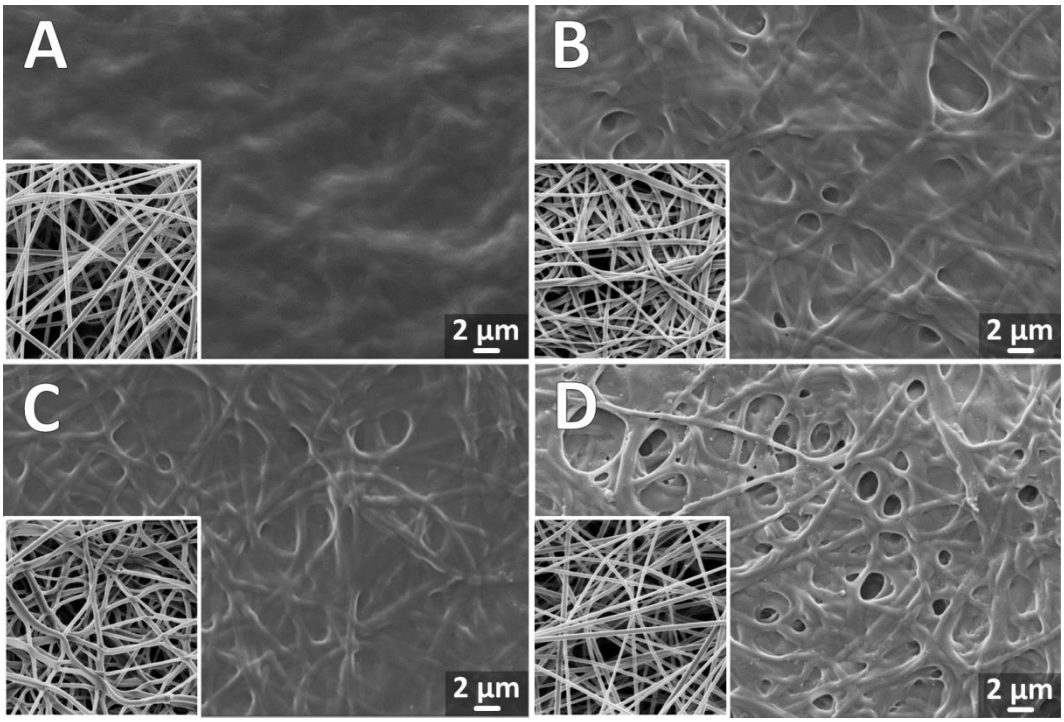


Figure 9: SEM images of the electrospun lysozyme-loaded PVA fibers after release experiments: A) untreated fibers B) MeOH treated fibers, C) GTA treated fibers, D) UV treated fibers. The initial state of the fibers is depicted in the small images for each formulation. Reproduced with permission from [59].

The next step was to investigate the effect of postmodification methods on the encapsulated lysozyme. One of the most reliable methods to investigate its activity is based on detecting the rate of its lytic activity on bacteria like *Micrococcus lysodeikticus* [61, 62]. The reduction of light absorbance of a suspension of *Micrococcus lysodeikticus* incubated with lysozyme can subsequently be used as an indicator for the activity of the protein. In this respect, a decrease in light absorbance correlates with high lysozyme activity. For our studies, lysozyme activity for each fiber mat sample was compared to the corresponding activity of freshly prepared solutions containing the same amount of lysozyme. For the untreated fibers, the activity of the encapsulated lysozyme was not affected, indicating the activity of the encapsulated protein was maintained during the electrospinning process (Figure 10A). Likewise, post-modification with MeOH did also not affect the activity of the encapsulated lysozyme (Figure 10A). In contrast, treatment with GTA vapor and UV light was associated with a loss of about 50 % of the initial protein activity (Figure 10A).

In a next step, potential effects of the different post-modification approaches on interactions with biological systems (cell cytotoxicity) were investigated by incubating cells with fiber mats and determining their cellular mitochondrial activity using methylthiazol tetrazolium (MTT) assays. The results show no significant reduction of the cell proliferation capacity for cells grown on MeOH treated fiber mats compared to untreated fibers (Figure 10B). Compared to cells maintained in standard two-dimensional polystyrene culture wells serving as positive control, only a slight reduction in absorbance values was observed for the cells cultivated on fibers. This is presumably related to the structural differences between the fiber mats and the two-dimensional culture wells. For GTA treated fibers, cells grown on these fibers showed hardly any mitochondrial cell activity and the results were much comparable to that of the negative control experiments (where cell death was induced by means of Triton X-100) (Figure 10B). The present results indicate that despite the relatively minimal exposure time to GTA vapor, residuals of GTA on the fiber surface could have implicate cytotoxic effect on the cells. For the fibers treated with UV light, significant reduction of the mitochondrial activity of the cells was found compared to the untreated fibers and the positive control (Figure 10B). This could be attributed to the UV induced formation of free radicals which can damage the cell structure and therefore affect cell viability [63, 64]. However, it has to be taken into considerations that MTT assays are normally performed on cell monolayers, which are much sensitive to external stimuli and are not sufficiently equivalent to the *in vivo* situation in the human body.

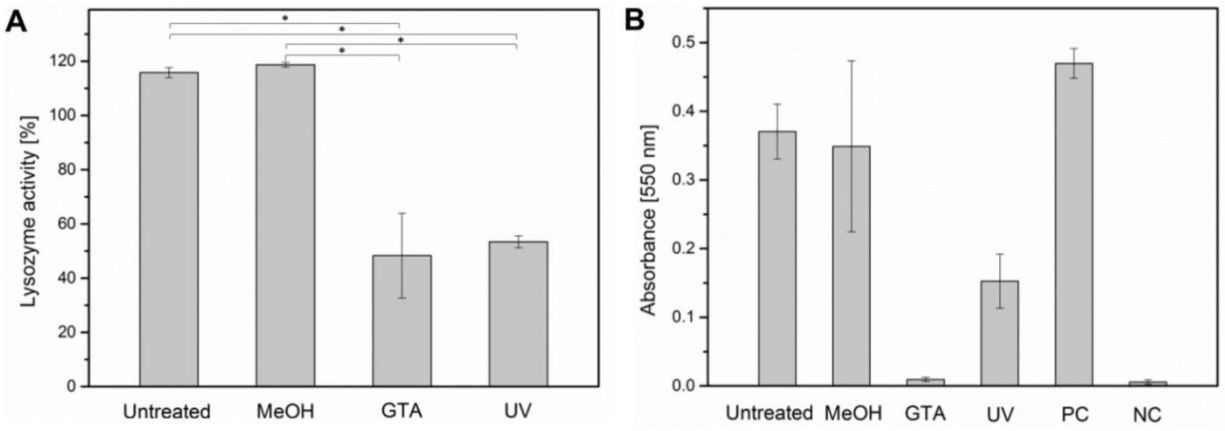


Figure 10: Analysis of the protein activity in the electrospun fibers (A) and cell viability testing of the fiber mats using an MTT assay (B) in which PC and NC represent the positive and negative controls, respectively. Results are expressed as the mean ± SD, n=3. Statistical significance was considered at $p<0.05$ (*). Reproduced with permission from [59].

In conclusion, the results of this study provide a deeper insight into the effect of post-modification of protein loaded electrospun fibers on protein release and activity, and on cytotoxicity of the fibers. While the protein release could successfully be prolonged by means of post-modification methods, crucial requirements for optimum therapeutic effectiveness of such protein-loaded delivery systems include maintaining the activity of the encapsulated protein and exclusion of any adverse effects to the human body. In this context, lysozyme activity was not affected by treatment with MeOH, whereas GTA and UV treatment considerably reduced its activity. Furthermore, cytotoxic effects on cultivated human cells were identified for fibers treated with GTA, as well as to a lesser extent for UV-treated fibers, whereas MeOH-treated fibers did not affect cell viability. These results elucidate the effects of fiber postmodification on release kinetics as well as on activity and biocompatibility of protein therapeutics, thus providing better insight into this option for the development of protein drug delivery systems based on electrospun fibers.

3.3. Establishment of novel approaches for *in situ* dissolution analysis of electrospun fibers

3.3.1. Introduction

Dissolution testing for electrospun fibers is generally performed in small vials by immersing the fiber mats in buffered solutions. Then, defined aliquots of dissolution medium are withdrawn at predefined time points and the dissolved drug is quantified. However, this procedure is associated with several drawbacks including inaccuracies in the case of frequent sampling and partial folding of the fiber mats upon contacting the dissolution media. This section presents the development of a predictive dissolution setup for electrospun fibers based on a fully automated fiber optics system for advanced *in situ* monitoring of drug release from electrospun fibers.

Corresponding article: IV.

3.3.2. Monitoring drug release from electrospun fibers using an *in situ* fiber optics system

Lysozyme was encapsulated within electrospun fibers based on PVA. For comparison, PVA films were prepared from the same solutions. Even though both systems were based on the same polymeric solution, a clear difference in the visual appearance could be observed (Figure 11). While the ultrafine substructure of the fiber network in the electrospun fibers led to the opaque white color appearance (Figure 11A and B) [23], the casted film exhibited a smooth surface with transparent appearance (Figure 11C and D). Although circular punches with the same diameter were acquired from films and fiber mats, the ultrafine substructure of the electrospun fibers increases the actual surface area of these samples, which can be expected to have an impact on the release kinetics of encapsulated drugs.

