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PRODUCTION OF NONWOVEN FABRICS BY USING SILK FIBRES VIA ELECTROSPINNING TECHNIQUE

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SUMMARY OF THE THESIS

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Abstract

This dissertation was concerned and focused a fabrication of a silk fibroin (SF) nonwoven sheet and its blending with polycaprolactone (PCL) via a needleless electrospinning technique (technology Nanospider™). The procedure concentrated on a novel method for the preparation of a spinning solution from silk fibroin, by using a mixture of formic acid and calcium chloride as a solvent. The role of the concentration of silk fibroin solution, applied voltage and spinning distance are investigated as a function of the morphology of obtained fibres and the spinning performance of the electrospinning process. Biocompatibility of the obtained fibre sheets that resulted from the experiment was evaluated by *in vitro* testing method, with 3T3 mouse fibroblasts, normal human dermal fibroblasts, MG-63 osteoblasts and human umbilical vein endothelial cells. Tensile strength and hydrophilicity as well as physical properties evaluation of electrospun fibre sheets were performed.

The solvent system consists of formic acid and calcium chloride that can dissolve silk fibroin at room temperature, a rate of 0.25 gram of calcium chloride per 1 gram of silk fibroin is required to obtain the completely dissolved silk fibroin solution. This solvent system could be potentially employed and used for a preparation of silk fibroin solution for a large-scale production of silk nanofibres, with a needleless electrospinning method.

The diameters of the silk electrospun fibres obtained from the formic acid-calcium chloride solvent system had a diameter ranging from 100 nm to 2400 nm depending upon the spinning parameters. Concentrations of silk fibroin in the range of 8 wt% to 12 wt% seem to be a suitable concentration for the preparation of a nanofibre sheet, with needleless electrospinning. Furthermore, increasing the concentration of the silk fibroin solution and the applied voltage improved the spinning ability and the spinning performance in needleless electrospinning. Pure silk fibroin electrospun fibres have poor mechanical properties while research indicates blending PCL with silk fibroin can improve mechanical properties significantly. The diameters of the blended SF/PCL electrospun fibres were smaller and the elasticity was greater than the pure silk fibroin electrospun fibres. However, an increase of PCL content in the blended solution affected the spinning performance of the process. The spinning performance of the electrospinning process tends to decrease as the polycaprolactone content in the blended solution increases.

Silk electrospun fibre sheets and its blends with PCL are promising materials for the biomedical applications such as wound dressing and bone tissue engineering. *In vitro* tests with living cells show very good biocompatibility of the electrospun fibre sheets, especially with MG 63 osteoblasts. In addition, the PCL/SF blended fibre sheets have been applied as supports for immobilisation of laccase from *Trametes versicolor*. The blended fibre sheets were suitable for enzyme immobilisation and the blended fibre sheets with the laccase immobilised showed very good results in the degradation of endocrine disrupting chemicals (bisphenol A and 17α-ethinyl estradiol). The laccase immobilisation onto the PCL/SF blended fibre sheets seems to be a promising system for bioremediation of wastewater treatment.

Keywords: silk fibroin, needleless electrospinning, formic acid, calcium chloride

Anotace

Tato dizertační práce se zabývá výrobou nanovlákenných vrstev z fibroinu z přírodního hedvábí (silk fibroin, dále jen SF), a směsí SF s polykaprolaktonem (PCL) připravené metodou bezjehlového elektrostatického zvlákňování (technologie Nanospider^{TM}). V procesu zvlákňování byla zkoumána inovativní metoda přípravy zvlákňovacího roztoku SF za použití rozpouštědla ve formě směsi kyseliny mravenčí a chloridu vápenatého. Výzkum byl zaměřen na vliv koncentrace roztoku fibroinu, použitého napětí a vzdálenosti elektrod na morfologii vzniklých vláken i na samotný proces zvlákňování. *In vitro* testy za použití 3T3 myších fibroblastů, lidských kožních fibroblastů, MG 63 osteoblastů a lidských endotelových buněk z pupečníkové žíly byly zvoleny pro hodnocení biokompatibility vlákenných vrstev. Dále byla sledována pevnost v tahu a hydrofilita spolu s dalšími fyzikálními vlastnostmi vytvořených vlákenných vrstev.

