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Novel blood protein based scaffolds for cardiovascular tissue engineering

Electrospinning of a novel autologous scaffold with enhanced hemocompatibility and appropriate biostability in aqueous solution

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Abstract: A major challenge in cardiovascular tissue engineering is the fabrication of scaffolds, which provide appropriate morphological and mechanical properties while avoiding undesirable immune reactions. In this study electrospinning was used to fabricate scaffolds out of blood proteins for cardiovascular tissue engineering. Lyophilised porcine plasma was dissolved in deionised water at a final concentration of 7.5% m/v and blended with 3.7% m/v PEO. Electrospinning resulted in homogeneous fibre morphologies with a mean fibre diameter of 151 nm, which could be adapted to create macroscopic shapes (mats, tubes). Cross-linking with glutaraldehyde vapour improved the long-term stability of protein based scaffolds in comparison to untreated scaffolds, resulting in a mass loss of 41% and 96% after 28 days of incubation in aqueous solution, respectively.

Keywords: biopolymer solution; biostability analysis; blood protein based scaffold; cross-linking; electrospinning; glutaraldehyde vapour; long-term stability; tissue engineering.

1 Introduction

Worldwide, 17.5 million people died of cardiovascular diseases in 2012. With a percentage of 46.2%, these diseases represent the largest cause of total deaths [1]. Current therapeutic approaches include alloplastic vascular prostheses (Dacron®, Teflon®) to replace or bypass the diseased vessel. However, the use of such prostheses represents several limitations like activation of foreign body reactions or chronic inflammation [2]. Therefore, the development of a suitable material for the replacement of diseased vascular tissue is desirable.

The aim of tissue engineering is a functional tissue replacement by culturing cells on suitable scaffolds. Therefore, it is important to develop biocompatible scaffolds for cardiovascular applications that have no undesirable effects on the surrounding tissue [3]. Electrospinning is a promising method for the fabrication of biocompatible fine-fibres and porous scaffolds [4–6]. To reduce undesired immune reactions for patients, the use of biopolymer solutions from blood proteins is desirable because proteins contain specific structural and functional information and are biodegradable. The biodegradable scaffolds will be gradually replaced by infiltrating and proliferating cells, leading to an effective tissue remodelling. A disadvantage of choosing electrospun protein based scaffolds is their high hydrolytic degradation rate in a physiological environment [7].

The aim of this study is to develop an electrospinning setup for the fabrication of blood protein based patient-specific scaffolds for cardiovascular applications. In addition, cross-linking with glutaraldehyde vapour will be performed and its influence on long-term biostability has to be analysed.

2 Material and methods

2.1 Biopolymer solution for electrospinning

Anti-coagulated porcine blood from the slaughterhouse was centrifuged with ≈ 2000 rcf (relative centrifugal force, Heraeus Megafuge 1 S-R, Thermo Fisher Scientific) for 10 min to separate blood components. Cell count (Advia120, Siemens) and protein concentration (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific) were

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Table 1: Composition and concentration of biopolymer solutions used for the fabrication of electrospun protein based scaffolds.

Solution	Concentration (mg/ml)			Concentration of heparin (µl/ml)
	PPlas	PEO	Span85	
I	–	37	–	–
II	100	37	–	0.4
III	75	37	–	0.3
IV	75	37	50	0.3
V	75	37	100	0.3

Polymers were dissolved in deionised water on a shaker for at least 72 h.

measured to characterise resulting blood plasma. Blood protein powder was created by lyophilisation. Biopolymer solutions were blended with non-toxic synthetic and water-soluble polyethylene oxide (PEO) to improve spinnability [7]. The lyophilised porcine plasma (PPlas) and PEO (400 kDa, Sigma-Aldrich) were dissolved in deionised water on a shaker for at least 72 h. Heparin (5000 IE/ml, Biochrom) was added to avoid coagulation. Different concentrations and blends were tested (see Table 1). Sorbitan trioleate (Span85, Sigma-Aldrich) was added for adjusting surface tension. Solution parameters like density, viscosity, surface tension and conductivity were measured to investigate their influence on the electrospinning process.

2.2 Scaffold fabrication and assessment of nanofibres morphology

Electrospinning was performed with a flow rate of 0.75 ml/h and an applied voltage of 20 kV. Two blunt cannulas (Ø = 0.4 mm) were arranged at 45° to the collector to improve process stability and efficiency. The distance between cannulas and the collector was set to 180 mm. A rotating-drum collector (b = 120 mm, Ø = 150 mm) was used at a rotation velocity of 250 rpm to produce fibre mats. Tubular scaffolds were fabricated with a mandrel collector (Ø = 6 mm). Fibre mats and tubular scaffolds were spun for 4 h. Morphological properties of protein based scaffolds were analysed via scanning electron microscopy (SEM, Hitachi S-3400N). Wall thickness and fibre diameter were measured using digital image processing software "AxioVision" (Carl Zeiss).