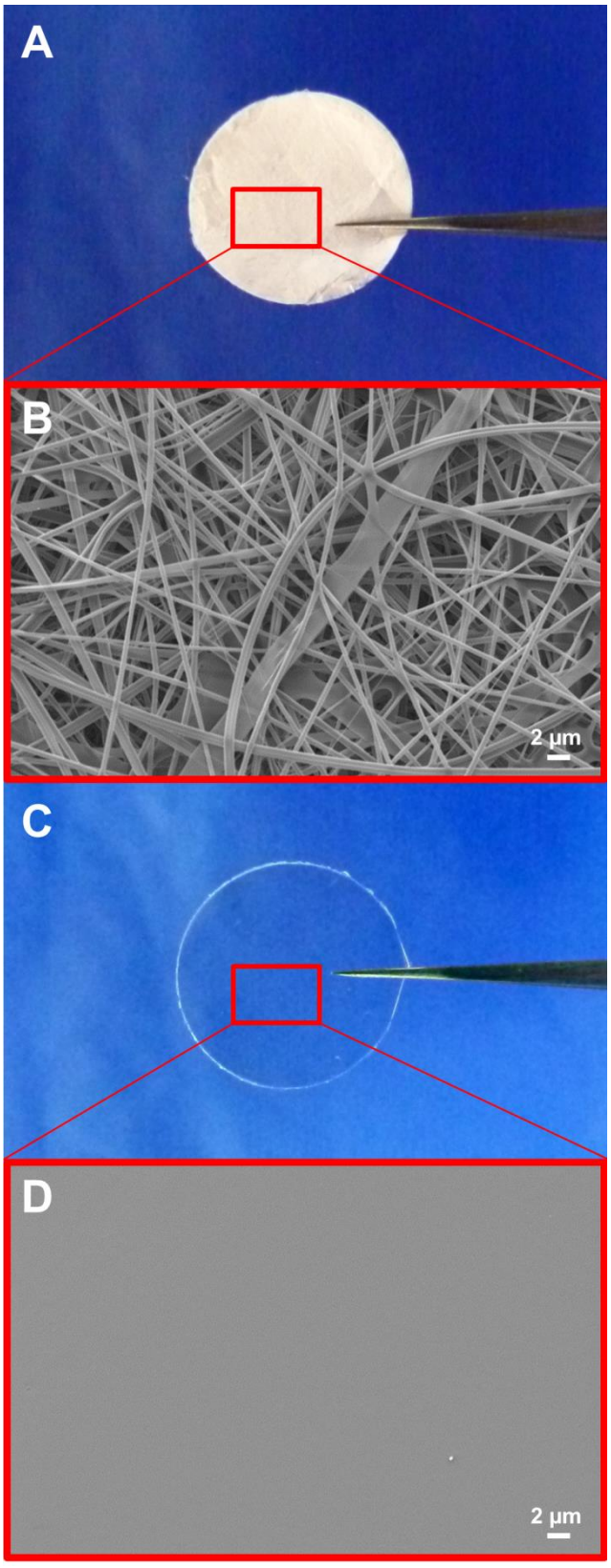


Figure 11: (A) Surface morphology of the electrospun fibers, (B) SEM image of the electrospun fibers, (C) surface morphology of a casted film, (D) SEM image of a casted film. Reproduced with permission from [65].

Conventional drug release experiments are generally performed by immersing the drug-loaded dosage form in buffered solutions and determining the concentration of

the released drug at predetermined time points. Although this method can be applied for thin polymeric dosage forms such as electrospun fiber mats, films, or transdermal patches, the thin and flexible nature of such systems make them susceptible to folding upon immersion in the release media as shown in Figure 12. This folding can eventually decrease the surface area of the investigated sample, leading to inaccurate determination of the release kinetics.

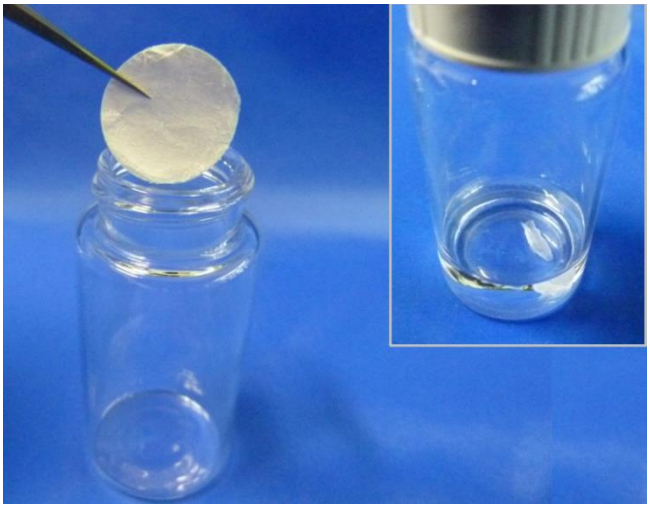


Figure 12: Electrospun fiber mat with glass vial for conventional drug release testing before immersion in dissolution medium, and in dissolution medium (magnification). Reproduced with permission from [65].

In this respect, it is necessary to preserve the flat structure of the sample during the release experiment for improve accuracy and reproducibility. For instance, dissolution testing from transdermal patches requires the fixation of the sample by using a strip of double-sided adhesive or an extraction cell [66]. The same concept was considered for polymeric films [67]. However, in the case of electrospun fibers release experiments are generally performed by simply immersing the fiber mats in buffer without any fixation of the sample [68]. The issue of sample folding was rarely addressed for electrospun fibers. For instance, in a study by Verreck et al. a modified Finn chamber was used to investigate drug release from electrospun fibers, thus keeping the sample from folding during dissolution [48].

In this study, we designed a novel flexible adapter to keep the sample fixed in a certain position during the release experiments. The designed adapter consisted of commercially available silicone and a nylon based net (Figure 13). While the mechanical flexibility of silicone allowed for convenient use, the nylon net provided the required support and at the same time unrestricted diffusion of the release

medium as well as released drug. Prior to the release experiments, interactions of net and drug were experimentally excluded (data not shown).

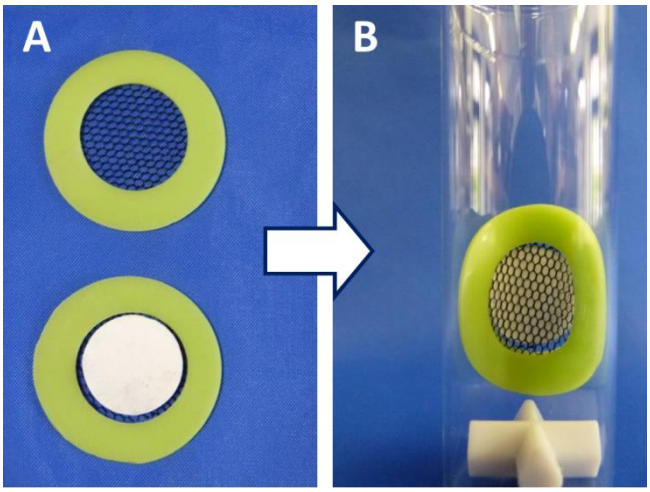


Figure 13: Sample mounting using the designed flexible adapter. (A) Adapter without (top) and with (bottom) electrospun mat, (B) adapter with fiber mat installed within the dissolution vessel. Reproduced with permission from [65].

Release experiments in PBS (pH 7.4) were performed with the electrospun fiber mats as well as with the casted films. The designed adapter was used to mount the samples to the wall of the dissolution vessel, preventing undesired sample folding (Figure 13). The dissolution setup was composed of fiber optics immersed in glass vials that were tightly sealed during the experiments to prevent volume changes due to evaporation of the media. Furthermore, each fiber optic channel (corresponding to a separate dissolution vial) was calibrated with its own standard curve prior to experiments, and a very good linearity was achieved as shown in the representative calibration curve (Figure 14A). Complete drug release from electrospun fibers and casted films was achieved after approximately 20 minutes, which can be attributed to the hydrophilic nature of the drug and the polymeric material. The results of the dissolution experiments are shown in Figure 14B. The high sampling intervals provided a deeper insight into the release process from the electrospun fibers as well as from the casted films. Lysozyme release from electrospun fibers occurred much faster compared to its release from casted films (Figure 14B). This can be attributed to the higher surface provided by the ultrafine fibrous structure, which facilitated the release into the dissolution media. Although the overall difference in the release kinetics can be considered marginal, the frequent sampling intervals allowed for the successful identification of this occurrence to be achieved.

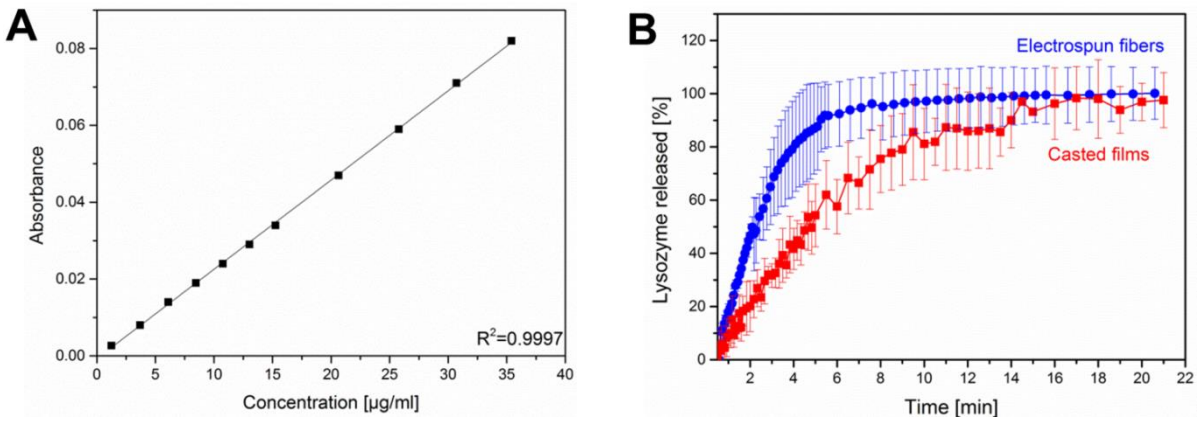


Figure 14: (A) Representative calibration curve and (B) lysozyme release from fiber mats as well as from films (mean ± SD, n=3). Reproduced with permission from [65].

In conclusion, fully automated fiber optics systems provide many advantages for the dissolution testing of electrospun fibers, not only in terms of saving time, but also in providing deeper insight into the release kinetics due to the high sampling frequency. Furthermore, the flexible adapter designed in this work provided the required support for the tested samples and prevented the undesired sample folding. The present study highlights the potential of applying fiber optics dissolution systems for investigating drug release from electrospun fiber mats, thus providing an advanced alternative to the conventional dissolution experimental setups.

3.4. Generation of novel electrospun fibers with tunable biomechanical properties for cultivation of human cells

3.4.1. Introduction

The fibrous three-dimensional structure of electrospun fibers resembles the extracellular matrix (ECM). This similarity initially encouraged the researches for applying electrospun fibers for cell cultivation and tissue engineering [36]. Electrospun fibers were shown to provide a unique comfortable environment for cells facilitating their attachments and proliferation [36]. In this section, novel hybrid electrospun fibers with tunable biomechanical properties combining natural and synthetic polymers were generated. Thorough characterization of these fibers was performed with respect to their morphology, biomechanical properties and interactions with primary human cells.

Corresponding article: V.

3.4.2. Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers.

In order to fabricate electrospun fibers as substrates for cultivation of human cells, three different types of electrospun fiber mats varying in their biomechanical properties were generated, followed by characterization and evaluation of their interactions with primary human cells. The first fiber mat was solely composed of PCL, a synthetic, FDA-approved, biocompatible and water-insoluble polymer providing mechanical stability and flexibility [69]. For the second fiber mat, blend electrospun fibers were fabricated by mixing PCL with gelatin as a natural, water-soluble polymer [70]. The aim of this approach was to increase the wettability of the fiber surface and to coordinate the biomechanical behavior using a multi-component system. The third fiber mat aimed at further modulating the fiber mat´s mechanical properties and facilitating its hydrophilic characteristics. For this purpose, gelatin fibers were simultaneously electrospun along with the blend fibers forming a binary fiber system. The fibers generated in this study are schematically illustrated in Figure 15.