Rozpouštědlový systém, který sestával z kyseliny mravenčí a chloridu vápenatého, byl schopen rozpustit SF za pokojové teploty při použití poměru 0,25 g chloridu vápenatého na 1 g SF. Tento rozpouštědlový systém je vhodný pro nanovláken metodou elektrostatického zvlákňování na poloprovozní jednotce Superlab.

Průměr vláken, získaných za použití zmíněného rozpouštědlového systému, se pohyboval v rozmezí 100 nm až 2400 nm v závislosti na parametrech zvlákňovacího procesu. Pro přípravu nanovláken prostřednictvím bezjehlového zvlákňování byla optimální koncentrace SF od 8% hmot. do 12% hmot. S rostoucí koncentrací a napětím se zlepšovala zvláknitelnost roztoku a produktivita zvlákňovacího procesu. Zatímco vlákna ze samotného SF měla špatné mechanické vlastnosti, ukázalo se, že ve směsi s PCL docházelo k výraznému zlepšení. Průměr směsných nanovláken byl nižší a pružnost těchto vrstev byla vyšší než v případě čistého SF. Se zvyšujícím se podílem PCL však docházelo ke zhoršení zvlákňovacího procesu.

Nanovlákenné vrstvy z čistého SF a ze směsi SF a PCL jsou materiály s potenciálem pro využití v biomedicínských aplikacích, jako jsou kryty ran nebo tkáňové inženýrství zaměřené na regeneraci kostních tkání. *In vitro* testy s živými buňkami, především MG-63 osteoblasty, potvrdily velmi dobrou biokompatibilitu připravených nanovlákenných vrstev. PCL/SF nanovlákna navíc našla své uplatnění jako nosič pro imobilizaci lakázy *Trametes versicolor*. Nejen že se tato směsná nanovlákna uplatnila jako nosič pro enzym, ale zároveň měla imobilizovaná lakáza velmi dobré výsledky v oblasti degradace endokrinních disruptorů (bisfenol A a 17α-ethinyl estradiol). Imobilizace lakázy na PCL/SF nanovlákna má potenciál pro využití při čištění odpadních vod.

Klíčová slova: přírodní hedvábí, bezjehlové elektrostatické zvlákňování, kyselina mravenčí, chlorid vápenatý

Content

1. Introduction

Polymer nanofibres have gained much attention as promising materials due to their unique properties, such as a high specific surface area, small pore diameters and ability to act as a barrier against microorganisms. They have shown enormous application potential in diverse areas, including filtration, energy storage, catalyst and enzyme carriers, drug delivery and release control systems and tissue engineering scaffolds. [1-3]. There are several methods to produce fibres at the nanoscale. One of these, electrospinning, has attracted a lot of interest in the last decade. Electrospinning was described as early as 1934 by Anton [5]. It is a simple but effective method to produce polymer fibres with a diameter in the range of several micrometres down to tens of nanometres, depending on the polymer and processing conditions [4-5].

