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Novel approach to use insoluble collagen type I for electrospinning of nanofibers and their characterization

Master Thesis

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Abstract

The emergence of nanotechnology and therefore the ability to study and produce nanoscale structures for regenerative medicinal applications has created big interest in the process of electrospinning. Electrospinning is able to form fibers in the nano- or microrange out of a suitable polymer solution. Hence the formation of biological scaffolds out of nanofibers which mimics extracellular matrix can be achieved. The extension of possible new polymers which can be electrospun is in full progress. Thus, in the herein work a novel biopolymer, insoluble collagen type I, is successfully electrospun into various structures such as round shaped, beaded or non beaded nanofibers with a diameter between 100 - 300 nm. The insoluble collagen was firstly solubilized using a proteolytic enzyme and blended with PEO. A protocol for fiber spinning was developed and the nanofibers are characterized using light microscope and SEM. Cross-linking experiments using glutaraldehyde vapor showed re-introduced biostability of the electrospun fibers, an important feature for future biomedical applications.

SDS-PAGE and FPLC experiments to analyse the polymer solution suggest that some fraction of the insoluble collagen with prolonging time of proteolytic treatment is cleaved even in the triple helical structure and not only in the telopeptide region as previous publications proposed.

Collagen nanofibers produced here in this work hold potential for various cell and tissue culture applications. Further experiments can be performed for assessing their ability to support growth of various cell types.

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Nomenclature

ECM	Extracellular matrix
SEM	Scanning electron microscopy
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
FPLC	Fast protein liquid chromatography
ES	Electrospinning
PLGA	Poly(lactic-co-glycolic acid)
PEO	Polyethylene glycol
ASC	Acid soluble collagen
PSC	(Acid) insoluble collagen
AA	Amino acid
ddH_2O	double distilled water
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
GA	Glutaraldehyde
AMCHF	Avitene^{TM} Microfibrillar Collagen Hemostat flour
PBS	Phosphate-buffered saline
RT	Room temperature
EDTA	Ethylenediaminetetraacetic acid
PTCS	Proteolytic treated collagen solution
SEI	Secondary electron imaging
WD	Working distance
GIMP	GNU imagine manipulation program
Tris	Tris(hydroxymethyl)aminomethane
APS	Ammonium persulfate
TEMED	Tetramethylethylenediamine
NA	Numerical aperture
PS	Polystyrene
PLC	Poly(caprolactone)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
CD	Circular dichroism
FTIR	Fourier transform infrared spectroscopy
DTT	Dithiothreitol

1 Introduction

Regenerative medicine is a branch of research providing novel therapies for replacing and restoring function to any organ as well as tissue within the human body. Working in an interdisciplinary research field including cell biology, molecular biology and tissue engineering many researchers try to force progress in supporting human body's own repair mechanisms.[1] Tissue engineering and 3D-printing approaches are the state of the art in regenerative medicinal applications. The emergence of nanotechnology and therefore the ability to study and produce nanoscale structures for the purpose of tissue regeneration, raised much attention in regenerative medicine[2] and led to a novel approach to produce nanomaterials, called electrospinning (ES). Loosely connected 3D porous scaffolds produced by electrospinning can mimic extra cellular matrix (ECM) and hence provide support for cells to regenerate new tissue.[3] The rise of ES in recent years is due to its simple and inexpensive setup (Figure 1) and its potential application in numerous fields like filtration membranes, fiber based sensors, catalytic nanofibers and tissue engineered scaffolds, for example in axonal regeneration.[4]

1.1 Electrospinning

Electrospinning (ES) is an application technique used to spin fibers out of a big variety of both natural and synthetic polymers in the nano- to micrometer range. It is a relatively old technique first observed by Rayleigh (late 1800s) and patented by Formhals[5] in 1934. This process of electrostatic fiber formation raised much attention in the last decade not only because of the usage of a big variety of polymeric substances as a source for ES but also because of the ability to consistently spin fibers in the submicron range. It would be hard to achieve by other fiber-spinning technologies. This advantage led to the usage of ES as a suitable technique for tissue engineering and therefore for regenerative medicine.[2]

1.1.1 Principle of electrospinning

In the process of ES, electrical repulsive forces are used to produce polymer fibers with diameters ranging from a few nanometers to several micrometers. Depending on the field of application both natural polymers, like silk fibroin or collagen as well as synthetic polymers, like poly(lactic-co-glycolic acid) (PLGA) are used for ES. The polymers are solubilized in a suitable solution. This solution is then subjected to a strong electrical field usually in the range between 1 to 30 kV.[6] The drop of polymer solution at the tip of the nozzle will become highly electrified. Due to the accumulation of charges on the drop surface the pendant drop will experience two major electrostatic forces, namely electrostatic repulsion between similar surface charges and Coulombic forces strived by the external electric field.[7] These forces give rise to a conical shaped drop, the so called Taylor cone.[8] As soon as the electrostatic forces exceed the surface tension of the polymer solution a charged jet of solution is ejected. As the jet is continuously elongated by whipping processes forming a thin and long thread the solvent is evaporated. Ruthledge *et al.*[9,10] stated that the high

frequency whipping of the jet, is due to three instabilities within the jet. The first instability, the "Rayleigh instability", is dominated by surface tension. The second and third instabilities, the two "conducting" modes, however, are dominated by electric forces. The resulting whipping causes bending and stretching of the ejected jet, which ultimately leads to diameter reduction from hundreds of micrometers to a few nanometers.

A grounded collector, e.g. a spinning mandrel is collecting the ejected thread (Figure 1).

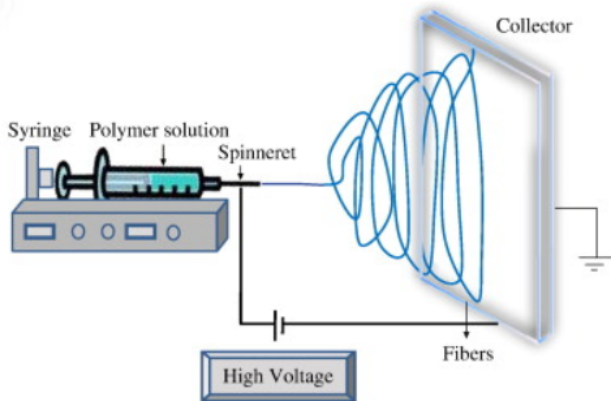

Figure 1: Illustration of an electrospinning setup: High voltage is applied to a nozzle filled with polymer solution. The solution is fed at a certain flow rate and the produced fibers are obtained at a grounded collector.[2]

Various parameters influence the process of electrospinning and therefore make the production of reproducible and uniform fibers more challenging. The parameters are classified in three branches: ambient parameters, process parameters and solution parameters. Ambient parameters including temperature and humidity can influence fiber diameter according to some studies.[11] Solution concentration, like polymer molecular weight or solution viscosity affects the morphology of electrospun fibers as well as processing parameters like applied voltage, feeding rate or tip to collector distance do. Diameter control of electrospun fibers by altering electrospinning parameters have been investigated in several publications.[12–14] According to these publications the major influence on fiber morphology has the polymer concentration, the electric field strength, the electrical conductivity of the solution and the feeding rate. While higher concentration and higher feeding rate seem to increase the diameter, higher applied voltage seems to decrease fiber diameter. However, the exact influence of one parameter is difficult to isolate since altering one parameter can affect other parameters (e.g. changing polymer molecular weight can also change the polymer solution viscosity).

The polymer of choice in the process of ES depends on the desired application. Different polymers provide the electrospun fibers with different properties and functions. Since these fibers are produced to mimic ECM components it would be desirable to use polymers which are neither inflammatory nor toxic but which are biodegradable and compatible with human tissue.[15] As mentioned earlier a big variety of natural as well as synthetic polymers have been studied as possible material for nanofiber spinning. Over the years a large number of polymers including

proteins,[16,17] nucleic acids[18] and even polysaccharides[19] have been successfully electrospun. To get more and more (bio-)polymers ready for electrospinning researchers are faced with some difficulties including limitation by their molecular weights or/and by their solubility. To surpass this issue an effective way is to blend polymers with other polymers which are well suited for ES, for example polyethylene glycol (PEO) or to modify the polymer using chemical reagents or enzymes to get the desired polymer solubilized.

Collagen type I, a fibrous protein of the ECM is a good example for electrospinning limitation. Acid soluble collagen (ASC) type I is heavily used in ES due to its favourable properties. But insoluble collagen (PSC) type I which accounts for roughly 95% of all human collagen type I, has not been used yet due to its insolubility. But since some studies, independently from ES purposes, described the dissolvation of insoluble collagen using proteolytic enzymes, this approach was taken to produce a polymer solution suitable for ES processes.

1.2 Collagen

About 25 % of the total body protein of vertebrate's accounts for collagen, and it is therefore the most abundant connective tissue protein in animals.[15] There are around 16 different types of collagen in the human body but 80 - 90 % of the body collagen consists of type I, II, and III. Beside the main function of structural support to the present tissue, it is also said to seclude important factors required for tissue regeneration and maintenance.[2] Its widespread occurrence, its favourable biochemical and biophysical properties like its biodegradability, its excellent biocompatibility, its low antigenicity and its haemostatic and cell-binding properties[20] are reasons for its extensively usage not only in electrospinning but also in regenerative medicine overall.[15]

1.2.1 Biophysical and biochemical properties of collagen type I

The basic molecular subunit is a rigid rod like structure, which is 2800 Å in length, 1.5 Å in width and has a molecular weight of 300 kDa. This tropocollagen called rigid rod consists of three coiled subunits: two $\alpha 1$ chains and one $\alpha 2$ chain, with each of this chains composing of around 1050 amino acids (AA). Together they wound around each other to form a right handed triple helical structure (Figure 2). The helical structure is due to a characteristic repeating motif: Gly-Pro-X (X for any AA) and a copiousness of hydroxyproline. The triple helix is stabilized by intramolecular hydrogen bonding built between the peptide bond NH of a glycine residue with a carbonyl group of an adjacent peptide chain. Furthermore, at both ends of the tropocollagen molecule non helical telopeptide regions exists (amino- and carboxytelopeptides).

Depending on which animal source the used collagen originates, the molecular weight of an α chain can differ strongly between 95 - 140 kDa. Physicochemical properties can vary strongly as well between different sources. Collagen monomeric chains (α chains) can form intermolecular cross-linking forming either dimers (β

chains) or even trimers (γ chains). From this results, that a collagen molecule can be composed of different subunits (α, β or γ chains) depending on its source.

Intermolecular interaction between short segments, the so called telopeptides, at both ends of the collagen chains lead to the formation of collagen fibrils. In this peptide region an unusual AA, the hydroxylysine forms with the help of the enzyme lysine oxidase and via an aldol cross reaction a covalent bond with another hydroxylysine of an adjacent collagen. This crosslinking is the reason for its (bio-)stability and its insolubility. It is so strong that only 5% of all collagen is soluble in acidic solutions and hence directly available for further biomedical applications like electrospinning.[21]

1.2.2 Collagen for electrospinning

The bigger portion of collagen (95%) has not been used for electrospinning applications yet. Although there are some publications which use proteolytic enzymes for collagen solubilization,[22,23] nobody has tried to use this solubilized collagen as base material for electrospinning in contrary to ACS which is a frequently used polymer. With ASC solutions it is already possible to form fiber scaffolds with different morphologies. Not only fiber diameter, from nano- to microscale can already be adjusted but also bead composition or porosity can be adjusted.[24–27] It can be used in different blends and its cytocompatibility and ability to support growth of cells has already been tested.[28] Often ASC is used in blends with PEO for electrospinning. PEO is a well examined polymer for electrospinning since it is non-toxic, in acidic solutions chemically stable and able to increase viscosity of the collagen solution.[7]

A second disadvantage of ASC, beside the small percentage in vertebrate, is the use of highly toxic 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) for ASC solubilization.

To bypass these problems an already known approach, meaning the proteolytic treatment of collagen, was executed for a novel method of electrospinning. Proteolytic enzymes break up the intermolecular bonding between collagens in the telopeptide region while leaving the basic triple helical structure intact.[22] It is known that several proteolytic enzymes like pepsin, papain, ficin, bromelain or pronase have been used for insoluble collagen solubilization. Therefore, in this thesis insoluble collagen was solubilized using pepsin and pronase. The aim was to get a spinnable collagen solution out of insoluble collagen modification.