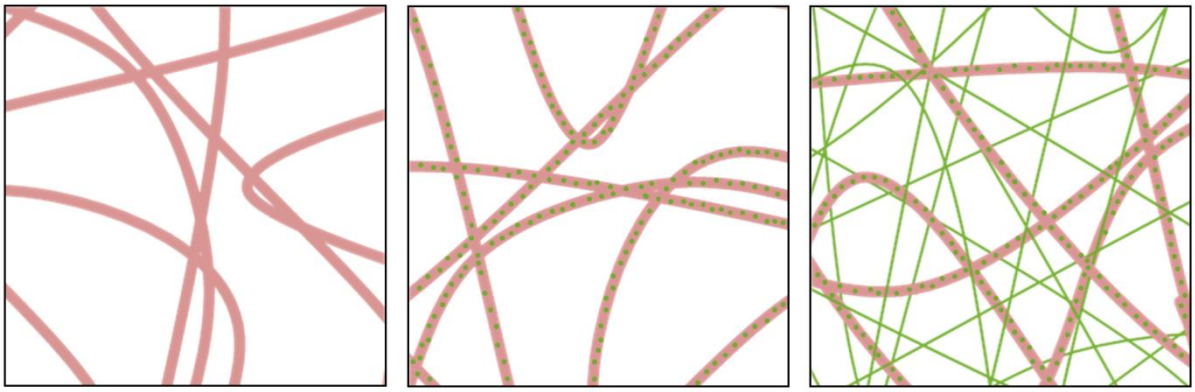


Figure 15: schematic illustration of the PCL, blend and hybrid fiber mats generated in this study. The red color represents PCL, while gelatin is represented in green.

All three types of fiber mats were compared to native human ECM which was decellularized after isolation from human skin. SEM imaging revealed that each fiber mat exhibited a homogenous fiber network with well-defined, interconnected porosity similar to the hierarchical architecture of native ECM and smooth surface (Figure 16A). Especially the hybrid fibers were closely comparable to native ECM in terms of fibers diameters. As the spatial distribution of different compounds in one fiber strongly affects the biomechanical properties of the prepared fibers, confocal Raman microscopy was used to investigate the distribution of PCL and gelatin within the blend fibers. Based on a z-stack analysis, virtual slices of the fibers in different focal planes allowed for spatially resolved, three-dimensional visualization of the fiber composition. One representative false-color image is depicted in Figure 16B. Interestingly, gelatin (green) is mainly found at the surface of the fiber, whereas the cores of the fibers mainly consist of pure PCL (red).

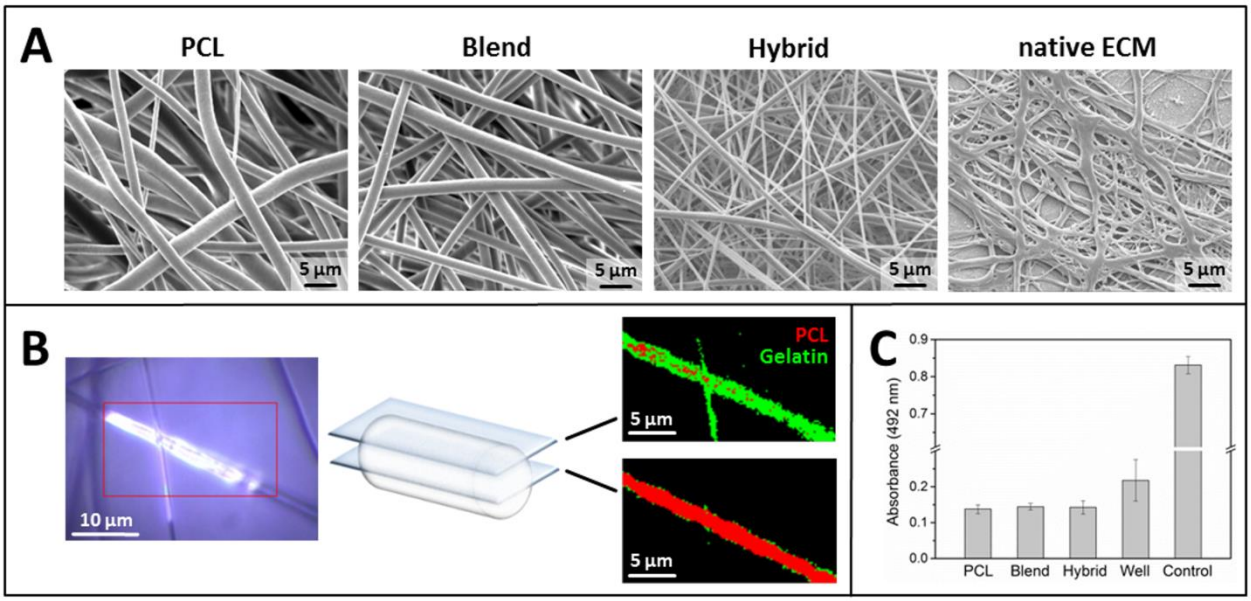
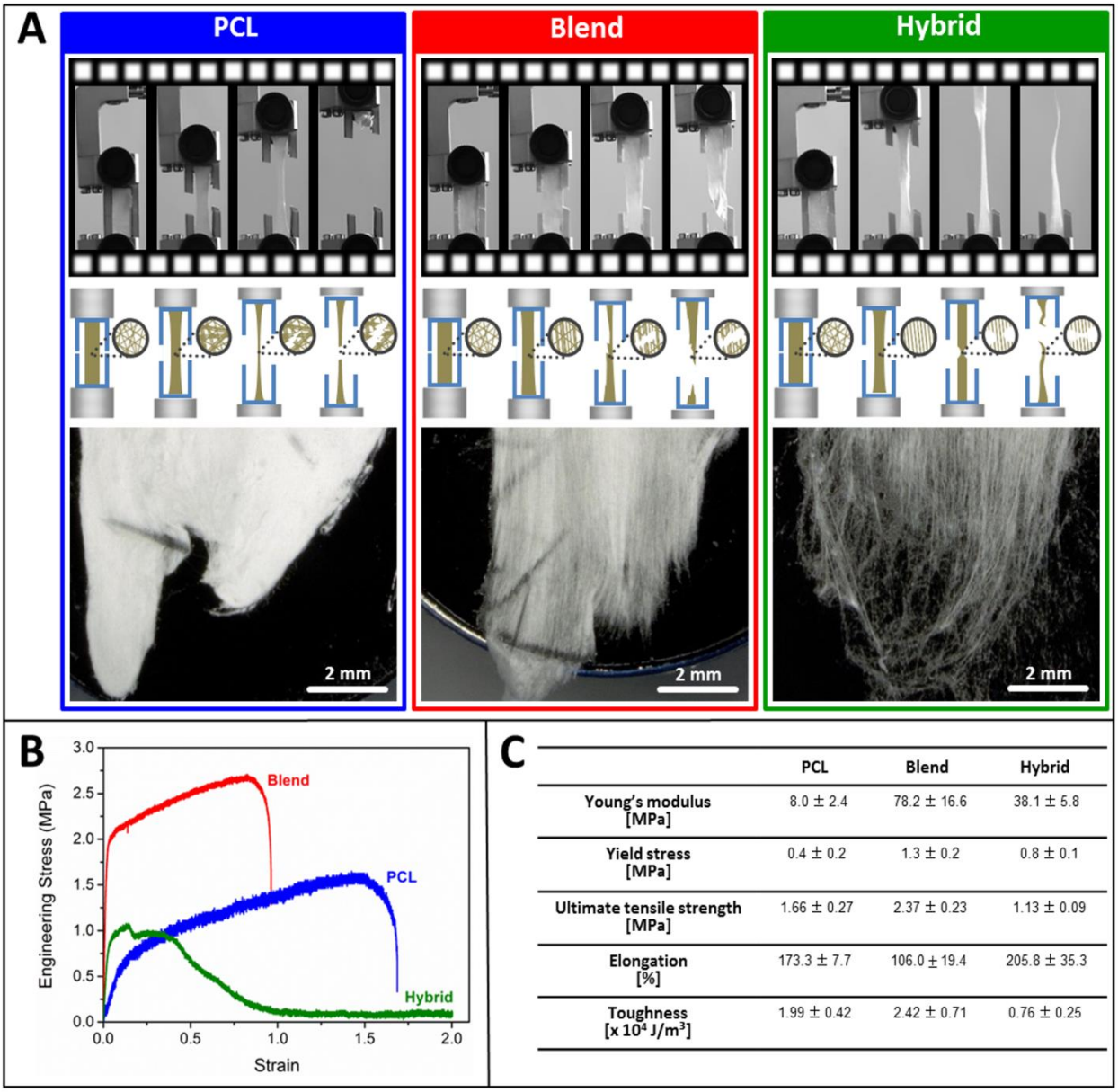


Figure 16: A) SEM images of the fabricated electrospun PCL, blend and hybrid fibers, and isolated ECM, B) Non-invasive, chemically selective Raman analysis of compound distribution (PCL – red, gelatin – green) in electrospun hybrid fibers. Reproduced with permission from [71].

Considering that the surface wettability is an important factor affecting the biological behavior of cells on material surfaces, contact angle measurements were conducted on the fabricated fiber mats. While PCL fibers exhibited a rather hydrophobic surface (contact angle of 119°), the gelatin containing fibers (namely blend and hybrid fibers) were shown to have a much improved wettability and hydrophilic properties with contact angle of 0°.

The next step in characterizing the generated electrospun fibers focused on testing their mechanical properties. In this respect, uniaxial tensile testing was used to record the structural changes of the fibers and their failure upon tensile loading. This test aimed at evaluating the fibers in terms of stiffness, flexibility, and toughness properties. Subsequently, SEM analysis of the investigated fibers was performed to provide better understanding of the fibers failure. For the PCL fiber mats, deformation started by extensive necking and continuous propagation along the tension axis, until the final failure was reached (Figure 17A). In the case of blend fiber mats, non-uniform elongation along the tensile axis was observed. Defects started to appear at different positions of the sample until a critical defect density led to failure (Figure 17A). The tearing failure started from sample edge with rapid propagation along the sample surface. In comparison to PCL fibers, the presence of gelatin in blend fibers contributed to improving the mechanical properties by increasing the toughness of

the blend fibers as shown by the higher Young's modulus [72]. This can be seen by the steep shift of the linear elastic phase within the stress-strain curve in y-direction (Figure 17B). Interstingly, the hybrid fibers, failed by gradual fiber breakage, where each failure event seemed to allow unraveling of the fibers and facilitated increased elongation as shown in Figure 17A. The continuous elongation of the hybrid fibers resulted in thinning of the parallely realigned fibers until breakage of the sample was induced by sequential failure of individual fibers. Further, the stress-strain curve revealed a brittle-to-ductile transition during fracture of hybrid fibers. A detailed characterizaiton of the mechanical properties of the generated fibers is summarized in Figure 17C.