Electrospinning technology can be divided into two branches: conventional or needle electrospinning and needleless electrospinning. Needle electrospinning setup normally comprises a high-voltage power supply and a syringe needle or capillary spinner connected to a power supply and a collector. During the electrospinning process, a high electric voltage is applied to the polymer solution. This leads to the formation of a strong electric field between the needle and the opposite electrode, resulting in the deformation of the solution droplet at the needle tip into a Taylor cone. When the electric force overcomes the surface tension of the polymer solution, the polymer solution is ejected from the tip of the Taylor cone to form a polymer jet. Randomly deposited dry fibres can be obtained on the collector due to the evaporation of solvent in the filament [5-6]. As a needle can produce only one polymer jet, needle electrospinning systems have very low productivity, typically less than 0.3 g/h per needle, making it unsuitable for practical uses [7]. Needleless electrospinning systems have been developed recently. In needleless electrospinning, instead of the generation of a polymer jet from the tip of the needle, polymer jets form from the surface of free liquid by self-organization [6-14]. For example, Jirsak et al. [9] invented a needleless electrospinning system using a roller or cylinder as the fibre generator, which was commercialized by Elmarco Co. (Czech Republic) with the brand name "Nanospider^{TM}". The roller electrospinning device contains a rotating cylinder electrode, which is partially immersed in a polymer solution reservoir. When the roller slowly rotates, the polymer solution is loaded onto the upper roller surface. Upon applying a high voltage to the electrospinning system, a number of solution jets are simultaneously generated from the surface of the rotating spinning electrode, thereby improving fibre productivity [5].

Silk is a fibrous protein produced by a variety of insects, including the silkworm. Silk fibres from silkworms have been used in textiles for nearly 5,000 years. The primary reasons for this longtime use have been the unique luster, tactile properties, high mechanical strength, elasticity, durability, softness and dyeability of silks. Silks also display interesting thermal and electromagnetic responses, particularly in the UV range for insect entrapment and form crystalline phases related to processing [15]. Silk fibres were used in optical instruments as late as the mid-1900s because of their fine and uniform diameter and high strength and stability over a range of temperatures and humidity. In addition to its outstanding mechanical properties, it is a candidate material for biomedical applications because it has good biological compatibility and oxygen and water vapour permeability, in addition to being

biodegradable and having minimal inflammatory reactions [16-19]. Silks have historically been used in medicine as sutures over the past 100 years and are currently used today in this mode along with a variety of consumer product applications. Commercially, silkworm cocoons are mass produced in a process termed ‘‘*Sericulture*’’ [15].

Although the silkworm spins its cocoon from a continuous filament of silk, the rest of the silk cocoon is unsuitable for reeling and is known as *silk waste*. Silk waste includes cocoons that are not suitable for reeling and waste silk from all stages of production from reeling through weaving. In Thailand, a large amount (36.6 tons) of this by-product is produced annually [20]. The composition of silk waste is similar to that of good silk, which is composed of an inner core protein called fibroin that is surrounded by a glue-like protein called sericin. Silk waste has been roughly characterized by scientists and showed a high value of remaining nutrients such as protein and lipid that could be transformed into high-value products. Many attempts have been emphasized on an application of these silk wastes for purposes, for example, handicraft, cosmetics, medical materials for human health and food additives according to its characteristics [20]. In order to discover the alternative way of value adding from silk waste in Thailand, this study interested in the fabrication of silk fibroin nanofibre sheets with needleless electrospinning techniques, concentrating on the effect of parameters on the electrospinning process.

2. Purpose and the aims of the thesis

The aim of this research is to fabricate silk fibroin nanofibres with a needleless electrospinning method, the experiment intensively concentrated on the effect of parameters on the needleless electrospinning process. In these studies, the role of the concentration of silk fibroin solution, applied voltage and working distance are investigated as a function of the morphology of the obtained fibres and the spinning performance of the electrospinning process. In addition, a new method for a preparation of the spinning solution by dissolving silk fibroin in a mixture of formic acid and calcium chloride is being used for a solution preparation instead of a ternary solvent system of $CaCl_2/C_2H_5OH/H_2O$, which has been widely used to dissolve silk fibroin. Furthermore, a characterisation of properties of the obtained electrospun fibre sheets and their interaction with living cells were also studied.