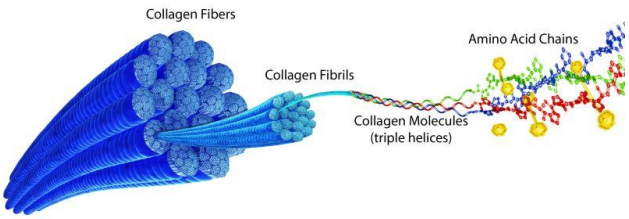

Figure 2: Illustrative drawing of collagen type I composition. From the smallest subunit, the α chains, whereupon three of which form a right handed triple helical structure, to cross-linked insoluble collagen fibers.[29]

1.2.3 Cross-linking by glutaraldehyde vapor

Potential regenerative medicinal or biomedical applications of electrospun collagen fibers requires a certain biostability in order to restore or replace tissue. If this property is not given, the fibers will be degraded before supporting damaged tissue long enough for regeneration. And since through proteolytic treatment of the collagen, exactly this biostability property is taken away, it has to be restored after successful electrospinning. One way and the most studied and employed chemical method of re-establishing cross-linking between the electrospun fibers is done by exposure of those fibers to glutaraldehyde (GA) vapor.[30,31] GA is able to cross-link collagen because the carbonyl groups of GA can covalently bind to an amide group of each collagen and therefore form a link between two collagen molecules (Figure 3). Several other chemical techniques to cross-link collagen-based fibers are available. Aldehydes, like formaldehyde or isocyanates,[32,33] like hexamethylene diisocyanate or genipin[34,35] could be used. A big disadvantage of the above mentioned agents, apart from genipin, is their high cytotoxicity. Genipin instead, which is a vegetal source derived chemical cross-linker, is an interesting potential replacement of GA because its low toxicity. To lower this potential toxicity of cross-linked fibers, the used chemical should be evaporated well after cross-linking.[36]

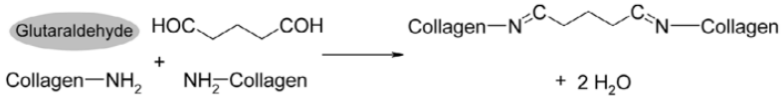

Figure 3: Schematic representation of cross-linking with GA vapor. Glutaraldehyde cross-links tropocollagen by binding covalently to an amide group of one tropocollagen and binding to a second amide group of another tropocollagen and hence link those two tropocollagen.[37]

1.3 Motivation for this project

Against this background, the prospective goal of this thesis was to establish a method/protocol for using a novel polymer as base material for electrospinning of nanofibers which can be used as biological scaffold for later axonal regeneration. Furthermore, fiber morphology and the influence of varying parameters are characterized.

Therefore, insoluble collagen type I was proteolytic treated to get it into solution. Further this process of collagen solubilization was optimized for the purpose of fastening and minimizing cost for this step. Out of this prepared solution nanofibers should further be electrospun and afterwards characterized using light microscopy, scanning electron microscopy (SEM) and cross-linking experiments. With these techniques fiber morphology as well as properties were identified. In order to test the proteolytic influence and hence the peptide composition of the prepared solution, sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) and fast protein liquid chromatography (FPLC) analysis was carried out.

2 Materials and Methods

2.1 Reagents

Pepsin (EC 3.4.23.1) and pronase (EC 3.4.24.4) were purchased from Sigma Aldrich Switzerland. 1,1,1,3,3,3-hexafluor-2-propanol (HFIP) and PEO (average M_w: 900 kDa) were purchased from Sigma Aldrich Switzerland as well. All other chemicals were either in the Guzman Lab, if not otherwise labelled, or purchased from chemical suppliers. Double distilled water (ddH_2O) 18.2 MΩ-cm was collected from PURELAB® Ultra (ELGA LabWater). Glass vials (with plastic snap-cap, 5 mL) were purchased from VWR International. Avitene™ Microfibrillar Collagen Hemostat flour (AMCHF, purified bovine corium collagen) was purchased from Bard Davol Inc. All required equipment was either available in the DBM facility, the pathology facility or the Department of Chemistry facility.

2.2 Prepare electrospinning solution

2.2.1 Non proteolytic treatment of collagen type I

In pursuance of solubilize Avitene™ Microfibrillar Collagen Hemostat flour, different amounts of this collagen flour was tried to be dissolved in the following chemicals or the mixture of following chemicals according to previous publications: HFIP (Sigma Aldrich), acetic acid (Sigma Aldrich), sodium acetate (Mallinckrodt Pharmaceuticals) or Phosphate-buffered saline (PBS; gibco Thermo Fisher Scientific).[38–43] Therefore, different amounts of collagen were weighed (Mettler AT261 Delta Range) and added to vials filled with varying solutions, which can be checked in Table 1. The solutions were kept at room temperature (RT) and stirred for 24 - 72 h. After this time period, the solutions were examined to find out, whether the collagen flour got dissolved or not.

Collagen % w/v	Chemicals	Ratio
12	Acetic acid:ethyl acetate:ddH_2O	4:3:3
19	PBS 10X:EtOH	1:1
6, 8, 10, 14	HFIP	Pure
0.1, 0.5, 10	Sodium acetate in acetic acid	0.65 M, pH 3.0
0.1, 1	Acetic acid	0.1 M
0.5	Acetic acid	0.2 M/0.5 M/1 M
0.5	HFIP:acetic acid	1:1/3:1/1:3/5:1/1:5

Table 1: List of tested non proteolytic treatment of AMCHF. In all tested conditions, which were tried out according to strategies of previous published publications, AMCHF did not dissolve, therefore another way of collagen solubilization had to be addressed.

2.2.2 Proteolytic treatment of collagen type I

In order to come up with a method to bring the insoluble collagen into solution and optimize this process, a big variety of collagen concentrations ($x\%\,w/v$),

enzyme/polymer ratios and conditions were tested. The first tried collagen concentrations were based on protocols of previous publications and patents, but since they were not made for later electrospinning processes, the protocols were altered to the project needs.

When the proteolytic enzyme pepsin, an enzyme with activity in the acid pH range, was used, AMCHF was added in different amounts (80 - 200 mg) per vial to 950 μL of a 10 mM aq. HCl (Sigma Aldrich) + 40 mM $CaCl_2$ (Mallinckrodt Pharmaceuticals) solution (pH 1.8) in each vial. The resulting mixtures were stirred for an hour at RT. Lyophilized salt free pepsin was reconstituted in 50 μL of the same 10 mM aq. HCl + 40 mM $CaCl_2$ solution as mentioned before. A varying amount of pepsin in each 50 μL was reconstituted to get different final enzyme/polymer ratios (0.1% $^{w}/_{w}$ - 2% $^{w}/_{w}$). The reconstituted pepsin was then added to the different concentrated polymer solutions to yield a final volume of 1 mL. Hence, collagen solution with concentration between 8 - 20% $^{w}/_{v}$ containing 0.1 - 2% pepsin by weight of dry collagen were produced. It was stirred for several hours (4 - 23 h) at RT. As soon as the polymer solution was transparent, meaning no visible debris and therefore all collagen dissolved, the solution was neutralized, to inactivate the enzyme, using 10 M NaOH aq. (Sigma Aldrich) solution until pH 7 was reached and the vials were placed at 4 °C until further use. The time periods, from enzyme injection to complete collagen dissolvation were noted and compared with each other and the pronase case, with relation to time duration and used enzyme/polymer ratios.

The second proteolytic enzyme examined was pronase. Pronase is a mixture of several nonspecific endo- and exoproteases with optimal activity in the neutral pH range. Similarly as described above, AMCHF (80 mg) was added to 950 μL of a 40 mM $CaCl_2$ (pH 6.2) solution in a vial. The resulting mixtures were stirred for an hour at RT. Lyophilized pronase was reconstituted in 50 μL of the same 40 mM $CaCl_2$ solution as recently mentioned. The reconstituted pronase was then added to the different concentrated polymer solutions to yield a final volume of 1 mL. Hence, collagen solutions with a concentration of 8 $^{w}/_{v}$ containing 0.1 - 2% pronase by weight of dry collagen were produced. It was stirred for several hours (4 - 23 h) at RT. As soon as the polymer solution were transparent, meaning no visible debris and therefore all collagen dissolved, ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA tetrasodium salt hydrate; Sigma Aldrich) was added to each vial in an amount equimolar to the calcium ion present and the vials were placed at 4 °C until further use. The time periods, from enzyme injection to complete collagen solubilization were noted and compared with each other and the pepsin case, with relation to time duration and used enzyme/polymer ratios.

2.2.3 PEO preparation for collagen/PEO blends

To increase the viscosity of the proteolytic treated collagen solution (PTCS) a 5% $^{w}/_{v}$ PEO (Sigma Aldrich) solution was prepared. Therefore, 50 mg of PEO was added to 1 mL double-destilled water (ddH_2O). The mixture was stirred overnight at RT till the PEO was dissolved. This prepared solution was used for blending with PTCS in different collagen/PEO ratios (Table 4).

2.3 Electrospinning

With regard to develop a novel approach for electrospinning using insoluble collagen not only the solution parameters had to be adjusted in a more or less trial and error procedure but also several other parameters, like processing or ambient parameter needed to be calibrated. Therefore, a big variety of conditions and condition combinations were tested. While only the brief general experimental procedure is described here, the exact protocol and tried conditions can be checked in Table 2 - 4. Before the electrospinning of collagen/PEO blends, electrospinning of PEO mixed with water was tested, to slowly approach conditions which would work and not waste too much PTCS.

Condition No.	PEO [% w/v]	ddH_2O [% v/v]	Ratio	Mandrel speed [rpm]	Feeding rate [mL/h]	Voltage [+kV/-kV]	Temp. [°C]	Humidity [%]
I.1	5	100	3:2	100	2	16/-2	24.8	31
I.2	5	100	3:2	500	2	16/-2	24.8	31
I.3	5	100	2:3	100	2	20/-2	24.8	31
I.4	5	100	2:3	500	2	20/-2	24.8	31

Table 2: Table of conditions tried with only blends of PEO with ddH_2O. This was carried out to slowly approach conditions which would work and not waste to much PTCS. 5% $^{w}/_{v}$ PEO solution was blended either 3:2 or 2:3 with ddH_2O. Feeding rate was set to 2 $^{mL}/_{h}$, applied high voltage to either 16 or 20 kV and mandrel speed to either 100 or 500 rpm. Humidity and temperature could not be varied since the electrospinning setup lacked a climate chamber.

The foregoing prepared PTCS (Section 2.2.2) were filled into a syringe (5 mL BD syringe Luer-Lok^{TM} tip; Becton Dickinson) mounted to a syringe pump (Single-syringe infusion pump 115 VAC; Cole-Parmer) and extruded at defined feeding rates (0.4 - 1.8 $^{mL}/_{h}$) at ambient conditions through a metal blunt tipped needle(1,1 x 0.25; 0.6 mm inner diameter). The needle was connected to the syringe via a hose (1 mm inner diameter). Temperature, pressure and humidity, each factors which are said to have impacts on fiber morphology, could not be influenced, since the used system did not have an environmental chamber. The blunt tipped needle itself was mounted to the electrospinning apparatus (Electrospinning machine EC-CLI; IME Technologies) and high voltage between 15 kV - 25 kV was applied to the needle tip. Electrospun fibers were collected on aluminum foil (Alumella, Alu-Vertriebsstelle AG) and on glass coverslips ($\oslash$ 22 μm, Fisher Scientific) which were both mounted to a turning negatively charged (-2 kV) mandrel. The speed of the turning mandrel was set between 100 - 1000 rpm in order to examine the impact of mandrel speed to the alignment of the electrospun fibers. Each tested condition (Tables 2 - 4) was preset on the electrospinning setup. Feeding rate was adjusted directly at the syringe pump, while applied voltage and mandrel speed could be inserted directly into the software of the EC-CLI. Before each new condition the collecting material was dismounted from the mandrel and exchanged with new collecting material. Deposition of eventual electrospun fibers was lasted for 3 - 5 min. All tested conditions were examined under light microscope as well as under SEM.

Condition No.	Collagen [% w/v]	Mandrel speed [rpm]	Feeding rate [mL/h]	Voltage [+kV/-kV]	Temp. [°C]	Humidity [%]
VII.17	8	100	0.8	20/-2	28.2	44
VII.18	8	100	1.8	20/-2	28.2	44
VIII.1	16	100	0.4	20/-2	24.9	55
VIII.2	14	100	0.8/0.4/2.8	22/-2	24.9	55
IX.1	10	100	0.80	22/-2	24.5	53
X.1	20	100	0.8/1.8	22/-2	24.5	53
XI.1	15	100	0.8	22/-2	24.5	53
XII.1	12	100	0.8	20/25/-2	24.5	53
XII.2	12	100	0.8	16/-2	24.5	53
X.1	14	100	0.8	16/-2	25.2	66
XIII.2	14	100	0.8	20/25/-2	25.2	66
XIII.3	14	100	1.8	25/-2	25.2	66
X.2	20	100	0.8	16/-2	25.2	66
X.3	20	100	0.8	25/-2	25.2	66
XII.3	12	100	0.8/1.8	14/16/18/25/-2	25.2	66
XIV.1	11	100	0.8/1.8	16/-2	25.2	66

Table 3: Table of conditions tried with only pure PTCS. Collagen concentration was varied from 8 - 20% w/v. Feeding rate was varied at 0.4/0.8/1.8 mL/h and applied high voltage between 14 - 25 kV. Mandrel speed was not varied since it has no influence on the actual electrospinning process. Humidity and temperature could not be varied since the electrospinning setup lacked a climate chamber.