	PCL	Blend	Hybrid
Young's modulus [MPa]	8.0 ± 2.4	78.2 ± 16.6	38.1 ± 5.8
Yield stress [MPa]	0.4 ± 0.2	1.3 ± 0.2	0.8 ± 0.1
Ultimate tensile strength [MPa]	1.66 ± 0.27	2.37 ± 0.23	1.13 ± 0.09
Elongation [%]	173.3 ± 7.7	106.0 ± 19.4	205.8 ± 35.3
Toughness [x 10^4 J/m^3]	1.99 ± 0.42	2.42 ± 0.71	0.76 ± 0.25

Figure 17: Mechanical properties of electrospun PCL, blend and hybrid fiber mats. (A) Visualization of the different failure modes based on acquired time-lapsed serial images upon tensile loading and representative optical micrographs of the fracture fiber surfaces after mechanical testing. (B) Mechanical properties calculated from the engineering stress-strain curves. (C) Typical engineering stress-strain curves for each type of fiber mats. Reproduced with permission from [71].

In order to evaluate the suitability of the fabricated fibers for the cultivation of human cells, primary human skin fibroblasts were cultivated on all three fiber formulations (PCL, blend, and hybrid fibers). Interestingly, significant differences in terms of cell morphology and density were observed among the fabricated fibers. For instance, the level of cell coverage on the PCL fibers was rather low (Figure 18A). This can be attributed to the rather hydrophobic surface of PCL fiber mats (water contact angle

119°, n= 5), low stiffness (8.0 MPa ± 2.5 MPa, n = 6), and moderate toughness (1.99 x 104 J/m³ ± 0.42, n = 6). In comparison, blend fiber mats showed a higher cell density and three-dimensional cell shaping (Figure 18B), which were induced by improved cell attachment properties due to the increased hydrophilicity (water contact angle 0°, n= 5) as well as substantial increase in stiffness (78.2 MPa ± 16.6 MPa, n = 6) and toughness (2.42 x 104 J/m³ ± 0.71, n = 6). The highest level of cell growth and three-dimensional cell organization was observed for the hybrid fiber mats, where cells could attach to several fibers due to their improved hydrophilicity and smaller pore sizes in the initial phase (Figure 18C).

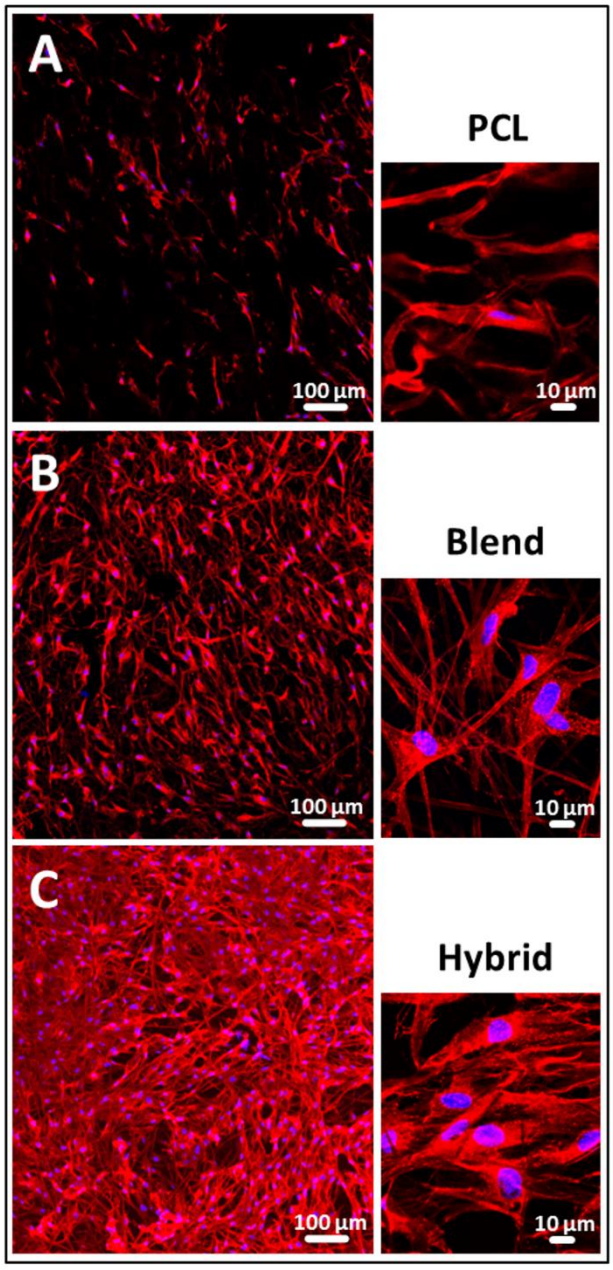


Figure 18: Evaluation of cell-matrix interactions by analysis of cell behavior as response to fiber surface. Fluorescence staining of cell membrane (red) and cell nuclei (blue) to visualize human dermal fibroblasts cultivated for 14 days onto (A) PCL, (B) blend and (C) hybrid fiber mats revealing considerable differences in cell density and three dimensional cell-shaping among these three electrospun fiber mats. Reproduced with permission from [71].

In order to follow up with the previous results, the next studies aimed at evaluating the potential of the hybrid fiber mats for the hierarchical three-dimensional cultivation of different human cells. In this context, fibroblasts (as cells forming the dermis in human skin) were cultivated on hybrid fiber mats in a multi-well plate assembly equipped with permeable membrane inserts. After two weeks of cultivation, keratinocytes (resembling the upper part of the skin, the epidermis) were seeded on

top of the same multi-well plate assembly. Then, the well plate inserts were lifted to the air-liquid interface to induce a stratification of the upper keratinocytes layers, thus mimicking the *in vivo* situation in the human body. Schematic illustration of the assembly is illustrated in Figure 19A. Fluorescence-based staining of the cell membrane (red) and cell nuclei (blue) revealed a three-dimensional cellular organization of the fibroblasts after two weeks of cultivation as well as high cell density of keratinocytes detected on the hybrid fiber mat surface after ten days of cultivation (Figure 19A).

The final step of this study was to compare the fiber mats with excised human skin by histological analysis. For this purpose, Hematoxylin/eosin stained cross sections were prepared (Figure 19B). While hematoxylin (blue) binds to cell nuclei, eosin (pink) stains collagenous structures as the main constituents of the dermis part. Direct comparison of hybrid fiber mat and excised human skin showed high comparability represented by the multilayered sheet of keratinocytes located on the outer surface of the fiber mat scaffold and the presence and growth of the fibroblasts within the fiber mat scaffold (Figure 19B). Furthermore, the barrier formation was verified using involucrin (green) as a specific immunofluorescence-based marker for terminal differentiation of the keratinocytes [73, 74]. Additionally, 4',6-diamidino-2-phenylindole staining (DAPI Staining) (blue) allowed visualization of the cell nuclei, which can be seen in high density in the viable epidermis. Thus confirming the localization of involucrin occurring towards the air exposed region above the viable part of the epidermis (Figure 19B). The presence of an intact barrier along the entire length of the fiber mat cross section is represented by the uniform consistent stratified layer as shown in the bottom left corner of Figure 19B.

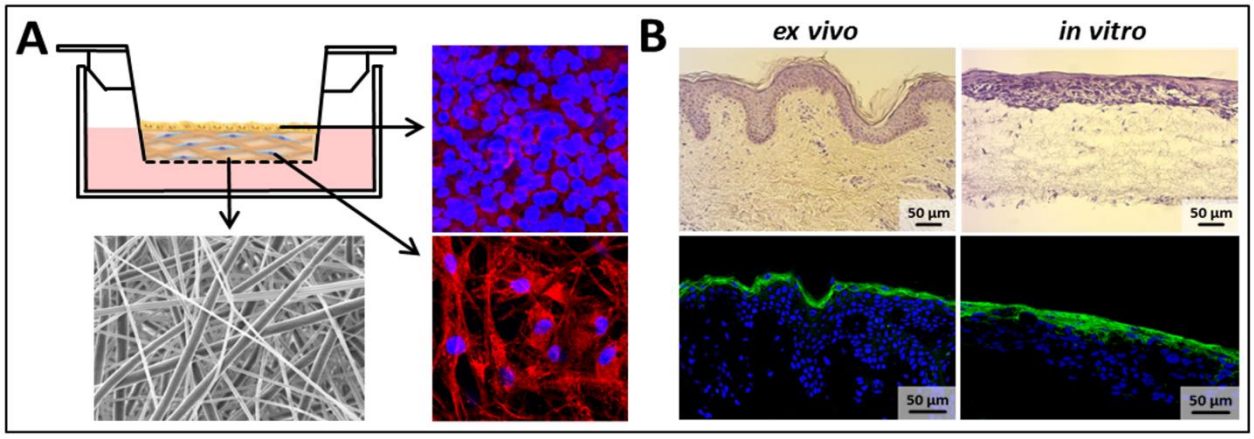


Figure 19: Evaluation of the applicability of electrospun hybrid fiber mats for hierarchical co-cultivation of human-derived skin cells. (A) Experimental setup for *in vitro* reconstruction of human skin tissue using a Transwell®-system by first seeding human primary dermal fibroblasts on electrospun hybrid fiber mats for 14 days, followed by cocultivation with human keratinocytes on top of the fiber mats surface for 10 days under submersed culture conditions and further cultivation at air-liquid interface for 14 days. (B) Example of *in vitro* reconstructed skin tissue visualized by hematoxylin/ eosin staining and evaluation of barrier formation by immunofluorescence-staining of terminal differentiated keratinocytes (green) using involucrin (right column) compared to ex vivo human skin tissue (left column). Reproduced with permission from [71].

To summarize, in this study we introduced a novel approach for designing and fabricating bio-inspired functional electrospun fibers derived from the native architecture of human ECM with tailor-made biomechanical properties favoring cell attachment. The three-dimensional fibrous network offered by hybrid electrospun fiber mat provided a unique bio-adaptive environment for cell attachment, migration, and proliferation within the matrix. The present results confirm the synergistic effect of tailored biomechanics, surface wettability, and biodegradation of the fiber mat on the cellular behavior, hence, providing a better understanding on the factors affecting the development of scaffolds based on electrospun fibers for application in cell cultivation and tissue engineering.