2.3 Biostability analysis and cross-linking method

To assess the *in vitro* degradation behaviour of electrospun protein based scaffolds, real time biostability analysis according to DIN EN ISO 10993-13:2010 [8] was performed with a test period of 1 to 28 days in a statically simulated body environment. Scaffolds were cross-linked with glutaraldehyde vapour to increase their long-term stability. In addition, untreated protein based scaffolds as well as treated and untreated PEO scaffolds served as control (see Table 2). PEO scaffolds were electrospun with comparable process parameters used for the protein based scaffolds. All scaffolds were placed in gas-tight boxes, which allowed to separate them from liquid glutaraldehyde agent (25%, Carl Roth) but leading to an incubation in glutaraldehyde vapour. They stayed in an incubator (Memmert) for 4 h with a chamber temperature of 37°C. Finally, the scaffolds were degassed for at least 12 h, cut into samples of ≈ 50 mg, and sterilized with ultraviolet radiation for 20 min. Afterwards each sample was incubated in 1.5 ml phosphate buffered saline containing 1% v/v penicillin/streptomycin-solution (10,000 IE/ml, 10,000 µg/ml, Biochrom).

3 Results and discussion

3.1 Biopolymer solution characteristics

According to Stokes formula, dynamic viscosity increases with lower fluid density. The lowest value of 2.69 MPa·s was detected for a concentration of 10% m/v PPlas and 3.7% m/v PEO (density: 1.0303 g/cm^3) and increased with decreasing mass fraction of PPlas (7.5% m/v, density: 1.0219 g/cm^3) to 3.37 MPa·s. The viscosity for the solution with Span85 5% m/v (density: 1.0191 g/cm^3) is 3.56 MPa·s and for 10% m/v (density: 1.0159 g/cm^3) 4.04 MPa·s. Pure PEO (3.7% m/v) has the highest value viscosity of 4.17 MPa·s (density: 1.0027 g/cm^3).

The surface tension of biopolymer solutions without Span85 is dominated by the surface tension of deionised water with 60.54 mN/m (10% m/v PPlas), 60.22 mN/m (7.5% m/v PPlas) and 60.00 mN/m (pure PEO). The addition of Span85 in a concentration of 5% m/v lead to a decrease in surface tension down to 36.11 mN/m (7.5% m/v

Table 2: Study design for biostability analysis.

	Day 1	Day 3	Day 7	Day 14	Day 28
i	n = 3	n = 4	n = 4	n = 5	n = 5
ii	n = 5	n = 4	–	–	–
iii	n = 3	n = 3	–	–	–
iv	n = 3	–	–	–	–

i: with glutaraldehyde vapour treated protein based samples; ii: untreated protein based samples; iii: with glutaraldehyde vapour treated pure PEO samples; iv: untreated pure PEO samples.

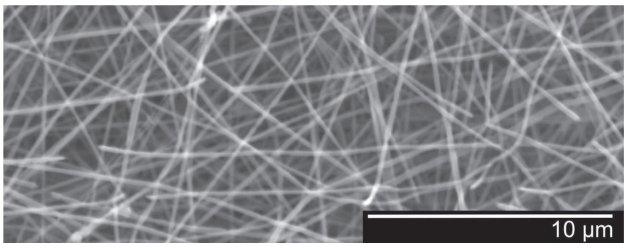

Figure 1: SEM-image of a resulting electrospun fibre mat with the concentration of 7.5% m/v PPlas and 3.7% m/v PEO. Visible are smooth and beadless nanofibres.

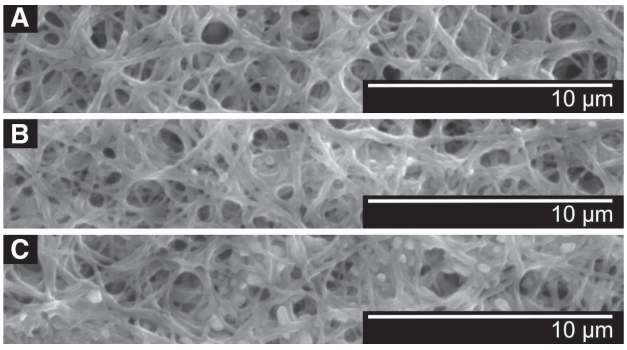

Figure 2: SEM-image of cross-linked protein based samples. A fibrous structure exist after biostability analysis over all time intervals (A: day 1; B: day 7; C: day 28). Salt crystal growth is observed after day 7.

PPlas) and with a concentration of 10% m/v Span85 to 33.23 mN/m (7.5% m/v PPlas). In low concentrations the surfactant reduces the interfacial tension between two phases by accumulate the surfactant molecules at the molecular interface [9]. The addition was done to adjust surface tension of the solution, without affecting its conductivity, due to the non-ionic property of Span85 [9]. However, high values of conductivity were measured for all biopolymer solutions, except pure PEO (2.15 µS/cm), with the highest value of 226.83 µS/cm for the 10% m/v PPlas and 3.7% m/v PEO solution. For the 7.5% m/v PPlas and 3.7% m/v PEO solution a value of 160.90 µS/cm was measured. With solution additive Span85, the conductivities were around this value (162.17 µS/cm for 5% m/v and 152.15 µS/cm for 10% m/v) and show the neutrality of this solution additive.