Condition No.	Collagen [% w/v]	PEO [% w/v]	Ratio	Mandrel speed [rpm]	Feeding rate [mL/h]	Voltage [+kV/-kV]	Temp. [°C]	Humidity [%]
III.0	8	5	1:2.5	100	1.8	16/-2	25.5	49
III.1	8	5	1:2.5	100	1.8	20/-2	25.5	49
III.2	8	5	1:2.5	1000	1.8	20/-2	25.5	49
III.3	8	5	1:2.5	1000	0.8	20/-2	25.5	49
III.4	8	5	1:2.5	100	0.8	20/-2	25.5	49
IV.5	8	5	2.5:1	100	1.8	20/-2	26.2	47
IV.6	8	5	2.5:1	1000	1.8	20/-2	26.2	47
IV.7	8	5	2.5:1	1000	0.8	20/-2	26.2	47
IV.8	8	5	2.5:1	100	0.8	20/-2	26.2	47
XV.1	2	5	1:1	500	0.8	20/-2	24.6	56
XV.2	2	5	1:1	1000	1.8	20/-2	24.6	56
XV.3	2	5	1:1	1000	6	18/-2	24.6	56
XVI.1	2	5	1:2	1000	1.8	18/-2	24.6	56
XVI.2	2	5	1:2	1000	0.8	20/-2	24.6	56
XVII.1	6	5	4:1	1000	0.8	20/-2	24.6	56
XVII.2	6	5	4:1	1000	1.8	20/-2	24.6	56

Table 4: Table of conditions tried with collagen/PEO blends. Humidity and temperature could not be varied since the electrospinning setup lacked a climate chamber.

2.4 Scanning electron microscopy

In order to characterise the shape and diameter of the fibers, SEM images were taken. Therefore, the nanofibers which were electrospun on aluminum foil were scanned using a SEM. The aluminum foil was cut into approx. $1x1$ cm squares and placed on specimen stages using double sided adhesive tape. The sample were sputtered (high vacuum-coater Leica EM ACE600, Leica Microsystems) 15 min with gold particles to a final film thickness of 10.48 nm. All specimen were analysed under a scanning electron microscope (Nova NanoSEM 230, FEI Company) operating at 15 kV in secondary electron imaging (SEI) mode. High resolution morphological images at three different magnifications, low (2000x), medium (5000x) and high (30000x), were digitally recorded and analysed with Fiji-ImageJ software. Working distance (WD) and exact magnification are depicted in each SEM micrograph separately.

2.5 Image analysis

SEM images of electrospun fibers were processed and analysed using Fiji-ImageJ software (Wayne Rasband). Fiber morphology analysis, like diameter, shape and alignment were conducted using Fiji-ImageJ freehand measurement tools. Statistical analysis was carried out using OriginPro 2017G software (Data Analysis and Graphing Software; OriginLab). All figures in this thesis are edited with GNU image manipulation program (GIMP).

2.6 Cross-linking

With regard to re-establish covalent cross-linking between collagen molecules and therefore make electrospun fibers again biostable after proteolytic treatment, the fibers are cross-linked by exposure to glutaraldehyde (GA) vapor.[27] Furthermore, the cross-linking should show that not only PEO gets electrospun out of the collagen/PEO blends. So glass coverslips and aluminum foil covered with electrospun fibers were placed on a ceramic grid inside a desiccator and exposed for 24 h at RT to the vapor of an 25% $^{v}/_{v}$ aq. solution of GA (glutaraldehyde solution 25%; Fisher Chemical). After this treatment the specimen were dried at ambient conditions for 2 h and afterwards submerged in ddH_2O for 4 h at RT. The impact of this submerge to the morphology of the fibers was analysed by light microscope and SEM.

2.7 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.[44] The gels were prepared to final polyacrylamide concentrations between 7% - 10% of the seperating gel and 4% of the stacking gel.

Therefore, for the 10% polyacrylamide separating gel, 1250 µL of a 40% polyacrylamide/bis solution (OmniPur Calbiochem), 2500 µL ddH_2O, 1250 µL of a 1.5 M tris(hydroxymethyl)-aminomethan (Tris) solution (pH 8.8), 50 µL of a 10% $^{w}/_{v}$ ammonium persulfate (APS; Sigma Aldrich) solution and 5 µL of tetramethylethylenediamine (TEMED; OmniPur Calbiochem) were mixed together to a final volume of 5 mL and final concentration of 10% polyacrylamide. This mixture was gently swirled and pipetted - while avoiding introducing air bubbles - into the space between two plates which were hold together by green holders and mounted on a rack. The surface was covered carefully with ddH_2O and the gel was let polymerized for 30 - 45 min.

For the 7% polyacrylamide separating gel, the volumina of each substance was adjusted accordingly. 875 µL of a 40% polyacrylamide/bis solution, 2750 µL ddH_2O, 1250 µL of a 1.5 M Tris solution (pH 8.8), 50 µL of a 10% $^{w}/_{v}$ APS solution and 5 µL of TEMED were mixed together to a final volume of 5 mL.

For the stacking gel 620 µL of a 40% polyacrylamide/bis solution, 3130 µL ddH_2O, 1250 µL of a 1.5 M Tris solution (pH 6.8), 50 µL of a 10% $^{w}/_{v}$ APS solution and 5 µL of TEMED were mixed together to a final volume of 5 mL. After blotting off

excess of water with filter paper and gently swirling of the above prepared stacking gel mixture - the mixture was pipetted, while avoiding introducing air bubbles - between the two plates on top of the polymerized separating gel, up to just below the edge of the shorter plate. A 15 well spacer was carefully put in place and the gel was let polymerized for 30 - 45 min.

After complete polymerization, the gel was mounted into a electrophoresis chamber (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell; BioRad Laboratories Inc.), Laemmli running buffer (3 g Tris {Trizma® Sigma Aldrich}, 14.4 g glycine {Sigma Aldrich} and 1 g SDS {OmniPur Calbiochem} dissolved in 1000 mL ddH_2O) was added up to the marked line and the samples which have to be separated were filled into the gel wells.

The samples out of the PTCS were prepared according to a self developed protocol (see A.1.1). The SDS-PAGE samples were prepared to reach a final concentration of 40 $\mu g/_{well}$ digested collagen, 2 $\mu g/_{well}$ pepsin and 10 $\mu g/_{well}$ ASC. The SDS-PAGE samples than were shaken at 500 rpm and heated to 95 °C (Thermomixer C; Eppendorf) for 10 min, cooled to RT and 16 μL were injected into each sample well and electrophoresed, along with a molecular weight marker (Precision Plus Protein™ Dual Color Standarts; BioRad #161073). The gel was electrophoresed using a high-current power supply (PowerPac™ HC High-Current Power Supply) for 40 - 60 min at 30 - 40 V until the dye front reaches the stacking gel/separating gel interface. After this point the voltage was increased to 100 - 110 V until the dye front reaches the end of the gel. The gel was dismounted from the electrophoresis apparatus and subsequently fixed by an already prepared fixing solution (50% MeOH, 40% ddH_2O and 10% glacial acetic acid) for 30 min to overnight at low speed on a shaking plate. Afterwards the fixing solution was supplanted with a previously prepared 0.1% Coomassie® Brilliant Blue R-250 dye (Merck) solution (0.1% Coomassie® Brilliant Blue R-250 dye, 50% ddH_2O, 40% MeOH and 10% glacial acetic acid) similar as described by Faribanks *et al.*[45] and shaked again. After around 30 - 45 min the staining solution was replaced by a destainining solution (50% MeOH, 40% ddH_2O and 10% glacial acetic acid) on a shaking plate for 30 - 60 min depending on how fast destaining occured. The destained gels were scanned analysed and kept at 4 °C in ddH_2O.

2.8 FPLC

Pepsin treatment influence on collagen was examined with fast protein liquid chromatography (FPLC). PTCS with a concentration of 8% $^{w}/_{v}$ and 0.25% pepsin by weight of dry collagen, from different timepoints (3 h, 4 h, 5 h, 6 h, 7 h) were diluted 1:2 with a 50 mM phosphate buffer pH 7 to inactivate the pepsin, filtered (Millipore GSWP 0.22 μm filter) and 50 μL injected into the ÄktaFPLC (GE Healthcare Life Sciences). The sample was pumped (Pump P-920; GE Healthcare Life Sciences) with a speed of 0.15 $^{mL}/_{min}$ through a separating column (Superose^{TM} 6 Increase 3.2/300 column) and UV-Vis spectra's (Monitor UPC-900; GE Healthcare Life Sciences) were taken. Graphical analysis of the taken spectra's were carried out using OriginPro 2017G software and Microsoft Excel software (Data Analysis and Graphing Software; OriginLab).

2.9 Light microscopy

Electrospun nanofibers were observed using an upright light microscope (BX43 Upright Microscope; Olympus Life Science) with its corresponding software cell Sens (Olympus Life Science) and a digital imaging setup (DP73 color camera; Olympus Life Science). Objectives of 10X (numerical aperture [NA] 0.40), 20 (NA 0.75), 40X (NA 0.95) and 60X (NA 0.90) magnification were taken.

3 Results and discussion

In the herein section the results of the asked questions are addressed and discussed. The process of solubilize insoluble collagen using a proteolytic enzyme was optimized concerning solubilization time and material cost. Further it could be shown that this prepared solution can be used for electrospinning purposes. Electrospinning of a solution into fibers proved to be a challenging task, since a lot of parameters, including solution, process and ambient parameters influence fiber spinning. A variety of conditions had to be tested and analysed to slowly attain successful electrospun fibers. Out of the findings of these tested conditions, a method was stated with the exact values or range of values of parameters where fibers were obtained. Characterization of those obtained fibers was carried out using light microscopic images, SEM micrographs and cross-linking experiments. SEM micrographs revealed that electrospun fibers were in the submicron range between 125 - 275 nm, round shaped, continuous and depending on the tested condition beaded or non beaded. SDS-PAGE and FPLC revealed that the collagen is solubilized by excision of the telopeptide regions during proteolytic treatment. Nevertheless, in contrary to previous publications, a certain portion is digested more leading to the assumption that pepsin can intrude the tight triple helical structure. However, cross-linking experiments of electrospun fibers displayed that the integrity of the collagen structure appears to be preserved even after proteolytic treatment, since covalent bonding and therefore re-establishing insolubility still is possible.

3.1 Proteolytic treatment of collagen for ES solution preparation

In the process of method development it distilled out that pepsin is the enzyme to use. It is not only faster in solubilizing collagen type I but also needs a lower enzyme/polymer ratio to do that. This faster solubilization with pepsin is due to higher specific activity of pepsin, which has 2500 $^{\text{units}}/_{\text{mg}}$ while pronase only has 7 $^{\text{units}}/_{\text{mg}}$. Another reason could be, that different proteolytic enzymes cleave at different sites of the telopeptide[23] and this sites are variably accessible for the enzyme to cleave.

A percentage of 0.25% pepsin by weight of dry collagen crystallized out to be the best adjustment to fulfil the need of the project. 6 - 7 h of solubilization time seemed to be appropriate. Higher ratios would have been faster but were more cost intensive due to higher pepsin amount. Lower pepsin amount would have increased the solubilization time to an impractical duration (Figure 4).

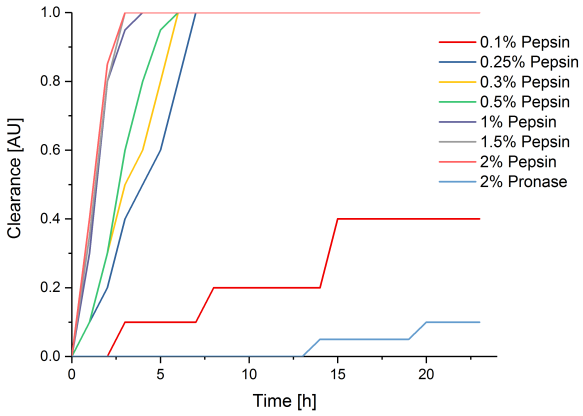

Figure 4: Graphical representation of proteolytic collagen treatment. The influence of different pepsin and pronase percentages by weight of dry collagen. While pronase needs almost 72 h for reaching full transparency, collagen dissolvation with pepsin occurs faster. 1 - 2% pepsin by weight of dry collagen reaches full transparency already after 3 h which is unnecessarily fast and uses a lot of pepsin. 0.25% appears to be the best with regard to the amount of time and the amount of needed pepsin.

3.2 Method for fiber electrospinning and their characterization

Electrospinning of pure collagen type I solutions was not possible. With no tried condition (Table 3) fibers could be obtained. The solution just dropped out of the blunt tipped needle or at most electrospraying could be observed. This failure of fiber electrospinning is probably due to the low viscosity of the PTCS.

Since with no examined pure collagen type I concentration (8% w/v - 20% w/v) fibers could be electrospun, blendings of collagen type I and PEO for further electrospinning were used (Table 4). After the addition of PEO to the enzymatically treated collagen solution, shortly before the electrospinning process, fiber formation could be observed. A solution with an amount of 8% w/v collagen type I blended with 5% w/v PEO in the ratio of 1:2.5 yielded beadless, round shaped fibers in the diameter range between 173 nm and 273 nm (Figure 5). A second solution with 8% w/v collagen type I blended with 5% w/v PEO in the ratio of 2.5:1 yielded beaded, round shaped fibers in the diameter range between 124 nm and 173 nm (Figure 6). This diameter measurements indicate that lower feeding rate leads to smaller fiber diameter at least in the PTCS with higher PEO concentration (Cond. III.0 - III.4). A second indication is that lower PEO concentration (Cond. IV.5 - IV.8) give rise to smaller fiber diameter (Figure 7).