4. General conclusion and perspective

Electrospinning shows great potential for broad spectrum of biomedical applications ranging from drug delivery to tissue engineering. Success in achieving the optimum results depends highly on the good understanding of the various parameters controlling the process of fibers fabrication and drug encapsulation. In this context, consideration of the physicochemical properties of both drug and polymer is of great importance for efficient drug encapsulation and controlled drug release. As for hydrophobic polymers, their slow degradation rate is an important advantage which allows for controlled drug delivery applications. However, using such type of polymers bears challenges if used for encapsulating hydrophilic drugs. In this case, inefficient drug encapsulation resulting in drug crystallization on (or near) the fibers outer surface can be a major problem as it leads to instant drug release. In this respect, increasing the polarity of solvent system was identified as a successful strategy for overcoming this undesired effect of drug crystallization.

In contrast to hydrophobic polymers, the adequate water solubility of hydrophilic polymers offers a more convenient alternative to encapsulate water soluble substances that are sensitive to organic solvents such as proteins. In general, hydrophilic polymers exhibit a rapid degradation rate in aqueous media. This feature allows for applications where instant drug release is desired (e.g. for fast dissolving oral films etc.), nevertheless, it can also be considered as a limitation if prolonged drug release is required. Based on that, this work investigated different post-modification methods to stabilize protein-loaded electrospun fibers using lysozyme as a model protein and poly(vinyl alcohol) as hydrophilic polymer. The applied post-modification methods included treatments with methanol, glutaraldehyde vapor, or ultraviolet light. These methods could prolong the release of the encapsulated substance at different extents. However, as the therapeutic effectiveness of such protein-loaded electrospun fibers requires activity maintenance of the encapsulated protein and exclusion of any adverse effects to the human body, future studies should focus on elaborating the mechanism by which such post-modification methods would affect the encapsulated protein. Further, the effect of post-modified protein-loaded fibers on *in vitro* 3D models has to be investigated in order to determine factors affecting cellular response to these fibers.

For establishing novel *in situ* dissolution analysis of electrospun fibers, the application of fully automated fiber-optic system was investigated. This approach provided many advantages over conventional dissolution testing as the higher sampling frequency allowed for a deeper insight into release kinetics. Further, to avoid partial folding of electrospun fibers that occurs upon contact with dissolution media, a novel adaptor was designed providing the required support for the tested samples and preventing the undesired sample folding.

As for applying electrospun fibers as substrates for the cultivation of human cells, novel biocompatible electrospun fibers were generated encompassing natural as well as synthetic polymers. Subsequently, the fibers biomechanical properties and fibers interaction with primary human cells were thoroughly investigated. The three-dimensional fiber network could successfully simulate the complex structure of the extracellular matrix providing a unique bio-adaptive environment, hence facilitating cellular attachment, migration, and proliferation.

In conclusion, this work provides a better understanding of various factors affecting the applicability of electrospun fibers for drug delivery and tissue engineering, by addressing several points of concerns including: drug stability, drug distribution within the fibers, drug release kinetics, and interactions of electrospun fibers with human cells. Thus, it offers a solid background for further research on electrospinning for biomedical applications.

For the near future, the possibility to encapsulate pharmaceutically active substances within electrospun fibers for tissue engineering applications has to be investigated. While the current work focused on either applying electrospun fibers for drug delivery or for tissue engineering, combination of these approaches can be very promising for advanced biomedical applications. For instance, encapsulating growth factors or anti-infectives within our hybrid electrospun fibers can be expected to provide an advance system for applications like chronic wounds. Characterization of these drug loaded fiber mats has to be complemented with thorough investigation by means of *in vitro* models followed by *in vivo* studies to evaluate their effectiveness.

5. References

1. Greiner, A. and J.H. Wendorff, *Electrospinning: A fascinating method for the preparation of ultrathin fibres.* Angew Chem Int Edit, 2007. **46**(30): p. 5670-5703.
2. Laudenslager, M.J. and W.M. Sigmund, *Developments in electrohydrodynamic forming: Fabricating nanomaterials from charged liquids via electrospinning and electrospraying.* Am Ceram Soc Bull, 2011. **90**(2): p. 22-26.
3. Gilbert, W., *De Magnete*. 1600: Dover publications (1958).
4. Formhals, A., *Process and apparatus for prepearing artificial threads*, U.S.P. Office, Editor. 1934: USA.
5. Taylor, G., *Disintegration of Water Drops in an Electric Field.* Proceedings of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 1964. **280**(1382): p. 383-397.
6. Li, D. and Y. Xia, *Electrospinning of Nanofibers: Reinventing the Wheel?* Adv Mater, 2004. **16**(14): p. 1151-1170.
7. Sill, T.J. and H.A. von Recum, *Electrospinning: applications in drug delivery and tissue engineering.* Biomaterials, 2008. **29**(13): p. 1989-2006.
8. Reneker, D.H. and A.L. Yarin, *Electrospinning jets and polymer nanofibers.* Polymer, 2008. **49**(10): p. 2387-2425.
9. Lushnikov, A., *Igor' Vasilievich Petryanov-Sokolov (1907–1996).* Journal of Aerosol Science, 1997. **28**(4): p. 545-546.
10. *On the 100th anniversary of the birth of I.V. Petryanov-Sokolov.* Izv. Atmos. Ocean. Phys., 2007. **43**(3): p. 395-395.
11. Lee, S. and S.K. Obendorf, *Use of electrospun nanofiber web for protective textile materials as barriers to liquid penetration.* Text Res J, 2007. **77**(9): p. 696-702.
12. Sas, I., et al., *Literature review on superhydrophobic self-cleaning surfaces produced by electrospinning.* J Polym Sci Pol Phys, 2012. **50**(12): p. 824-845.
13. Spasova, M., et al., *Electrospun biohybrid materials for plant biocontrol containing chitosan and Trichoderma viride spores.* J Bioact Compat Pol, 2011. **26**(1): p. 48-55.
14. Ramakrishna, S., et al., *Electrospun nanofibers: solving global issues.* Mater Today, 2006. **9**(3): p. 40-50.
15. Braghirolli, D.I., D. Steffens, and P. Pranke, *Electrospinning for regenerative medicine: a review of the main topics.* Drug discovery today, 2014. **19**(6): p. 743-53.
16. Agarwal, S., J.H. Wendorff, and A. Greiner, *Use of electrospinning technique for biomedical applications.* Polymer, 2008. **49**(26): p. 5603-5621.
17. Laurencin, C.T., et al., *Recent Patents on Electrospun Biomedical Nanostructures: An Overview.* Recent Patents on Biomedical Engineering, 2008. **1**(1): p. 68-78.
18. Meinel, A.J., et al., *Electrospun matrices for localized drug delivery: Current technologies and selected biomedical applications.* European Journal of Pharmaceutics and Biopharmaceutics, 2012. **81**(1): p. 1-13.
19. Zamani, M., M.P. Prabhakaran, and S. Ramakrishna, *Advances in drug delivery via electrospun and electrosprayed nanomaterials.* Int J Nanomedicine, 2013. **8**: p. 2997-3017.

20. Hu, X., et al., *Electrospinning of polymeric nanofibers for drug delivery applications.* J Control Release, 2014. **185**: p. 12-21.
21. Bolgen, N., et al., *In vivo performance of antibiotic embedded electrospun PCL membranes for prevention of abdominal adhesions.* J Biomed Mater Res B, 2007. **81B**(2): p. 530-543.
22. Kim, H.S. and H.S. Yoo, *MMPs-responsive release of DNA from electrospun nanofibrous matrix for local gene therapy: In vitro and in vivo evaluation.* J Control Release, 2010. **145**(3): p. 264-271.
23. Kenawy, E.R., et al., *Release of tetracycline hydrochloride from electrospun poly(ethylene-co-vinylacetate), poly(lactic acid), and a blend.* J Control Release, 2002. **81**(1-2): p. 57-64.
24. Maretschek, S., A. Greiner, and T. Kissel, *Electrospun biodegradable nanofiber nonwovens for controlled release of proteins.* J Control Release, 2008. **127**(2): p. 180-187.
25. Chew, S.Y., et al., *Sustained release of proteins from electrospun biodegradable fibers.* Biomacromolecules, 2005. **6**(4): p. 2017-2024.
26. Sun, Z., et al., *Compound Core–Shell Polymer Nanofibers by Co-Electrospinning.* Adv Mater, 2003. **15**(22): p. 1929-1932.
27. Jiang, H.L., et al., *A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents.* J Control Release, 2005. **108**(2-3): p. 237-243.
28. Lu, Y., et al., *Mild immobilization of diverse macromolecular bioactive agents onto multifunctional fibrous membranes prepared by coaxial electrospinning.* Acta Biomater, 2009. **5**(5): p. 1562-1574.
29. Srikar, R., et al., *Desorption-limited mechanism of release from polymer nanofibers.* Langmuir : the ACS journal of surfaces and colloids, 2008. **24**(3): p. 965-974.
30. Jiang, H.L., et al., *Modulation of protein release from biodegradable core-shell structured fibers prepared by coaxial electrospinning.* J Biomed Mater Res B, 2006. **79B**(1): p. 50-57.
31. Saraf, A., et al., *Regulated non-viral gene delivery from coaxial electrospun fiber mesh scaffolds.* J Control Release, 2010. **143**(1): p. 95-103.
32. Townsend-Nicholson, A. and S.N. Jayasinghe, *Cell electrospinning: a unique biotechnique for encapsulating living organisms for generating active biological microthreads/scaffolds.* Biomacromolecules, 2006. **7**(12): p. 3364-9.
33. Choi, J.S., K.W. Leong, and H.S. Yoo, *In vivo wound healing of diabetic ulcers using electrospun nanofibers immobilized with human epidermal growth factor (EGF).* Biomaterials, 2008. **29**(5): p. 587-596.
34. Casper, C.L., et al., *Coating electrospun collagen and gelatin fibers with perlecan domain I for increased growth factor binding.* Biomacromolecules, 2007. **8**(4): p. 1116-1123.
35. Nie, H., et al., *Three-dimensional fibrous PLGA/HAp composite scaffold for BMP-2 delivery.* Biotechnology and bioengineering, 2008. **99**(1): p. 223-234.
36. Sun, T., et al., *Development of a 3D cell culture system for investigating cell interactions with electrospun fibers.* Biotechnology and bioengineering, 2007. **97**(5): p. 1318-28.
37. Karp, G., *Cell Biology*. 2010: Wiley.
38. Del Gaudio, C., A. Bianco, and M. Grigioni, *Electrospun bioresorbable trileaflet heart valve prosthesis for tissue engineering: in vitro functional assessment of a pulmonary cardiac valve design.* Ann I Super Sanita, 2008. **44**(2): p. 178-186.