3. Overview of the current state of the problem

The silk fibroin protein is a structuring molecule that has the ability to be processed into numerous forms through a variety of techniques. Several different material morphologies can be formed from aqueous or organic solvent formulations of the natural fibre form of silk for utilization in biomaterials for biomedical applications. Natural silk fibres dissolve only in a limited number of solvents because of the presence of a large amount of intra- and intermolecular hydrogen bonds in fibroin and its high crystallinity. Consequently, hydrogen bonds have an important effect on the conformation and structure of fibroin. The influence of hydrogen bonding on the stability of fibroin molecules can be seen by the ease with which protein dissolution occurs in known hydrogen bond-breaking solvents. Silk fibroins are insoluble in water, dilute acids, alkali and the majority of organic solvents but only swells to 30-40%. Dissolution methods used for solubilizing the degummed silk fibroin fibres generally rely on strong chaotropic agents, including concentrated acids (hydrochloric acid,

phosphoric acid and sulphuric acid) and in high ionic strength aqueous salt solutions (such as lithium thiocyanate, lithium bromide, calcium chloride, zinc chloride, and magnesium chloride) to neutralize the hydrogen bonds stabilizing the silk crystal structure. Consequently, the conditions of the dissolution process can influence the chemical composition and the molecular structure of the silk protein, affecting its biomaterial properties [21-22].

The most ubiquitous method to produce regenerated silk fibroin solution is through the use of heavy salts like LiBr or $CaCl_2$. The degummed fibres can be dissolved using LiBr (9.3 M) solution or a ternary solvent system of $CaCl_2/C_2H_5OH/H_2O$ (1/2/8 mole ratio) to disrupt hydrogen bonding between the fibroin protein chains. The solution should then be allowed to thoroughly dissolve for up to 4 hours at 60 ^{O}C to ensure complete dissolution. The heavy salts can then be removed from the silk solution through dialysis against deionized water over a period of 72 hours. Typically, the molecular weight cut-off for the dialysis membrane is 3,500 Da, which is permeable enough to allow for the salts and water to travel freely while retaining the fibroin light and heavy protein chains, respectively. Final silk solution concentrations range from 6% to 10% (w/v) content. The solvency of aqueous salt systems depends on the salt concentration, although premature reprecipitation is a problem unless the solutions are kept at low temperature. The main disadvantage of a salt-containing aqueous solvent is the long preparation time because aqueous solutions of fibroin have to be dialyzed for several days to remove the salts and to recover the polymer as films, sponges, or powder from the aqueous solution by dry forming. In some organic solvents (e.g. hexafluoroacetone and hexafluoroisopropanol), silk fibroin can be dissolved only after preliminary activation by dissolution in aqueous salt systems followed by the recovery [21-23].

Electrospinning silk solution is a favored processing methodology for producing nanometer- to micron-scale fibres that result in a high degree of available surface area for use in creating scaffolds for tissue engineering and regenerative medicine purposes [23]. In order to utilize silk waste, as well as achieve large-scale production of electrospun fibre sheets. It is necessary to be able to regenerate silk fibres through a simple but efficient spinning process. A needleless electrospinning was chosen as a technique for a preparation of electrospun fibre sheets for these studies owing to a capability to fabricate nanofibre layers in a mass industrial scale. However, a needleless electrospinning is a new technique and most research on an electrospinning of silk fibroin has been focused on the needle electrospinning system. There was a little information on an electrospinning of silk fibroin with a needleless system; therefore, parameters of the spinning process with have not been identified.

4. Materials and methods

4.1 Materials

Waste cocoons of *Bombyx mori* Linn. Thai silkworm (Nang-Noi Srisakate 1) were supplied from Chan farm, Amphoe Mueang Chan, Si Sa Ket Province, Thailand. Silk fibroin was obtained by a degumming process. Briefly, raw silk cocoons were degummed twice with 1.0% of sodium carbonate (Merck Ltd.) and 0.5% of a soaping agent at 100 ^{O}C for 30 minutes, rinsed with warm water to remove the sericin from the surface of the fibre, and then dried at room temperature. ECE phosphate reference detergent FBA free (Union TSL Co., Ltd., Thailand) was used as a soaping agent in the degumming process. The chemicals

used for the preparation of the spinning solutions were calcium chloride (Fluka AG, Switzerland) and 98% formic acid (Penta, Czech Republic). Polycaprolactone (PCL, M_n 70,000~90,000 g/mol) was purchased from Sigma Aldrich (Germany).