Condition No.	Collagen [% w/v]	PEO [% w/v]	Ratio	Mandrel speed [rpm]	Feeding rate [mL/h]	Voltage [+kV/-kV]	Temp. [°C]	Humidity [%]
III.1-4	8	5	1:2.5	100/500/1000	0.8/1.8	20 /-2	25.5	49
IV.5-8	8	5	2.5:1	100/500/1000	0.8/1.8	20/-2	26.2	47

Table 5: List of conditions and the corresponding parameter values under which fiberspinning was successfully carried out. Applied voltage has to be set to exactly 20 kV. Deviations from this value, did not show any reproducible electrospinning. Feeding rate can either be preset to 0.8 mL/h or 1.8 mL/h, at which lower feeding rate appeared to lead to overall smaller fiber diameter. Increased mandrel speed increased the fiber alignment but does not seem to have an impact on fiber diameter or beading. Ambient conditions should be considered as well.

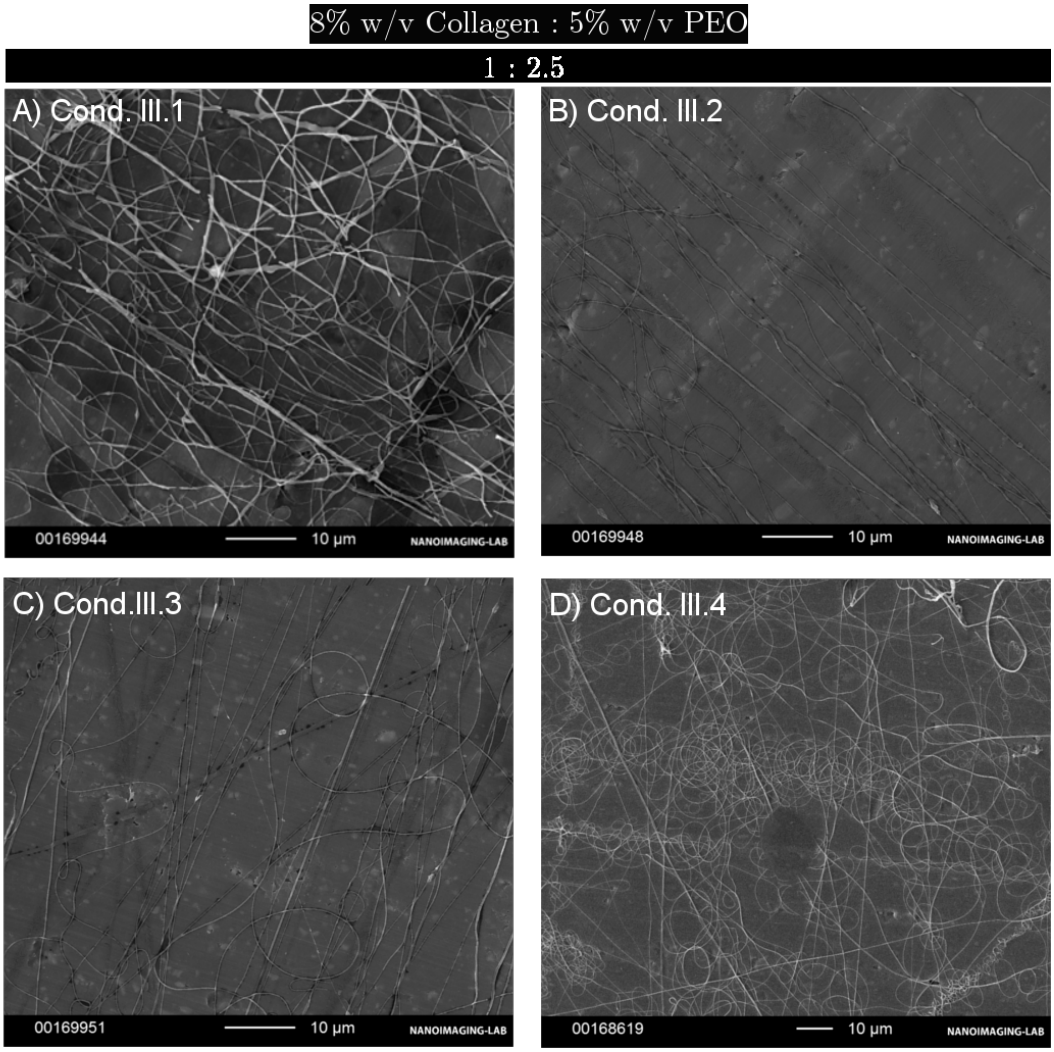

Figure 5: SEM micrographs of all conditions for successful fiber electrospinning out of a 1:2.5 (8% w/v collagen solution: 5% w/v PEO solution) blend. Image A (Cond. III.1), image B (Cond. III.2), image C (Cond. III.3), and image D (Cond. III.4) displays non beaded, round shaped electrospun fibers with an average fiber diameter of 273 nm, 231 nm, 173 nm, and 192 nm, respectively (15 kV for images A - D, WD (Image A, B) = 6.7 mm, WD (Image C) = 10.6 mm, WD (Image D) = 11.0 mm. Higher magnified micrographs of all conditions are displayed in A.3.

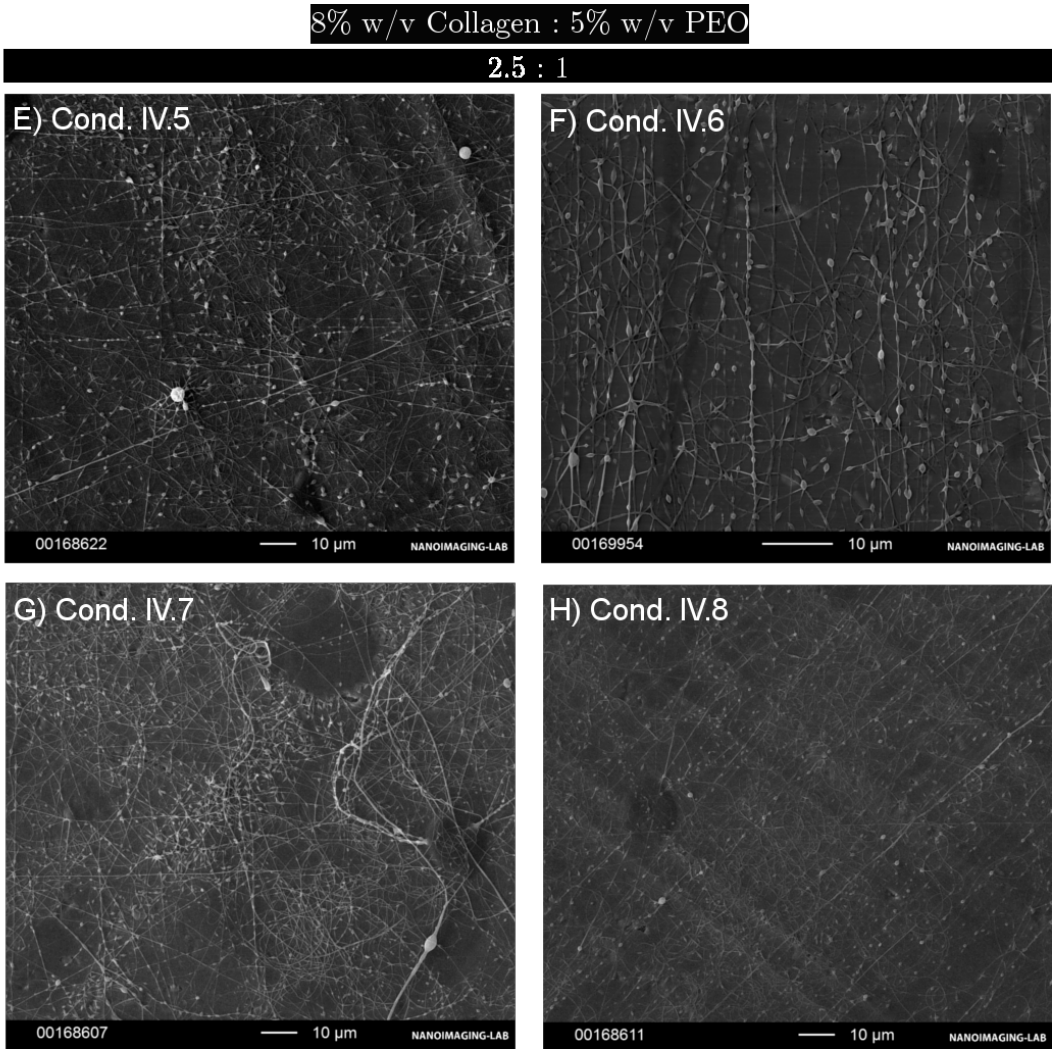

Figure 6: SEM micrographs of all conditions for successful fiber electrospinning out of a 2.5:1 (8% w/v collagen solution: 5% w/v PEO solution) blend. Image E (Cond. IV.5), image F (Cond. IV.6), image G (Cond. IV.7), and image H (Cond. IV.8) displays beaded, round shaped electrospun fibers with an average fiber diameter of 173 nm, 124 nm, 124 nm, and 159 nm, respectively (15 kV for images E - H, WD (Image E) = 11.0 mm, WD (Image F) = 6.8 mm, WD (Image G) = 11.4 mm, WD (Image H) = 11.3 mm). Higher magnified micrographs of all conditions are displayed in A.3.

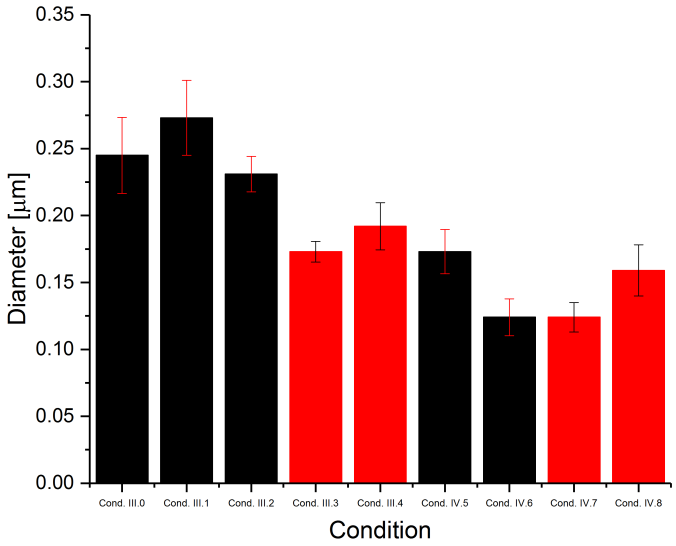

Figure 7: Graphical representation of average fiber diameter for conditions III.0 - IV.8. It appears that lower feeding rate (red bars) leads to smaller fiber diameter at least in the PTCS with higher PEO concentration (Cond. III.0 - III.4). Furthermore, lower PEO concentration (Cond. IV.5 - IV.8) appears to lead to smaller fiber diameter.

PEO blends were necessary because blending with PEO increased the viscosity of the used PTCS and made forming of fibers during electrospinning possible. Fine tuning of this concentration is necessary as well, since too high PEO concentration and therefore too high viscosity prohibited spinning and too low PEO concentration and therefore too low viscosity led to bead formation (Figure 6) or at very low PEO concentration to electrospraying. Bead formation is said to be a result of low viscosity of the electrospun collagen solution. Increasing the viscosity using PEO increases the surface tension of the solution and hence prevents the capillary breakup of the jet.[26]

Bead characterization using SEM micrographes and Fiji-ImageJ software yielded that beads from a 2.5:1 (8% $^{w}/_{v}$ collagen solution: 5% $^{w}/_{v}$ PEO solution) had an average diameter of 0.725 μm in Cond IV.5, 0.751 μm in Cond. IV.6, 0.623 μm in Cond. IV.7 and 0.662 μm in Cond. IV.8 (Figures 8 and 9). It can be concluded that lower feeding rate (Cond. IV.7/IV.8, 0.8 $^{mL}/_{h}$) leads to smaller bead diameter. Since these beads were not very uniform within one condition, this conclusion has to be treated with caution. The shapes of the beads are spherical to spindle-like.

Considering that bead formation is not uniform even within a tested condition, it can only be observed but not statistically displayed that bead formation in electrospun fibers appeared to follow a certain pattern. Bead spacing and bead shape seems to be related to the overall fiber diameter, meaning that thinner fibers have a shorter distance between the beads and the shape of the beads changes from spherical to spindle-like when fiber diameter is getting thicker. This would correspond to previous publications, where the correlation between bead spacing/shape and fiber diameter is reported.[46,47] Correlation between bead diameter and fiber diameter was not observed.

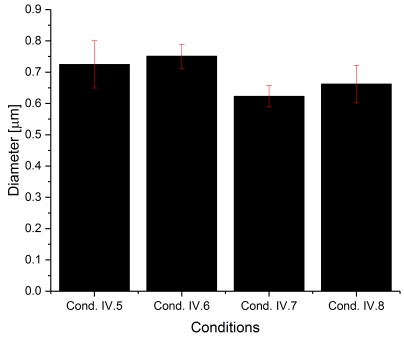

Figure 8: Graphical representation of average bead diameter for conditions IV.5 - IV.8. Conditions can be checked in Table 4. It seems like lower feeding rate (0.8 $^{mL}/_{h}$) in Cond. IV.7/IV.8 yields overall smaller bead diameter compared to higher feeding rate (1.8 $^{mL}/_{h}$) in Cond. IV.5/IV.6.