39. Boland, E.D., et al., *Electrospinning collagen and elastin: preliminary vascular tissue engineering.* Frontiers in bioscience : a journal and virtual library, 2004. **9**: p. 1422-32.
40. Prabhakaran, M.P., J.R. Venugopal, and S. Ramakrishna, *Mesenchymal stem cell differentiation to neuronal cells on electrospun nanofibrous substrates for nerve tissue engineering.* Biomaterials, 2009. **30**(28): p. 4996-5003.
41. Ghasemi-Mobarakeh, L., et al., *Electrospun poly(epsilon-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering.* Biomaterials, 2008. **29**(34): p. 4532-9.
42. Yoshimoto, H., et al., *A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering.* Biomaterials, 2003. **24**(12): p. 2077-82.
43. Li, C., et al., *Electrospun silk-BMP-2 scaffolds for bone tissue engineering.* Biomaterials, 2006. **27**(16): p. 3115-24.
44. Norouzi, M., et al., *Advances in skin regeneration: application of electrospun scaffolds.* Adv Healthc Mater, 2015. **4**(8): p. 1114-33.
45. Jiang, T., et al., *Electrospinning of polymer nanofibers for tissue regeneration.* Prog Polym Sci, 2015. **46**: p. 1-24.
46. Zeng, J., et al., *Influence of the drug compatibility with polymer solution on the release kinetics of electrospun fiber formulation.* J Control Release, 2005. **105**(1-2): p. 43-51.
47. Garsuch, V. and J. Breitkreutz, *Novel analytical methods for the characterization of oral wafers.* Eur J Pharm Biopharm, 2009. **73**(1): p. 195-201.
48. Verreck, G., et al., *Incorporation of drugs in an amorphous state into electrospun nanofibers composed of a water-insoluble, nonbiodegradable polymer.* J Control Release, 2003. **92**(3): p. 349-360.
49. Kim, T.G., D.S. Lee, and T.G. Park, *Controlled protein release from electrospun biodegradable fiber mesh composed of poly(epsilon-caprolactone) and poly(ethylene oxide).* Int J Pharm, 2007. **338**(1-2): p. 276-83.
50. Seif, S., L. Franzen, and M. Windbergs, *Overcoming drug crystallization in electrospun fibers--Elucidating key parameters and developing strategies for drug delivery.* Int J Pharm, 2015. **478**(1): p. 390-7.
51. Wee-Eong, T., I. Ryuji, and R. Seeram, *Technological advances in electrospinning of nanofibers.* Sci Technol Adv Mat, 2011. **12**(1): p. 013002.
52. Zong, X., et al., *Structure and process relationship of electrospun bioabsorbable nanofiber membranes.* Polymer, 2002. **43**(16): p. 4403-4412.
53. Lee, K., et al., *Characterization of nano-structured poly (ε-caprolactone) nonwoven mats via electrospinning.* Polymer, 2003. **44**(4): p. 1287-1294.
54. Son, W.K., et al., *The effects of solution properties and polyelectrolyte on electrospinning of ultrafine poly (ethylene oxide) fibers.* Polymer, 2004. **45**(9): p. 2959-2966.
55. Sahoo, S., et al., *Growth factor delivery through electrospun nanofibers in scaffolds for tissue engineering applications.* J Biomed Mater Res A, 2010. **93A**(4): p. 1539-1550.
56. Taepaiboon, P., U. Rungsardthong, and P. Supaphol, *Effect of cross-linking on properties and release characteristics of sodium salicylate-loaded electrospun poly (vinyl alcohol) fibre mats.* Nanotechnology, 2007. **18**(17): p. 175102.

57. Kenawy, E.R., et al., *Controlled release of ketoprofen from electrospun poly(vinyl alcohol) nanofibers.* Mat Sci Eng a-Struct, 2007. **459**(1-2): p. 390-396.
58. Yao, L., et al., *Electrospinning and stabilization of fully hydrolyzed poly(vinyl alcohol) fibers.* Chem Mater, 2003. **15**(9): p. 1860-1864.
59. Seif, S., V. Planz, and M. Windbergs, *Controlling the Release of Proteins from Therapeutic Nanofibers: The Effect of Fabrication Modalities on Biocompatibility and Antimicrobial Activity of Lysozyme.* Planta medica, (EFirst).
60. Walker, J.M., *The Bicinchoninic Acid (BCA) Assay for Protein Quantitation*, in *Basic Protein and Peptide Protocols*, J.M. Walker, Editor. 1994, Humana Press: Totowa, NJ. p. 5-8.
61. Gorin, G., S.F. Wang, and L. Papapavlou, *Assay of lysozyme by its lytic action on M. lysodeikticus cells.* Analytical biochemistry, 1971. **39**(1): p. 113-127.
62. Liao, Y.H., M.B. Brown, and G.P. Martin, *Turbidimetric and HPLC assays for the determination of formulated lysozyme activity.* J Pharm Pharmacol, 2001. **53**(4): p. 549-54.
63. Choi, S.S., et al., *Fabrication and characterization of electrospun polybutadiene fibers crosslinked by UV irradiation.* J Appl Polym Sci, 2006. **101**(4): p. 2333-2337.
64. Bellomo, G., *Cell damage by oxygen free radicals.* Cytotechnology, 1991. **5**(Suppl 1): p. 71-3.
65. Seif, S., et al., *Monitoring Drug Release from Electrospun Fibers Using an In Situ Fiber-Optic System.* Dissolution Technologies, 2016. **23**(2).
66. *Dissolution test for transdermal patches (2.9.4.)*, in *European Pharmacopoeia*. 2008, Council of Europe.
67. Preis, M., et al., *Design and evaluation of bilayered buccal film preparations for local administration of lidocaine hydrochloride.* Eur J Pharm Biopharm, 2014. **86**(3): p. 552-61.
68. Srikar, R., et al., *Desorption-limited mechanism of release from polymer nanofibers.* Langmuir : the ACS journal of surfaces and colloids, 2008. **24**(3): p. 965-74.
69. Woodruff, M.A. and D.W. Hutmacher, *The return of a forgotten polymer—Polycaprolactone in the 21st century.* Prog Polym Sci, 2010. **35**(10): p. 1217-1256.
70. Huang, Z.-M., et al., *Electrospinning and mechanical characterization of gelatin nanofibers.* Polymer, 2004. **45**(15): p. 5361-5368.
71. Planz, V., et al., *Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers.* Integrative biology : quantitative biosciences from nano to macro, 2016. **8**(7): p. 775-84.
72. Zhang, Y., et al., *Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2005. **72**(1): p. 156-165.
73. Fiona, M. and D. Watt, *Involucrin and other markers of keratinocyte terminal differentiation.* J Invest Dermatol, 1983. **81**: p. 100-103.
74. Candi, E., R. Schmidt, and G. Melino, *The cornified envelope: a model of cell death in the skin.* Nature reviews Molecular cell biology, 2005. **6**(4): p. 328-340.

6. Original publications

6.1. Overcoming drug crystallization in electrospun fibers – Elucidating key parameters and developing strategies for drug delivery

Overcoming drug crystallization in electrospun fibers – Elucidating key parameters and developing strategies for drug delivery.

S. Seif, L. Franzen, M. Windbergs.

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Pharmaceutical nanotechnology

Overcoming drug crystallization in electrospun fibers – Elucidating key parameters and developing strategies for drug delivery

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ABSTRACT

For the development of novel therapeutics, uncontrolled crystallization of drugs within delivery systems represents a major challenge. Especially for thin and flexible polymeric systems such as oral films or dermal wound dressings, the formation and growth of drug crystals can significantly affect drug distribution and release kinetics as well as physical storage stability. In this context, electrospinning was introduced as a fabrication technique with the potential to encapsulate drugs within ultrafine fibers by rapid solvent evaporation overcoming drug crystallization during fabrication and storage. However, these effects could so far only be shown for specific drug-polymer combinations and an in-depth understanding of the underlying processes of drug-loaded fiber formation and influencing key parameters is still missing.

In this study, we systematically investigated crystal formation of caffeine as a model drug in electrospun fibers comparing different polymers. The solvent polarity was found to have a major impact on the drug crystal formation, whereas only a minor effect was attributed to the electrospinning process parameters.

Based on an in-depth understanding of the underlying processes determining drug crystallization processes in electrospun fibers, key parameters could be identified which allow for the rational development of drug-loaded electrospun fibers overcoming drug crystallization.

The full text of this article is available online at:

http://www.sciencedirect.com/science/article/pii/S0378517314008606?via%3Dihub

6.2. Controlling the release of natural proteins from therapeutic nanofibers – the effect of fiber fabrication on pharmacological activity and biocompatibility

Controlling protein release from hydrophilic electrospun fibers - the effect of post-modification on therapeutic activity and biocompatibility.