4.2 Analytical methods and apparatus

4.2.1 Characterisation of spinning solution properties

Conductivity and surface tension properties were determined by a conductivity meter (CON 510 Bench Conductivity/TDS Meter, Eutech Instruments, Netherlands) and a tensiometer (Digital Tensiometer K9, Krüss, Germany) using a plate method. Rheological properties of spinning solutions were measured by a rheometer (HAAKE™ RotoVisco™ 1 Rotational Rheometer, Thermo Scientific, Germany) using a plate-plate method. All measurements were conducted at room temperature (approximately 22-25 ^{O}C).

4.2.2 Morphology analysis and fibre diameter

The morphological appearance of the electrospun fibres was observed with a scanning electron microscope (SEM) Vega 3 (Tescan, Czech Republic) at an accelerated voltage of 20. All the samples were sputter-coated (Q150R ES, Quorum Technologies Ltd., England) with gold at a thickness of 7 nm. The diameter of nanofibres was measured by counting image pixels with image software, NIS-Elements AR software (LIM s.r.o., Czech Republic). The average fibre diameter and its distribution were determined from 200 random fibres obtained under each spinning condition.

4.2.3 Spinning performance of the electrospinning process

The spinning performance of the electrospinning process was calculated from the mass per unit area and width of the electrospun fibre sheets and the velocity of the backing material, using the equation (1) [24].

$$P = \frac{G \times W \times V_f}{L_r} \quad (1)$$

Where P is a spinning performance (g/min/m), G is a mass per unit area of electrospun fibres sheet in gram per square metre (g/m^2), W is a width of fibre layer in metre (m), V_f is a take-up cylinder speed in metre per minute (m/min) and L_r is a length of spinning electrode in metre (m).

4.2.4 Physical properties

- Tensile properties of the electrospun fibre sheets were examined with a tensile tester (Labor-Tech) at an extension rate of 3 mm/min with 15 mm gauge length. Ten samples of rectangular shape (10 mm width x 40 mm length) were cut along the same direction of width and length in the fibre sheet for each sample. The result of tensile strength and elongation at break were averaged and reported.

- Hydrophilicity of the electrospun fibre sheets was evaluated by water contact angle measurements using an instrument for contact angle measurement (See System, Advex Instruments, s. r. o., Czech Republic). A distilled water droplet size of ~20 μL was placed carefully onto the surface of the scaffolds at room temperature. After a period of 20 seconds, the contact angle was recorded. The mean value and standard deviation were calculated through testing at ten different positions on the same sample.

4.3 Experimental process

4.3.1 Preliminary investigation of the effect of calcium chloride on dissolution behavior of silk fibroin and morphology of silk fibroin electrospun fibres

Firstly, in order to find out the appropriate amount of calcium chloride for dissolution of silk fibroin in formic acid. Degummed silk fibres were directly dissolved in formic acid (98%) with various amounts of calcium chloride to prepare 8 wt% of silk fibroin solution. The ratio of silk fibroin to calcium chloride was 1:0.15, 1:0.20, 1:0.25, 1:0.30, 1:0.35, 1:0.40, 1:0.45 and 1:0.50 (w/w), respectively. All solutions were magnetically stirred at room temperature for 6 hours and then solutions were electrospun into fibre sheets.