Besides the solution parameters, process parameters were adjusted as well. The values for feeding rate [$^{mL}/_{h}$], applied voltage [kV] and mandrel speed [rpm] were set like following during successful electrospinning: Applied voltage [kV] was set to 20 kV, feeding rate [$^{mL}/_{h}$] was set to two values, namely 0.8 $^{mL}/_{h}$ or 1.8 $^{mL}/_{h}$ and mandrel speed [rpm] to 100, 500 or 1000 rpm (Table 5). Deviation from 20 kV does not yield any fibers, only in Cond. III.0, a condition which was not reproducible afterwards. Whereas it seems that lower feeding rate leads to overall smaller fiber diameter especially in the conditions tried with higher PEO concentration (Cond. III.1-III.4) and smaller bead diameter. Mandrel speed [rpm] clearly led to higher alignment of fibers (Figure 10). With even higher mandrel speed alignment is assumed to be increased further. If at one point the mandrel speed is to high and the ejected jet is broken upon collection on it, has to be verified in an additional study. The successful increase of electrospun fiber alignment is an important property for future medical assignments. Biomedical applications sometimes demands alignment of supporting tissue, if for example the tissue, which has to be regenerated needs some form of guidance or if the fiber alignment has to mimic this tissue.

To get to these parameters, a lot of adjustment and fine tuning work was carried out. Further optimization of these parameters to improve fiber morphology with respect to smaller diameter, more or less beads or more alignment would have gone beyond the scope of this thesis. Furthermore, ambient parameters like humidity and temperature couldn't be adjusted due to a missing climate chamber, which was not applied to the electrospinning setup. The values for humidity and temperature during the process were still written down, because according to previous publications these parameters show influence in fiber morphology and successful electrospinning.

There would be several other important parameters which have to be addressed but in the course of this thesis fine tuning of the most important parameters turned out to be more time consuming than it appeared in the beginning. Successful electrospinning requires not only fine tuning of different parameters but also the interplay of these parameters needs to be adjusted. Slight variation of one parameter could impede formation of fibers.

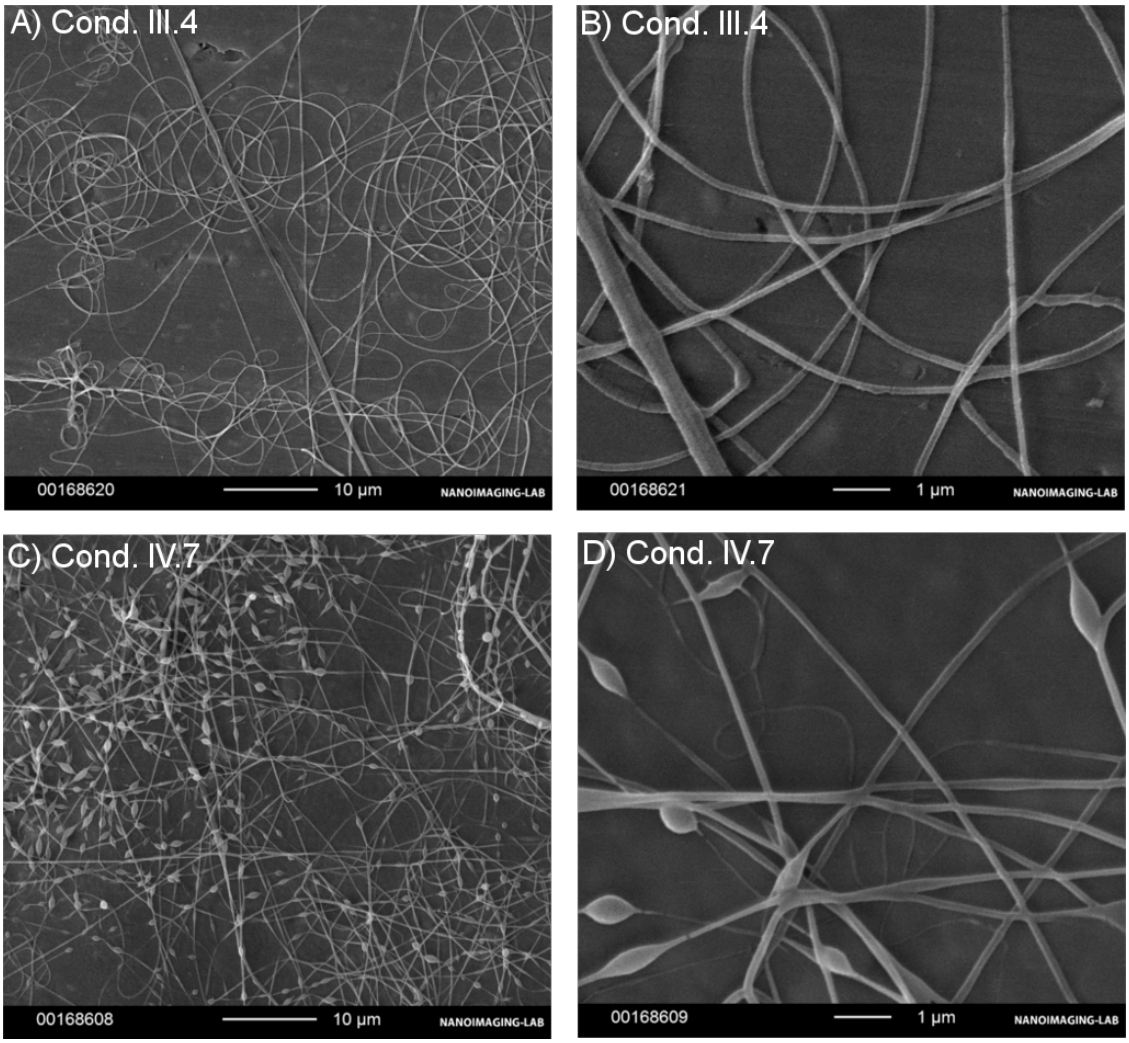

Figure 9: SEM micrographs showing the influence of different PTCS blending. Image A (Cond. III.4) displays non beaded electrospun fibers with an average fiber diameter of 192 nm (15 kV, WD = 11.0 mm). Image B (Cond. III.4) displays higher magnification of non beaded electrospun fibers (15 kV, WD = 11.0 mm). Image C (Cond. IV.7) shows beaded electrospun fibers with an average fiber diameter of 124 nm and an average bead diameter of 623 nm (15 kV, WD = 11.4 mm). Image D (Cond. IV.7) shows higher magnification of beaded electrospun fibers (15 kV, WD = 11.4 mm). It can be concluded that higher PEO concentration in the PTCS/PEO blend gives rise to non beaded fibers (Cond. III.4).

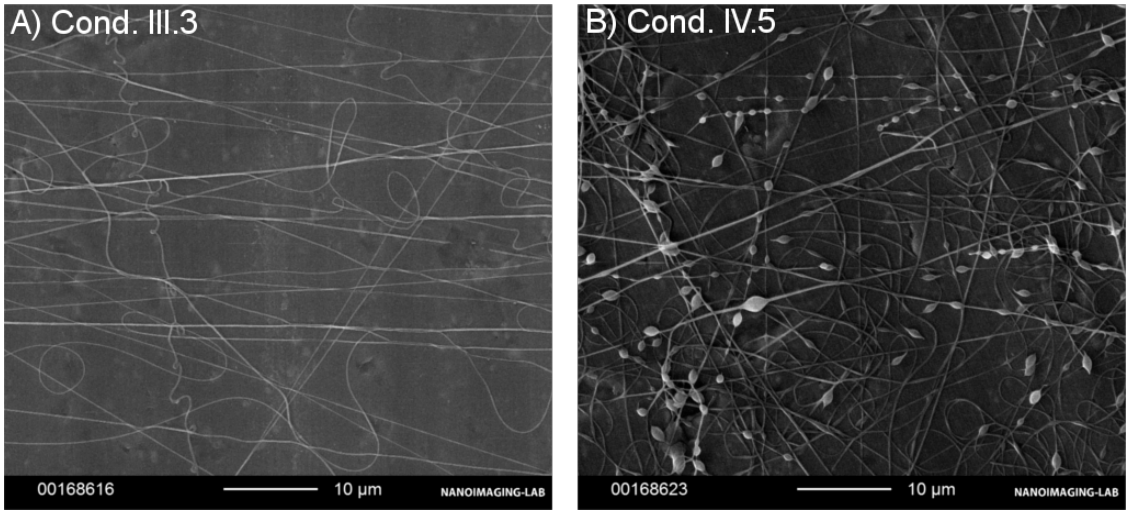

Figure 10: SEM micrographs showing the influence of mandrel speed on fiber alignment. Image A (Cond. III.3; 15 kV, WD = 11.2 mm) shows higher alignment of electrospun fibers than image B (Cond. IV.5; 15 kV, WD = 11.1 mm) presumably due to higher mandrel speed in Cond. III.3 (1000 rpm) than in Cond. IV.5 (100 rpm).

3.3 Cross-linking influences fiber stability in aqueous solutions

Cross-linking experiments with GA revealed that after exposure of the already electrospun fibers, which are spun out of proteolytically dissolved collagen, the collagen re-establishes covalent bonding in that range that it gets insoluble again. Figure 11 shows that fibers, which are electrospun on aluminum foil and subsequently cross-linked, can persist being submerged in ddH_2O, compared with a non GA treated sample of electrospun fibers on aluminum foil, where the fibers disappear after water treatment. Slight morphological changes of the fibers after GA treatment are observed in consistence with other ASC nanofiber publications.[38] The round shaped fibers appear to flatten upon treatment.

This regained biostability displays that in Cond.III - Cond.IV the fibers must be composed of intrinsically intact collagen or at least intrinsically intact small collagen peptides otherwise the GA could not act as cross-linker. Thus, for electrospun collagen fibers as potential substance for biomedical applications, like as scaffold material for regenerative purposes, cross-linking of the collagen appears to be necessary. Possible usage of the fibers to replace and support tissue requires biostability to some extend in order to support the tissue long enough before fiber degradation.

Furthermore, the control experiment in which fibers electrospun out of a water/PEO blend (Cond. I.1 - 4) is exposed to GA showed that biostability is only regained if the fibers are composed out of collagen. If the fibers are composed exclusively out of PEO those fibers are dissolved after submerge in water (Figure 11).

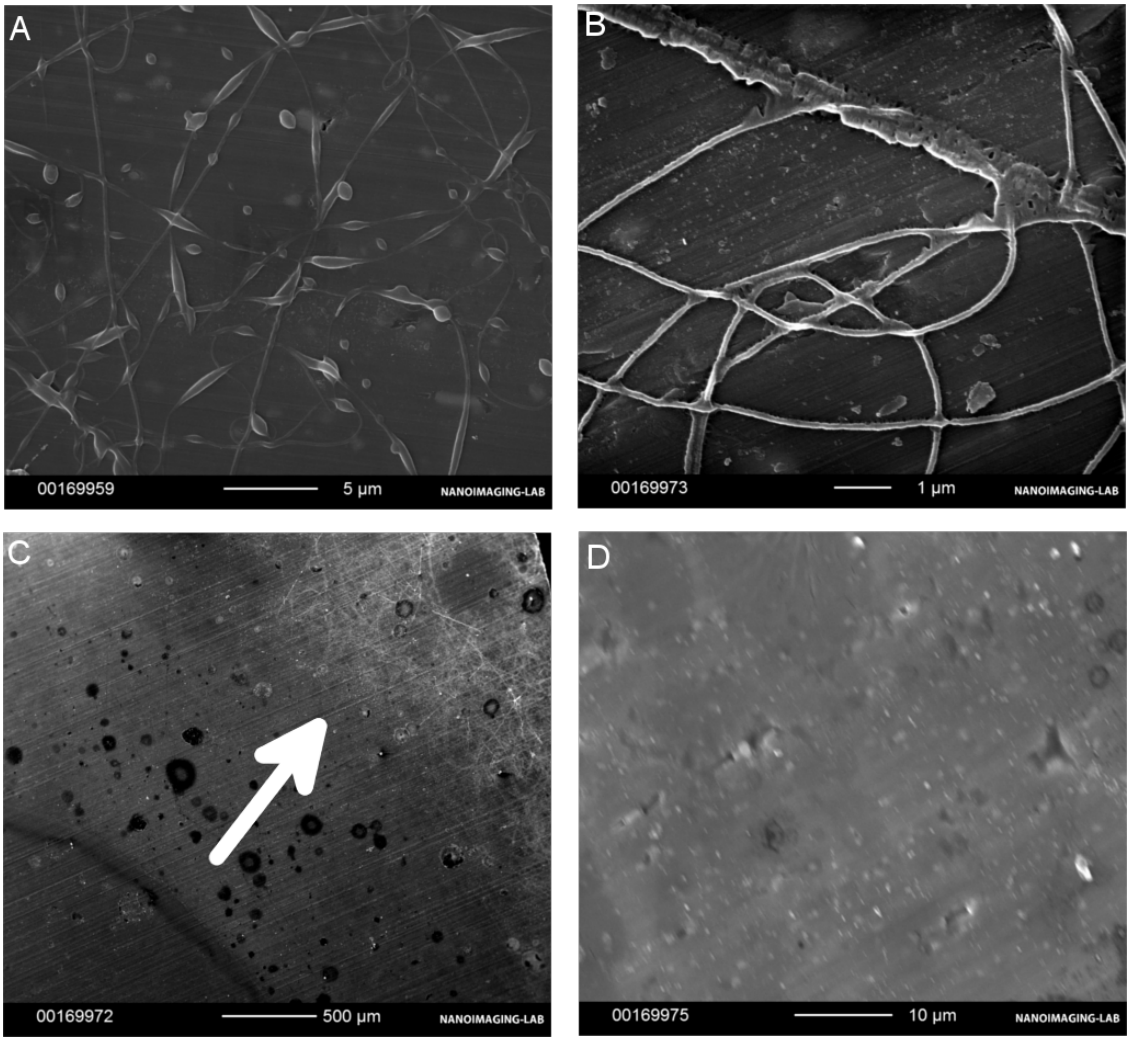

Figure 11: SEM micrograph images of cross-linking experiment. Images A (Cond. IV.7) and B (Cond. III.4) show the water treated cross-linked electrospun fibers on an aluminum foil. Fibers were exposed to GA vapour and therefore cross-linking occured. Fiber morphology changes slightly from round shaped fibers to more flat fibers. Image C (Cond. IV.4) shows the interface between a water treated and water untreated section of non cross-linked electrospun fibers on an aluminum foil, marked by the white arrow. Fibers were not exposed to GA vapour beforehand and therefore no cross-linking occurred which led to the dissolvation of fibers . Image D (Cond. I) serves as control, that if only PEO is electrospun, the fibers are even after GA vapour exposure not insoluble. After water treatment no fibers persist on the aluminum foil.