S. Seif, V. Planz, M. Windbergs.

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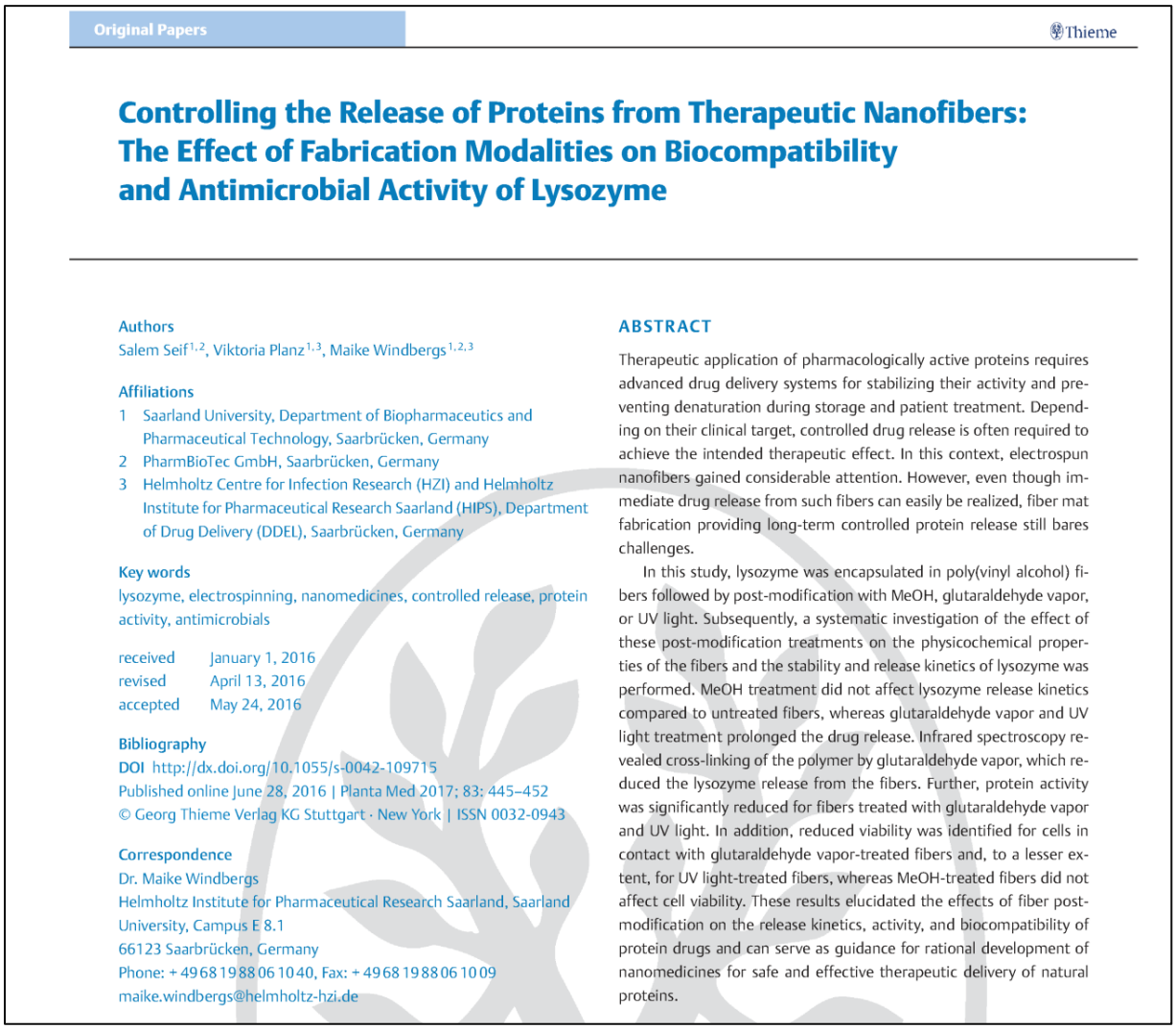

Original Papers

Thieme

Controlling the Release of Proteins from Therapeutic Nanofibers: The Effect of Fabrication Modalities on Biocompatibility and Antimicrobial Activity of Lysozyme

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ABSTRACT

Therapeutic application of pharmacologically active proteins requires advanced drug delivery systems for stabilizing their activity and preventing denaturation during storage and patient treatment. Depending on their clinical target, controlled drug release is often required to achieve the intended therapeutic effect. In this context, electrospun nanofibers gained considerable attention. However, even though immediate drug release from such fibers can easily be realized, fiber mat fabrication providing long-term controlled protein release still bares challenges.

In this study, lysozyme was encapsulated in poly(vinyl alcohol) fibers followed by post-modification with MeOH, glutaraldehyde vapor, or UV light. Subsequently, a systematic investigation of the effect of these post-modification treatments on the physicochemical properties of the fibers and the stability and release kinetics of lysozyme was performed. MeOH treatment did not affect lysozyme release kinetics compared to untreated fibers, whereas glutaraldehyde vapor and UV light treatment prolonged the drug release. Infrared spectroscopy revealed cross-linking of the polymer by glutaraldehyde vapor, which reduced the lysozyme release from the fibers. Further, protein activity was significantly reduced for fibers treated with glutaraldehyde vapor and UV light. In addition, reduced viability was identified for cells in contact with glutaraldehyde vapor-treated fibers and, to a lesser extent, for UV light-treated fibers, whereas MeOH-treated fibers did not affect cell viability. These results elucidated the effects of fiber post-modification on the release kinetics, activity, and biocompatibility of protein drugs and can serve as guidance for rational development of nanomedicines for safe and effective therapeutic delivery of natural proteins.

The full text of this article is available online at:

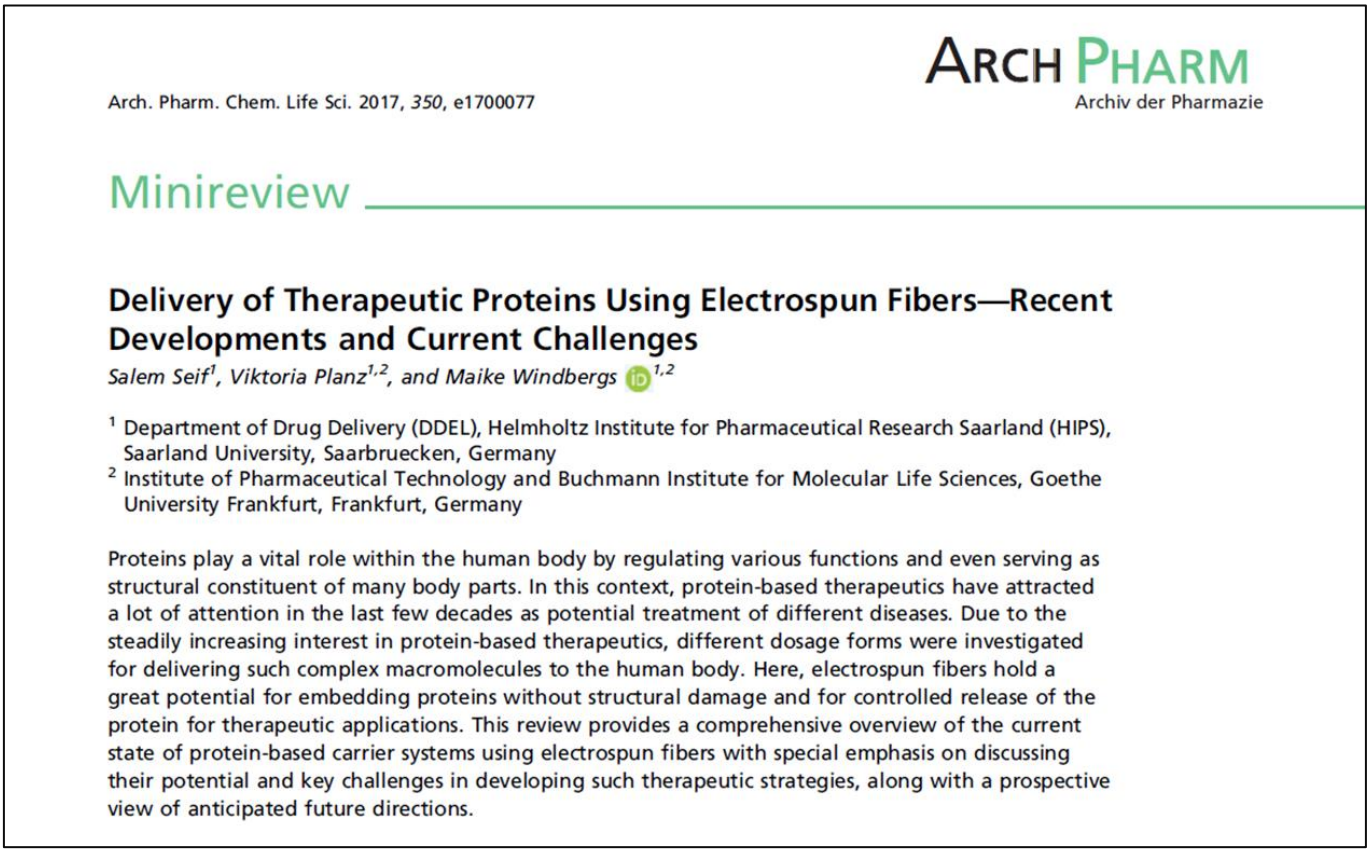
https://www.thieme-connect.com/products/ejournals/abstract/10.1055/s-0042-109715?lang=de

6.3. Delivery of therapeutic proteins using electrospun fibers – recent developments and current challenges

Delivery of therapeutic proteins using electrospun fibers – recent developments and current challenges.

S. Seif, V. Planz, M. Windbergs.

Archiv der Pharmazie, 2017. 350. DOI: 10.1002/ardp.201700077.



Arch. Pharm. Chem. Life Sci. 2017, *350*, e1700077

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Minireview

Delivery of Therapeutic Proteins Using Electrospun Fibers—Recent Developments and Current Challenges

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Proteins play a vital role within the human body by regulating various functions and even serving as structural constituent of many body parts. In this context, protein-based therapeutics have attracted a lot of attention in the last few decades as potential treatment of different diseases. Due to the steadily increasing interest in protein-based therapeutics, different dosage forms were investigated for delivering such complex macromolecules to the human body. Here, electrospun fibers hold a great potential for embedding proteins without structural damage and for controlled release of the protein for therapeutic applications. This review provides a comprehensive overview of the current state of protein-based carrier systems using electrospun fibers with special emphasis on discussing their potential and key challenges in developing such therapeutic strategies, along with a prospective view of anticipated future directions.

The full text of this article is available online at:

http://onlinelibrary.wiley.com/doi/10.1002/ardp.201700077/full

6.4. Monitoring Drug Release from Electrospun Fibers Using an *In Situ* Fiber-Optic System

Monitoring Drug Release from Electrospun Fibers Using an In Situ Fiber-Optic System.

S. Seif, F. Graef, S. Gordon, M. Windbergs.

Dissolution Technologies, 2016. 23(2). doi.org/10.14227/DT230216P6.

dx.doi.org/10.14227/DT230216P6

Monitoring Drug Release from Electrospun Fibers Using an In Situ Fiber-Optic System

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ABSTRACT

Electrospun fiber mats are currently gaining attention as advanced drug delivery systems. Dissolution testing for such systems is generally performed in small vials by immersing the fiber mats in buffered solutions. Defined aliquots of dissolution medium are withdrawn at predefined time points, and the dissolved drug is quantified. However, this procedure is associated with several drawbacks. The method is not automated, and as such requires manual sampling, which potentially leads to inaccuracies particularly in frequent sampling intervals as required for characterization of rapid drug release. Further, the sheet-like fiber mats tend to partially fold upon contact with the dissolution medium, which may potentially affect the release kinetics and reproducibility of the acquired release data.

In this study, we investigated the application of a fully automated fiber-optics based dissolution testing system for in situ monitoring of drug release from electrospun fiber mats. Electrospun poly(vinyl alcohol) fibers loaded with lysozyme were used as a model system. To prevent folding of the fiber mats and ensure a fixed position in the dissolution vessel throughout the experiment, a flexible adapter was developed to allow for the attachment of the fiber mats to the vessel walls. Lysozyme release from the fiber mats was compared with the release from cast films with the same composition. Even though the release processes were rather fast and differences in release kinetics of the two systems were marginal, the fiber-optics based dissolution setup allowed for the successful detection of released protein in both cases. The present study, therefore, highlights the potential for the utilization of fully automated fiber-optics based dissolution testing systems for advanced in situ monitoring of drug release from electrospun fibers.