3.2 Electrospinning of blood protein based solution

Homogeneous electrospun fibre mats could be fabricated with a solution of 7.5% m/v PPlas and 3.7% m/v PEO. Fibre mats showed a microstructure made of beadless nanofibres with a mean fibre diameter of 151 nm (see Figure 1).

3.3 Degradation of electrospun scaffolds

After chemical treatment with glutaraldehyde vapour, the samples showed colour differences. Chemically treated protein scaffolds (7.5% m/v PPlas and 3.7% m/v PEO) were brownish while untreated samples remained white. This is an indication for the reaction of glutaraldehyde vapour with the amino groups of the protein molecules. Colour differences within one sample can be explained by local inhomogeneities with areas of increased PEO or protein concentration.

Samples were removed from the fluid after specified time intervals and weight loss of the samples and protein concentrations of the fluids were measured. Treated and untreated samples of pure PEO and untreated protein scaffolds degraded within a short period of time. After the first study interval (day 1 and 3) they showed a mean weight loss of 96%. The mean protein concentration of the degradation solutions was nearly 10 mg/ml for all untreated protein based samples. The results show that PEO is not affected by cross-linking with glutaraldehyde vapour and untreated plasma proteins remain water-soluble after electrospinning. In contrast, cross-linking of protein based samples prevented the complete degradation. This was confirmed by the SEM-images (see Figure 2), showing a fibrous structure for all time points.

The mean weight loss of 41% after 28 days and the mean protein concentrations of 3.3 mg/ml proof a good

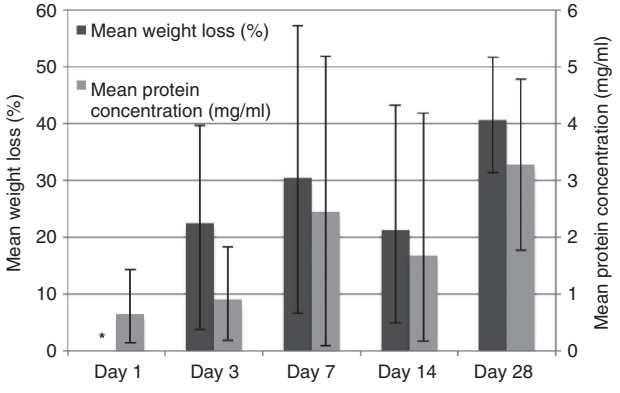

Figure 3: Plot of the measured mean values of weight loss (dark gray) and protein concentration (light gray) of cross-linked protein based samples with details of the minimum and maximum values (* measurement error, n = 3–5).

Figure 4: Electrospun tubular protein based scaffold treated with glutaraldehyde vapour (diameter: 6 mm, length: 20 mm). ©: Daniel Kühnhold, 2016.

biostability of protein based scaffolds (see Figure 3). During the first days of incubation non-cross-linked proteins and water-soluble PEO would go in solution. After 3 or rather 7 days the mean weight loss corresponds approximately to the incorporated mass of PEO (33% m/v). In addition the final weight loss of 41% was only 8% higher compared to the incorporated weight of PEO with 33% m/v. This stands for low protein release, proving an efficient cross-linking method.

3.4 Tubular electrospun scaffold

A tubular scaffold was successfully spun from a 7.5% m/v PPlas and 3.7% m/v PEO biopolymer solution and cross-linked with glutaraldehyde vapour (see Figure 4). The mean wall thickness of the scaffold was 56.37 µm and the length about 20 mm.

4 Conclusion and outlook

The aim of this study was to develop a setup for the electrospinning of blood protein based solutions to create patient-specific scaffolds. Furthermore, a cross-linking method to increase the long-term stability of such water-soluble protein based scaffolds was established. Based on a biopolymer solution with 7.5% m/v lyophilised porcine plasma and 3.7% m/v PEO electrospinning led to homogeneous scaffold morphology. Scaffolds showed a fibrous network with beadless fibres in the nanometer scale ($Ø = 151$ nm). A biostability analysis showed that the cross-linking of protein based samples prevented their complete degradation over the study period of 28 days. Furthermore a tubular protein based scaffold could be electrospun.

The experimental results of this study are promising for cardiovascular tissue engineering and further investigations into fabricating patient-specific tubular scaffolds via electrospinning of blood protein solutions blended with PEO. For an optimization of hemocompatibility further tests should be considered for cross-linking with native products, like thrombin, without the use of potentially toxic glutaraldehyde vapour. To raise cell proliferation it is possible to enhance electrospinning of protein based solution with the use of platelet rich plasma.

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