3.4 Analysis of proteolytic treated collagen

Since several previous papers concluded different influences of pepsin treatment on insoluble collagen type I, SDS-PAGE and FPLC analysis of the in this work used PTCS was carried out, to show this effect. Preservation of characteristic features of collagen like low immunogenicity and high mechanical strength is of paramount importance for future biomedical applications. Sato *et al.*[23] and Sun *et al.*[48] reported that pepsin treatment of insoluble collagen does not affect the integrity of the triple helical collagen structure despite the excision of its telopeptide region and that pepsin cannot digest the β chain (dimers) into two α chains (monomers). Meanwhile Ogawa *et al.*[49] concluded that not only telopeptide regions are digested

but dimers into monomers as well. In Figure 12 a SDS-PAGE gel is shown in which PTCS of different timepoints along with a molecular weight marker and controls were electrophoresed. This electrophoresis was executed in order to display the peptide composition of a to be spun solution. The different digestion timepoints showed several bands of peptides in the electrophoresed gel. The most prominent bands are in the region of 220 kD and around 37 kD, whereas the lower band gets more visible after longer proteolytic treatment. Indicated by this high molecular weight band at 220 kD and compared with the control (line 1) the biggest fraction of collagen could actually be β chain collagen which is not digested into its corresponding monomers. And the weight loss of telopeptide region is comparatively negligible with regard to overall molecular weights. The SDS-PAGE of untreated AMCHF (line 1) reveals that it consists of dimer collagen. Since no SDS-PAGE study on AMCHF exists and the producing company doesn't share any information on possible existing studies no comparison can be made. With prolonged time low molecular weight protein bands between 15 - 37 kD appeared. The appearance of these bands denotes that the tropocollagen is apparently not only cleaved at the telopeptide site but also cleavage within the triple helix occurs, leading to smaller collagen peptides. This is contrary to previous literature. Nevertheless not all collagen gets cleaved to smaller peptides due to proteolytic treatment, but the fraction of high molecular weight protein decreases over time. This is, besides time and cost efficiency, another indicator for optimizing the process of proteolytic collagen treatment. However, cross-linking experiment (Subsection 3.3) suggests that the integrity of the triple helical collagen structure is not affected since electrospun fibers regain biostability. And it is even possible that the small collagen peptides retain their intrinsic properties as well or at least it does not impede cross-linking between unaffected collagen. This assumption cannot be examined since PTCS was not separated into a high molecular weight PTCS fraction and a low molecular weight PTCS fraction and then electrospun and cross-linked separately.

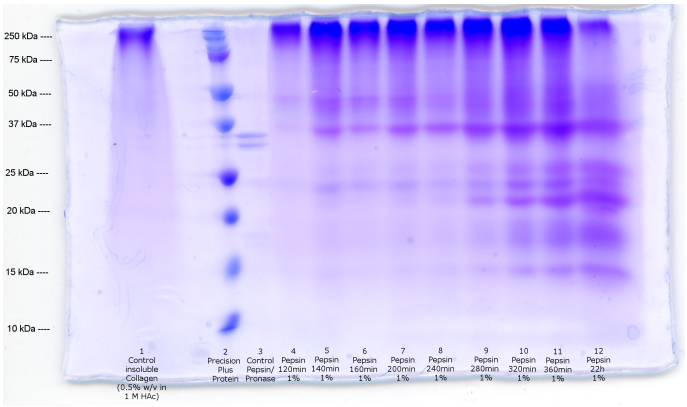

Figure 12: SDS-PAGE of PTCS from different timepoints. Pepsin treatment leads to collagen cleavage even in the helical structure and not only in the telopeptide region, which is indicated through the low molecular weight bands between 15 - 37 kD. Still, the biggest fraction is in the high molecular weight at least after 6 h when collagen is fully dissolved (Figure 4) and ready for electrospinning.

A second analysis was executed by FPLC. The FPLC elution graph (Figure 13) displays several peaks for one PTCS timepoint and therefore the PTCS composition

has to contain a mixture of different molecular weight collagen peptides. This result coincides with the above SDS-PAGE analysis. Calculation of the different molecular weights was done with a standard curve ($M_w = -187001 * (elutionvolume) + 417966; R^2 = 0.9808$; see A.2). This standard curve was computed out of the elution volume of eight enzymes with known molecular weights. By inserting the elution volume of the peaks of the unknown collagen mixture into the standard curve equation, three main molecular weight ranges could be calculated. β chains are assumed to be represented by the peaks in the range of 200 - 220 kDA. Small collagen peptides are assumed to be represented by the peaks in the range of 105 - 125 kDa and 25 - 75 kDa.

These FPLC results does not fully correspond with the above SDS-PAGE analysis, maybe due to different accuracy or sensitivity of this two methods. Obviously further investigations in the composition of PTCS has to be done.

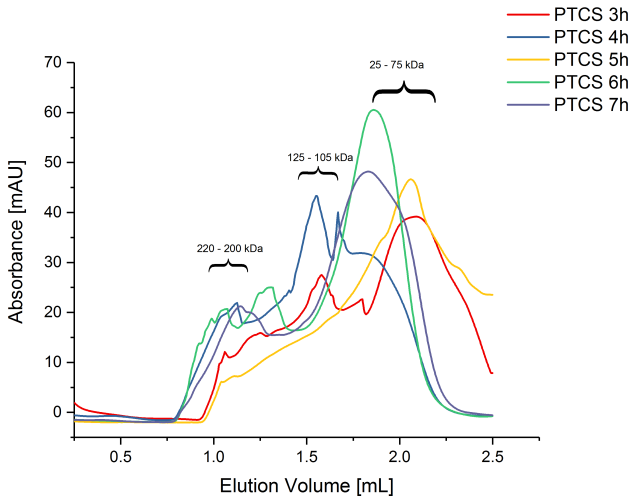

Figure 13: Graphical representation of FPLC experiment. The molecular weight of the peaks calculated with the standard curve show, that several peptide species are in the PTCS.

4 Conclusion

The process of electrospinning for fiber formation is an ambitious task. The optimization of the interplay of several influencing parameters in order to obtain a certain reproducibility and uniformity of fibers is challenging. Preparing a new method using a not yet used base material is even more challenging since parameters from already known materials are not one to one transferable onto the new substance.

In this thesis a novel method using insoluble collagen type I as a base material was developed. Firstly the insoluble collagen was solubilized using a proteolytic enzyme, then the pepsin treated collagen type I solution, with a collagen concentration of 8% $^{w}/_{v}$ was blended with 5% $^{w}/_{v}$ PEO in the ratio 1:2.5 and 2.5:1. Successful electrospinning, to get beaded as well as non beaded fibers with an average diameter between 125 - 275 nm, was possible using following parameters: 20 kV applied voltage, 0.8 $^{mL}/_{h}$ or 1.8 $^{mL}/_{h}$ feeding rate and 100 - 1000 rpm mandrel speed. The ambient conditions which could not be influenced were between 25.5 - 26.2 °C and 47 - 49 % humidity. The effect of parameter adjustment revealed manifold morphological changes. While high voltage modification impedes fiber formation completely, lower feeding rate appears to yield smaller fiber and bead diameter and higher mandrel speed increases the fiber alignment.

Since these tested parameters and several untested parameters seem to have such a big influence on the fiber formation, there will be still plenty of room for further parameter adjustment to get desirable fibers.

Making electrospun fibers more biostable for a better use in regenerative medicine was achieved by cross-linking with glutaraldehyde vapor. Fibers which were electrospun on glass or aluminum foil and exposed to GA vapor were not anymore soluble when submerged for 4 h in ddH_2O in contrast to non exposed fibers. This observation leads to the conclusion that the collagen is still able to re-establish intramolecular covalent bonds even after proteolytic treatment and appliance of high voltage during the ES process. This assumption is additionally supported by SDS-PAGE and FPLC analysis, which revealed the influence of pepsin treatment on collagen. SDS-PAGE showed that the majority of collagen is only cleaved in the non helical, telopeptide region and therefore the intrinsic properties are not lost. Nevertheless, FPLC and SDS-PAGE unveiled that still some fraction is digested into smaller collagen peptides, which means that pepsin can cleave collagen within its tight triple helical structure. This fraction increases with prolonging time.

In this thesis it was successfully shown that insoluble collagen type I after the right treatment can be used as a base material for electrospinning. The fibers are in nanometer range and would be ready for further applications.

4.1 Relevance of the thesis

In recent years the regenerative medicine and their medical applications gained more and more attention in research. Using biological or synthetic materials in novel therapies for replacing and restoring body own functions is of great promise to

advance the medical field. Since there are a lot of biomedical applications already in research and since scientist become more and more interested in producing nanoscale structures which can mimic tissue or other structures, electrospinning is a very versatile and promising way of generating this nanoscale structures. This thesis provides a method for using a never before used base material for electrospinning and get nanoscale fibers out of it. Insoluble collagen type I enqueues itself into a long list of already published materials for electrospinning. This developed method gives a guidance for a process, which requires easily available, non cost intensive and biocompatible materials. This reasons could be very important for future regenerative medicine applications.

4.2 Outlook

The production of fibers out of insoluble collagen type I can still be improved with regard to fiber uniformity and reproducibility. The addressed parameters in this thesis can be expanded with other parameters like solution conductivity as Huang *et al.*[7] showed with ASC or ambient parameters as Casper *et al.*[50] with polystyrene (PS) did. All these parameters have to be examined to fully control the electrospinning of insoluble collagen type I. In order to simplify the process, different blendings, besides collagen/PEO could be tested. For example collagen/poly(caprolactone) (PCL) or collagen/poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) blends. These polymers are already tested in blends with ASC.[51,52]

Furthermore, the prepared collagen solutions needs to be analysed in order to check if the integrity of the triple helical collagen structure is not affected and therefore its favourable biophysical properties are preserved. Circular dichroism (CD) or fourier transform infrared spectroscopy (FTIR) could serve as analysis methods for example.

Proceeding the next step of fiber application would be to test the biocompatibility or fiber/cell interaction. Biological assays with different cell types on electrospun fiber scaffolds, to study if the purpose of these fibers to mimic ECM endures, is needed for future biomedical applications.

Big interest lies in electrospun nanofibers for biomedical applications, since already commercial solutions, like cell culturing multiwell plates with integrated nanofibers, are available on the market.[53] Using a novel base material, as indicated in this thesis, which is said to have even better biocompatibility than already used synthetic solutions, could be used for own commercial applications.

5 References

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A Appendix

A.1 Protocols

A.1.1 SDS-PAGE sample preparation protocol

Per well 16 μL SDS-PAGE sample is loaded to reach a final protein amount per well of 20 $\mu g/_{well}$ ASC, 2 $\mu g/_{well}$ purified protein (pepsin) and 40 $\mu g/_{well}$ insoluble collagen type I. Therefore, following procedure was performed:

1. Take out 40 μL of PTCS 8% $w/_v$ at different time points and dilute/neutralize it with 280 μL (1:7) phosphate buffer (pH 7).

   For the pepsin/pronase control dilute 1.5 mg pepsin/pronase in 3 mL phosphate buffer (pH 7).

   For the insoluble collagen control prepare a0.5% $w/_v$ in 1 M acetic acid solution and neutralized it to pH 7 with 2 - 6 μL of a 10 M NaOH solution.

   This prepared solutions are mixed according to the following table to get a final volume of 32 μL of each sample:

C_{PPP}	$C_{ins\ Col}$	C_{Pepsin}	$C_{Pronase}$	PTCS $_{y\ min}$
4 µL PPP 28 µL ddH_2O	16 µL neutralized ins. Collagen 6 µL ddH_2O 8 µL 4X buffer	8 µL prepared pepsin solution 16 µL ddH_2O 8 µL 4X buffer	8 µL prepared pronase solution 16 µL ddH_2O 8 µL 4X buffer	8 µL prepared 1:7 PTCS 16 µL ddH_2O 8 µL 4X buffer

Table A.6: List for the right preparation of SDS-PAGE samples.