KEYWORDS: Fiber optic; electrospun fiber mats; dissolution.

The full text of this article is available online at:

www.dissolutiontech.com/issues/201605/DT201605_A01.pdf

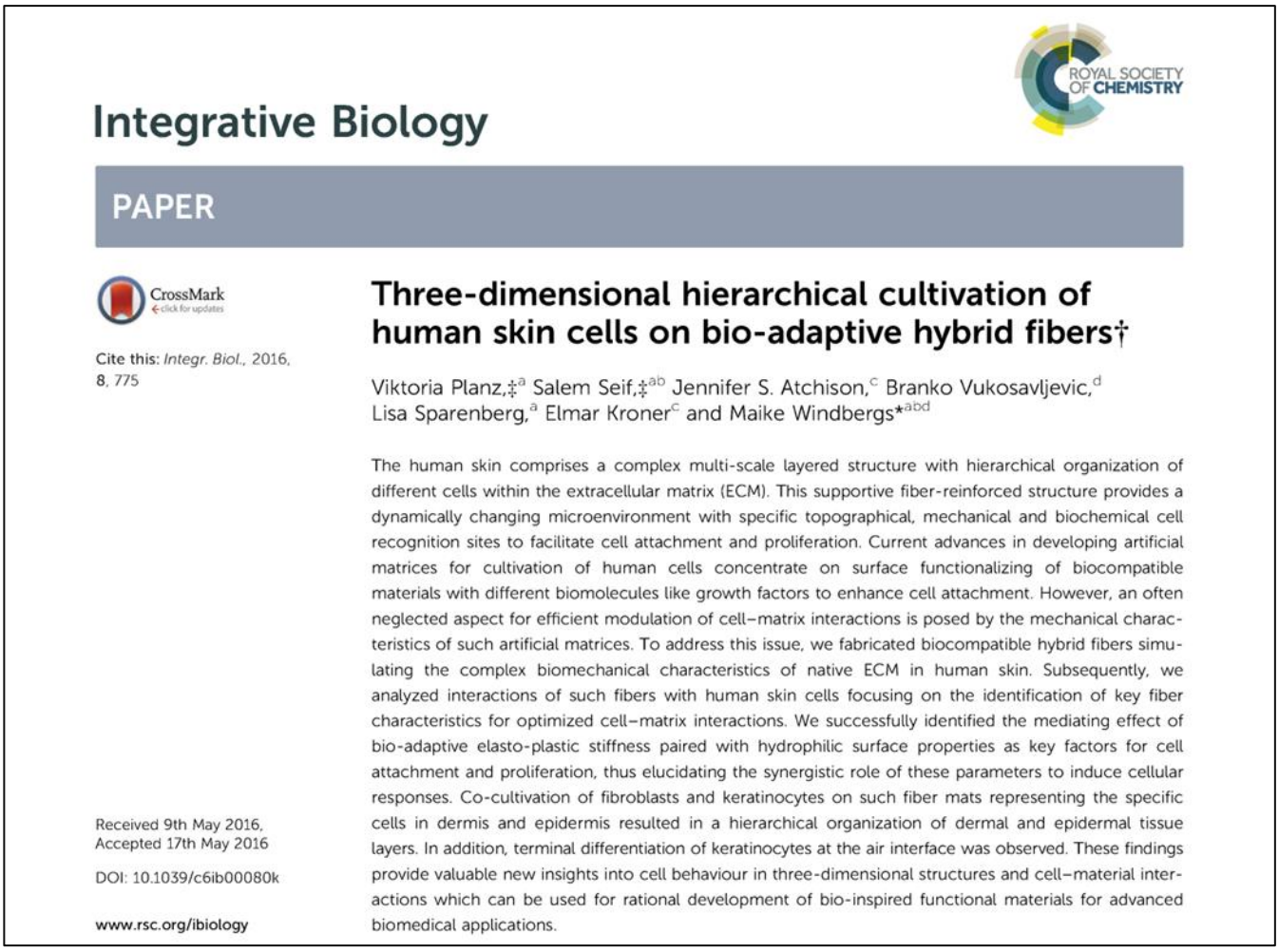
6.5. Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers

Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers.

V. Planz*, S. Seif*, J. Atchison, B. Vukosavljevic, L. Sparenberg, E. Kroner, M. Windbergs

Integrative Biology, 2016. 8: p. 775-784. DOI: 10.1039/C6IB00080K.

**These authors contributed equally to this work*



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Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers†

Viktoria Planz,‡[a] Salem Seif,‡[ab] Jennifer S. Atchison,[c] Branko Vukosavljevic,[d] Lisa Sparenberg,[a] Elmar Kroner[c] and Maike Windbergs*[abd]

The human skin comprises a complex multi-scale layered structure with hierarchical organization of different cells within the extracellular matrix (ECM). This supportive fiber-reinforced structure provides a dynamically changing microenvironment with specific topographical, mechanical and biochemical cell recognition sites to facilitate cell attachment and proliferation. Current advances in developing artificial matrices for cultivation of human cells concentrate on surface functionalizing of biocompatible materials with different biomolecules like growth factors to enhance cell attachment. However, an often neglected aspect for efficient modulation of cell–matrix interactions is posed by the mechanical characteristics of such artificial matrices. To address this issue, we fabricated biocompatible hybrid fibers simulating the complex biomechanical characteristics of native ECM in human skin. Subsequently, we analyzed interactions of such fibers with human skin cells focusing on the identification of key fiber characteristics for optimized cell–matrix interactions. We successfully identified the mediating effect of bio-adaptive elasto-plastic stiffness paired with hydrophilic surface properties as key factors for cell attachment and proliferation, thus elucidating the synergistic role of these parameters to induce cellular responses. Co-cultivation of fibroblasts and keratinocytes on such fiber mats representing the specific cells in dermis and epidermis resulted in a hierarchical organization of dermal and epidermal tissue layers. In addition, terminal differentiation of keratinocytes at the air interface was observed. These findings provide valuable new insights into cell behaviour in three-dimensional structures and cell–material interactions which can be used for rational development of bio-inspired functional materials for advanced biomedical applications.

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7. Curriculum Vitae

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Since Nov. 2011	PhD in Pharmaceutical Technology, Saarland University, Saarbrücken - Germany
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2004	Completed high school education, Al-Andalus school, Damascus - Syria

List of publications

S. Seif, V. Planz, M. Windbergs. Delivery of therapeutic proteins using electrospun fibers – recent developments and current challenges. *Archiv der Pharmazie*, 2017. 350. DOI: 10.1002/ardp.201700077.

S. Seif, V. Planz, M. Windbergs. Controlling the release of proteins from therapeutic nanofibers: the effect of fabrication modalities on biocompatibility and antimicrobial activity of lysozyme. *Planta Medica*, 2017. 83(05): p. 445-452. DOI: 10.1055/s-0042-109715.

V. Planz*, S. Seif*, J. Atchison, B. Vukosavljevic, L. Sparenberg, E. Kroner, M. Windbergs. Three-dimensional hierarchical cultivation of human skin cells on bio-adaptive hybrid fibers. *Integrative Biology*, 2016. 8: p. 775-784. DOI: 10.1039/C6IB00080K.
*These authors contributed equally to this work

S. Seif, F. Graef, S. Gordon, M. Windbergs. Monitoring drug release from electrospun fibers using an in situ fiber optics system. *Dissolution Technologies*,2016. doi.org/10.14227/DT230216P6.

S. Seif, L. Franzen, M. Windbergs. Overcoming drug crystallization in electrospun fibers – Elucidating key parameters and developing strategies for drug delivery. International Journal of Pharmaceutics, 2015. 478(1): p. 390-397. DOI: 10.1016/j.ijpharm.2014.11.045.

S. Seif, S. Hansen. Measuring the stratum corneum reservoir: Desorption kinetics from keratin. Journal of Pharmaceutical Sciences, 2012. 101 (10), p. 3718-3728.

Conference Contributions:

Oral presentations:

S. Seif, M Windbergs. Protein encapsulation and controlled release from electrospun fibers. 7th PSSRC annual symposium. Lille, France. 2013.

S. Seif, C.-M. Lehr. Working with proteins - from the kitchen to the lab. Old timer meeting of Prof. Dr. Hans Junginger. Marburg, Germany. 2013

Poster presentations:

S. Seif, M Windbergs. Tailor-made electrospun fibers for drug delivery and tissue engineering. Nano meets future “size matters 2015”. Saarbrücken, Germany. 2015.

S. Seif, M Windbergs. Towards the fabrication of electrospun fibers for controlled drug release: controlling the release kinetics by overcoming drug crystal formation. 1st European Conference on Pharmaceutics: Drug Delivery. Reims, France.2015

V. Planz, S. Seif, M. Windbergs. New perspectives for fabrication and analysis of biomimetic electrospun scaffolds for skin tissue engineering applications. Reims, France. 2015

V. Planz, S. Seif, L. Sparenberg, B. Vukosavljevic and M.Windbergs. Tracking the nature´s route – Electrospun fibers as biomimetic extracellular matrix for three-dimensional skin cell cultivation. 4th Galenus Workshop on Drug Delivery to Human Skin. Saarbrücken, Germany. 2015

V. Planz, L. Sparenberg, S. Seif, M. Windbergs. Establishment of a three-dimensional nanofibrous cell culture model for human skin wound analysis. 14th International Conference Perspectives in Percutaneous Penetration (PPP). La Grande Motte, France. 2014.

S. Seif, V. Planz, L. Sparenberg, M. Windbergs. Electrospun nanofibrous scaffold as biomimetic extracellular matrix substitute for three-dimensional skin cell cultivation. 4th HIPS Symposium on Pharmaceutical Sciences Devoted to Infection Research. Saarbrücken, Germany. 2014

S. Seif, M Windbergs. Investigating drug release from electrospun fibers for skin wound applications. 9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology (PBP). Lisbon, Portugal. 2014.

S. Seif, M Windbergs. Electrospun protein-loaded ultrafine fibers as novel drug delivery systems.3rd HIPS Symposium on Pharmaceutical Sciences Devoted to Infection Research. Saarbrücken, Germany. 2013.

S. Seif, C-M. Lehr, S. Hansen, Prediction of stratum corneum depot by means of desorption kinetics from keratin. Controlled release society German chapter annual meeting. Würzburg, Germany. 2012.

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