2. Shake the final prepared SDS-PAGE samples at 500 rpm while heating to 95 °C for 10 min.
3. Allow the heated samples to cool to RT.
4. Fill the SDS-PAGE samples into the wells of the gel and run the electrophoresis.

A.1.2 Phosphate buffer preparation

The in the thesis used 50 mM phosphate buffer (pH 7) was prepared according to the following recipe:

1. Dissolve 1.56 g $NaH_2PO_4 \cdot 2\ H_2O$ in 180 mL ddH_2O.
2. Titrate solution with a monovalent base (NaOH) until pH 7 is reached.
3. Add ddH_2O to a final volume of 200 mL.

A.1.3 4X sample buffer preparation

1. Stir together 0.8 mL of 1.5 M Tris/HCl pH 6.8 with 4.8 mL of a 50% $^{w}/_{v}$ glycerol solution.

2. Add 480 mg SDS, 370 mg dithiothreitol (DTT) and 4.2 mg bromophenol to the stirring mixture.

3. Add ddH_2O to a final volume of 6 mL. Store at -20 °C.

A.2 FPLC standard curve calculation

There is a linear correlation between elution volume and molecular weight, therefore with the elution graphs of proteins with known molecular weights, one can calculate a standard curve for the molecular weight calculation of unknown proteins. Herein, the FPLC graph of known proteins and the calculated elution peaks is shown. Computing was carried out using OriginPro 2017G and Microsoft Ecxel software:

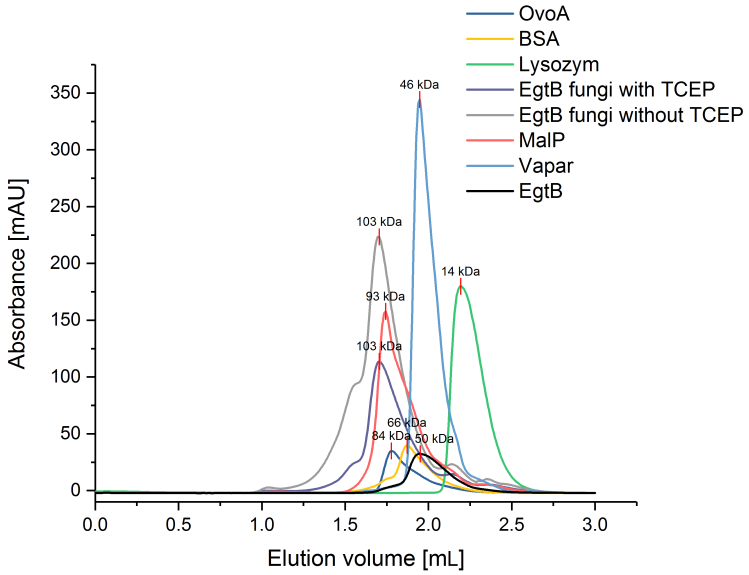


Figure A.14: Graphical representation of the FPLC elution of proteins with known molecular weights.

Enzyme	Elution volume [mL]	Molecular weight [kDa]
Lysozym	2.193	14.000
Vapar	1.947	46.258
EgtB	1.950	49.843
BSA	1.872	66.463
OvoA	1.778	84.470
MalP	1.743	92.685
EgtB fungi with TCPE	1.704	102.692
EgtB fungi without TCPE	1.704	102.692

Table A.7: List of proteins with known molecular weight and their corresponding elution volume.

A standard curve for molecular weight calculation of unknown proteins is computed using the regression line tool of Excel.

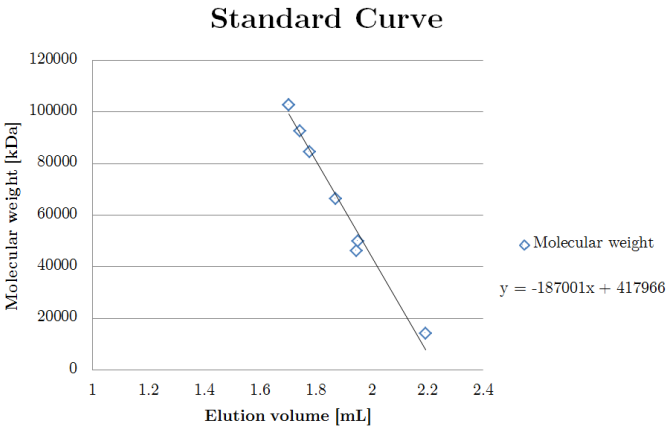
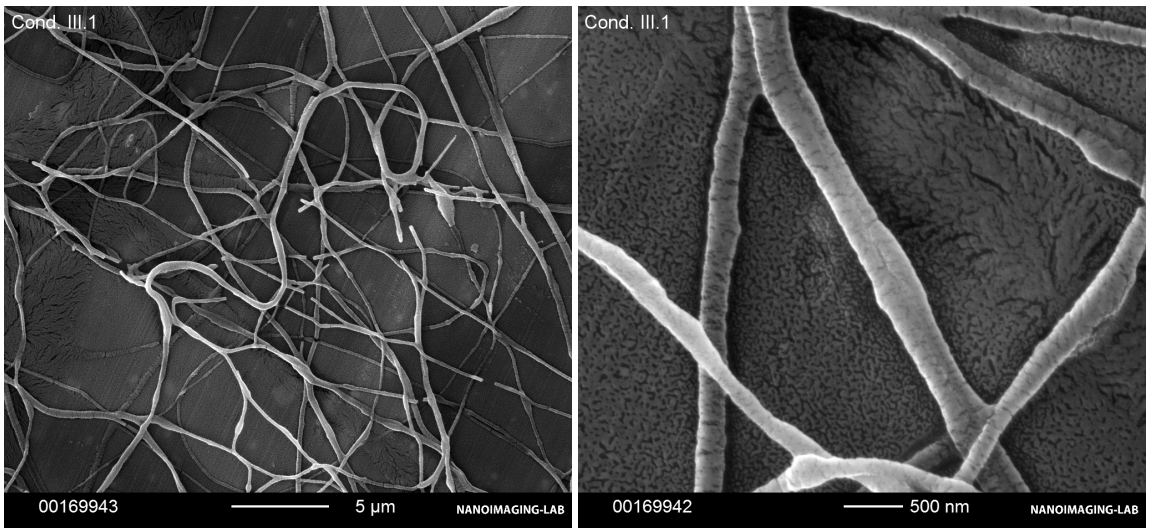
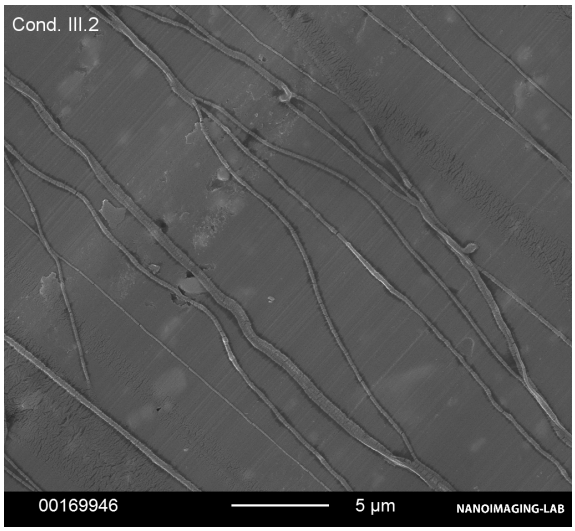


Figure A.15: Computing of standard curve for molecular weight calculation of unknown proteins.

A.3 Higher magnified SEM micrographs

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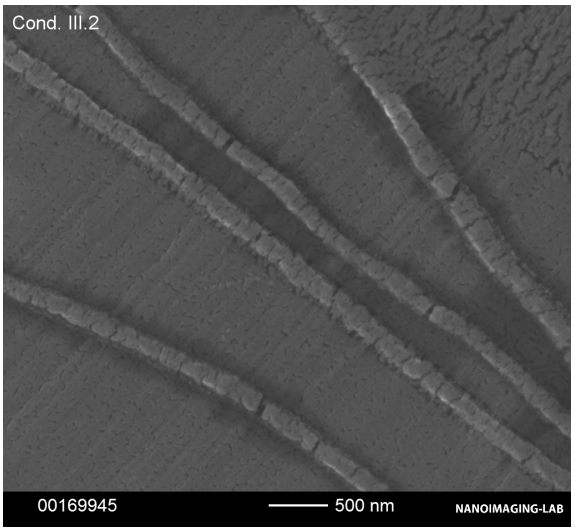
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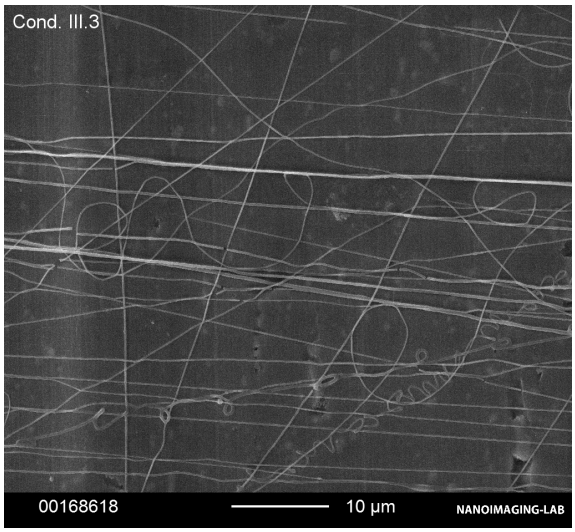
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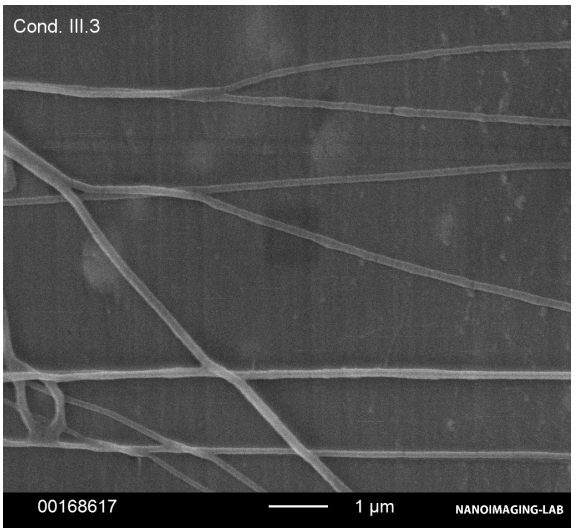
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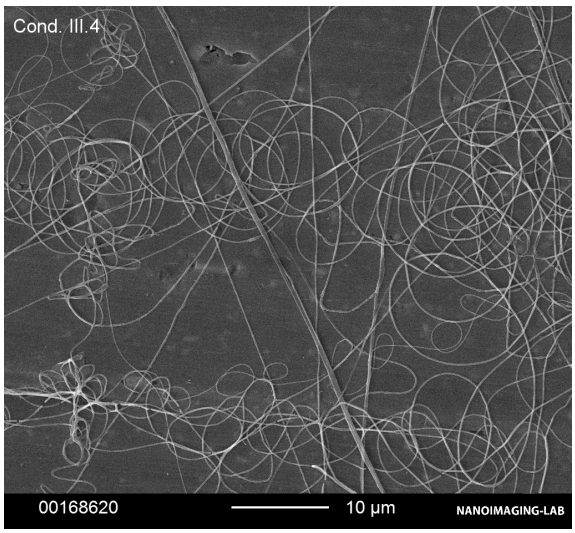
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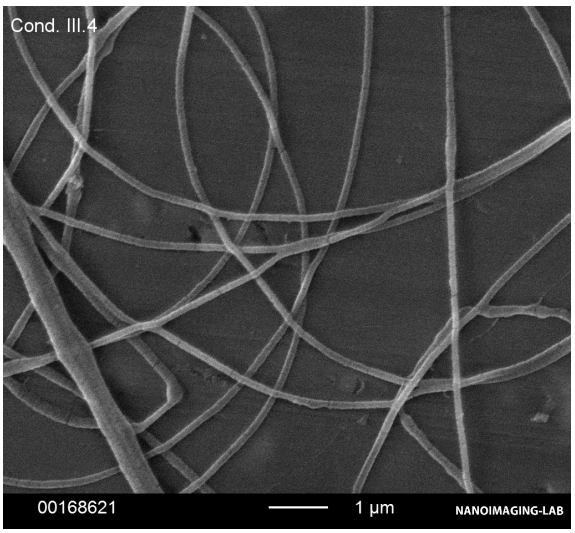
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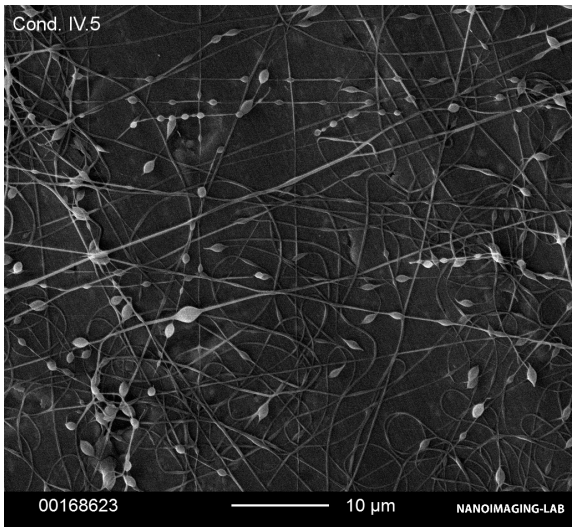
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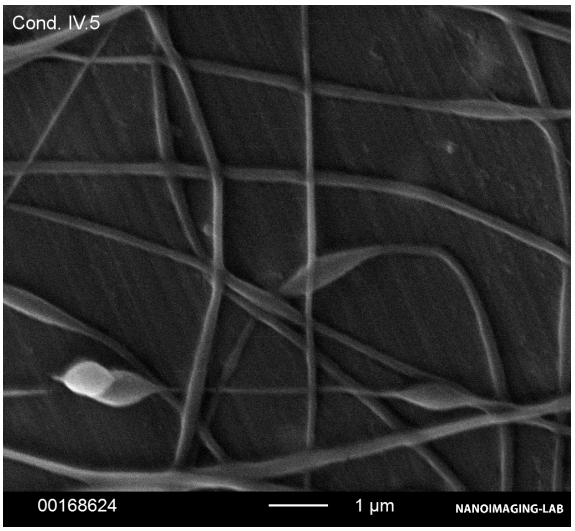
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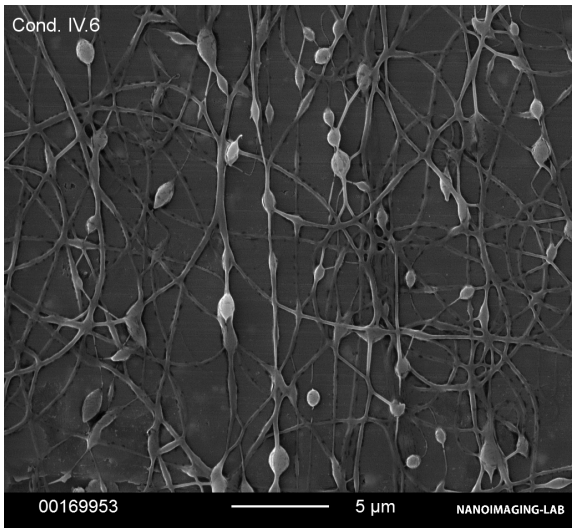
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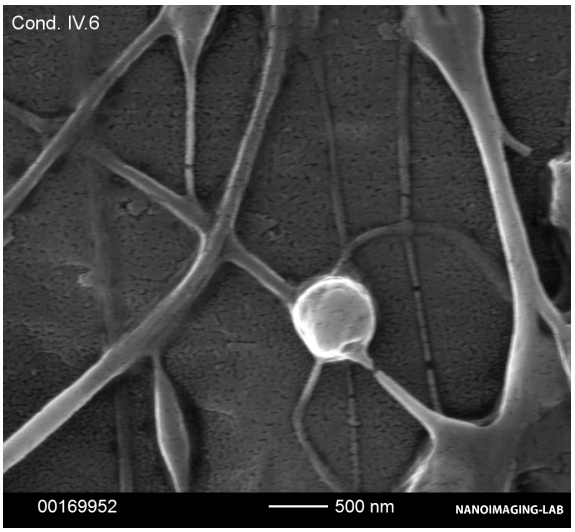
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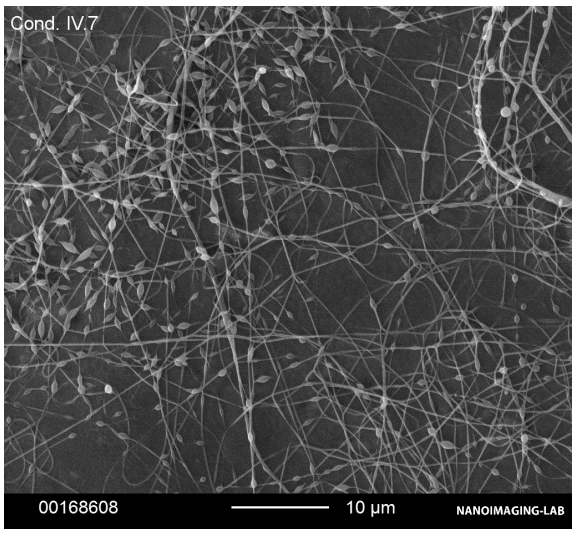
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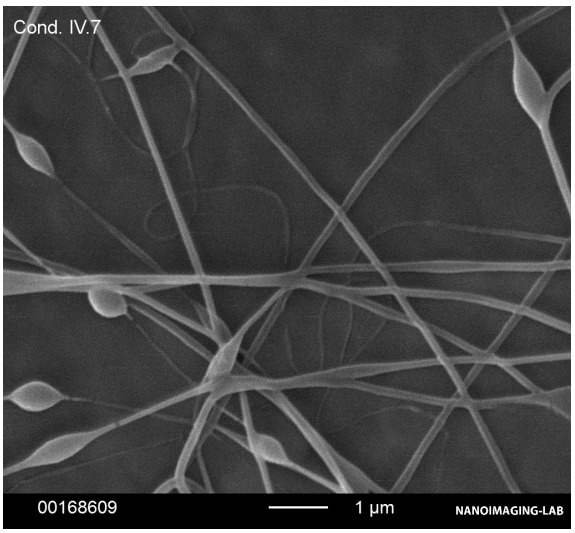
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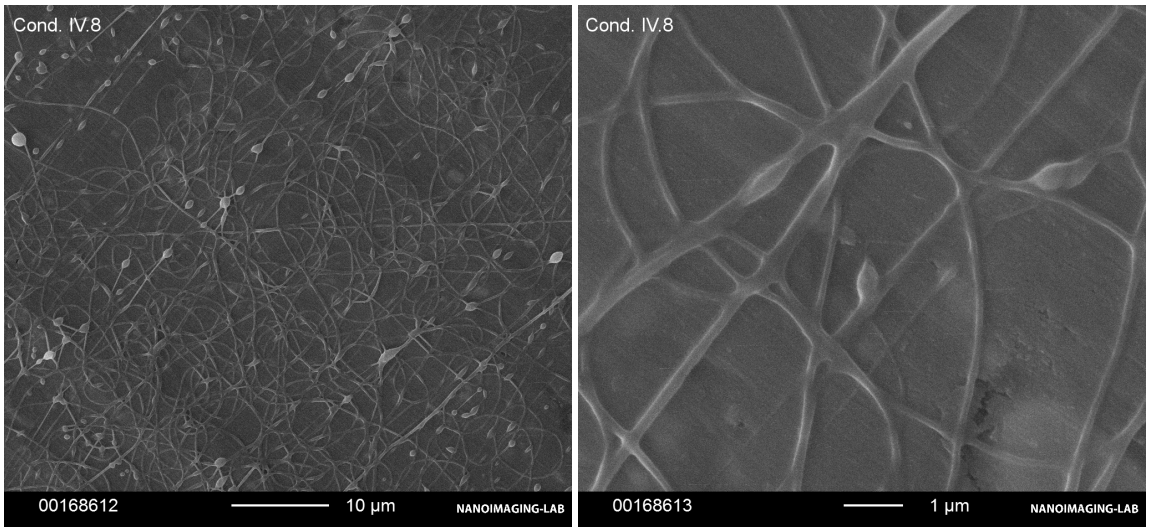

Figure A.16: SEM micrograph with higher magnification. Conditions are described directly in the images. Conditions III.1-4 depict round shaped, non beaded fibers out of a 1:2.5 (8% w/v collagen solution: 5% w/v PEO solution) blend. Conditions IV.5-8 depict round shaped, beaded fibers out of a 2.5:1 (8% w/v collagen solution: 5% w/v PEO solution) blend.

A.4 Light microscopy images

Light microscopy images were used to quickly identify if fibers were obtained on the collecting material or if the tried condition did not work. For further fiber characterization the resolution was too low.

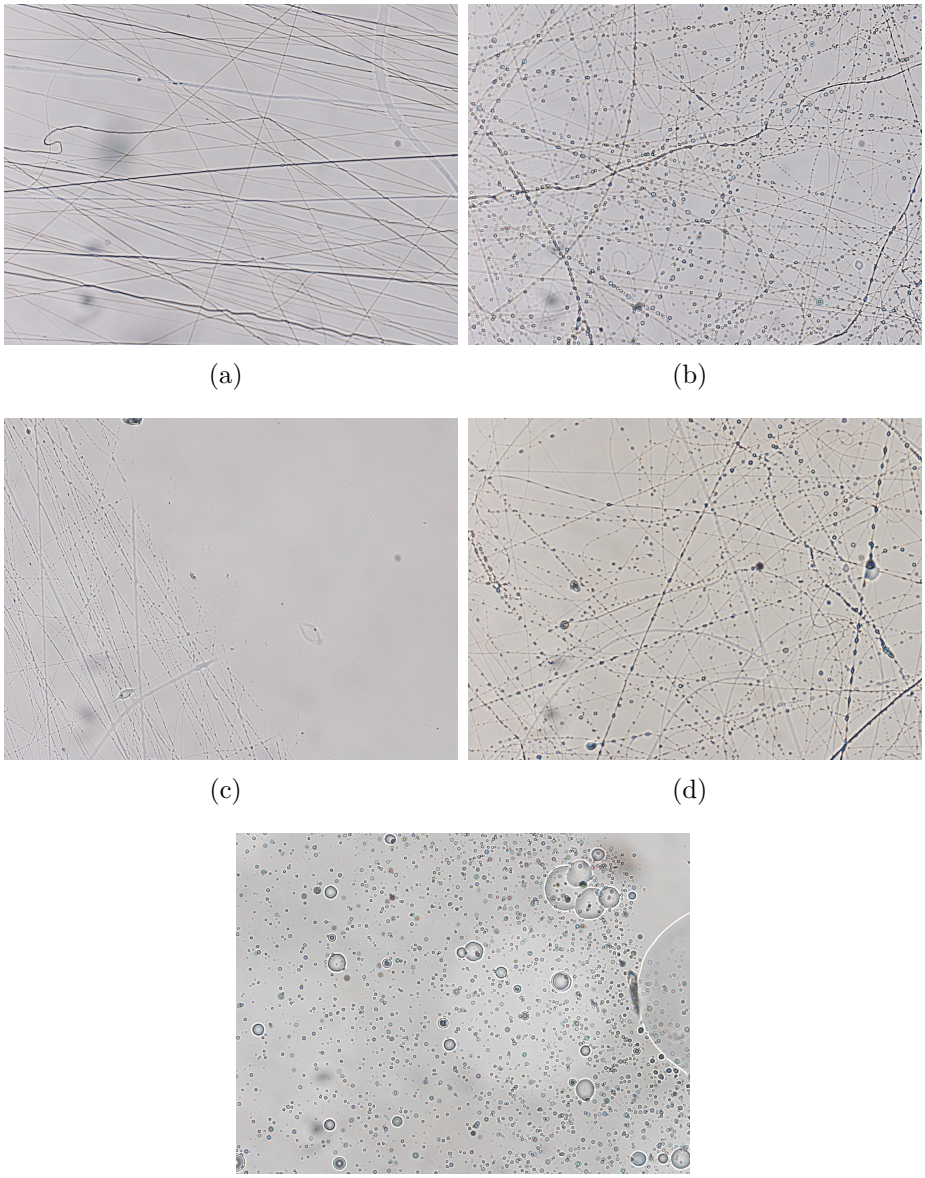

Figure A.17: Light microscope images of electrospun fibers. Image (a) (Cond. III.3, 60X) shows non beaded, aligned nanofibers. Image (b) (Cond. IV.7, 60X) displays beaded electrospun fibers. Image (c) (Cond. III., not cross-linked, 40X) and image (d) (Cond. III.2, cross-linked, 60X) show the impact of previous cross-linking with GA on the biostability of the electrospun fibers when exposed to ddH_2O for several hours. Image (e) (Cond. XIII.2, 40X) shows electrospraying.

UNIVERSITÄT BASEL

PHILOSOPHISCH-NATURWISSENSCHAFTLICHE FAKULTÄT

Erklärung zur wissenschaftlichen Redlichkeit
(beinhaltet Erklärung zu Plagiat und Betrug)

~~Bachelorarbeit~~ / Masterarbeit *(nicht Zutreffendes bitte streichen)*

Titel der Arbeit *(Druckschrift)*:

Novel approach to use insoluble collagen type I for electrospinning of nanofibers and their characterization

Name, Vorname *(Druckschrift)*: Züger Fabian

Matrikelnummer: 12-055-950

Hiermit erkläre ich, dass mir bei der Abfassung dieser Arbeit nur die darin angegebene Hilfe zuteil wurde und dass ich sie nur mit den in der Arbeit angegebenen Hilfsmitteln verfasst habe.

Ich habe sämtliche verwendeten Quellen erwähnt und gemäss anerkannten wissenschaftlichen Regeln zitiert.

Diese Erklärung wird ergänzt durch eine separat abgeschlossene Vereinbarung bezüglich der Veröffentlichung oder öffentlichen Zugänglichkeit dieser Arbeit.

☐ ja ■ nein

Ort, Datum: 15.09.2017

Unterschrift: F. Züger

Dieses Blatt ist in die Bachelor-, resp. Masterarbeit einzufügen.

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