A schematic representation of the equipment used in the experiment is illustrated in Figure 1. During the spinning process, the silk fibroin solution was placed on the surface of the spinning electrode (10 mm in diameter), which was connected to a high-voltage DC power supply (Spellman SL150). A high voltage of 50 kV was applied to the silk solution. The electrospun fibres were collected on a collector, which was placed at a distance of 100 mm from the electrode. The processes were carried out at room temperature and 40±2% humidity.

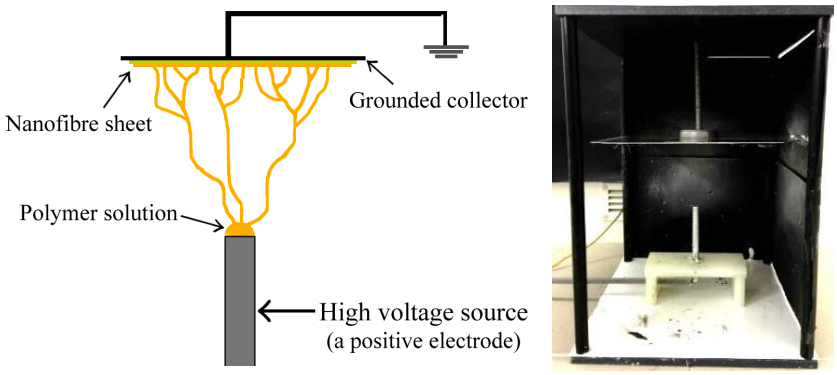


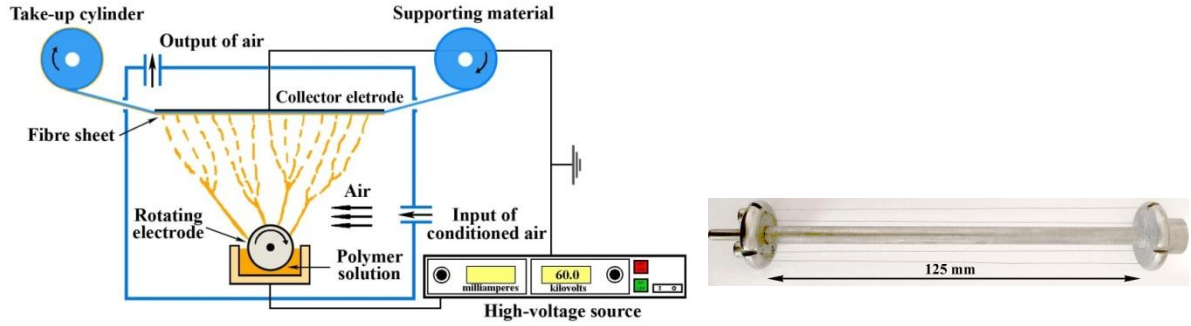
Figure 1 Schematic of a simple electrospinning experiment.

4.3.2 Fabrication of silk fibroin nanofibres by needleless electrospinning method

The spinning solutions were prepared by the dissolution of degummed silk fibres in a mixture of formic acid and calcium chloride. The ratio of silk fibre to calcium chloride was 1:0.25 (w/w), which is used throughout the remaining experiment. All solutions were magnetically stirred at room temperature overnight. The properties of spinning solutions were measured. Subsequently, the solution was electrospun into fibre sheets without any further treatment.

A schematic representation of the equipment used in the spinning process is depicted in Figure 2. The electrospinning device contains a rotating electrode, the electrode was made of stainless steel wire with 125 mm in length (wire diameter 0.02 mm) and a solution reservoir. The solution reservoir, which has a high voltage connected to the bottom of the solution bath, was filled with the silk fibroin solution. The process parameters are shown in Table 1. The electrospun fibres sheet was collected on the backing material (polypropylene nonwoven fabric) moving along the collector electrode. After spinning, the electrospun fibre sheets were treated with alcohol to achieve the solvent-induced crystallization of the silk fibroin and to reduce the water solubility of the fibre sheets. Briefly, the obtained fibre sheets were immersed in ethanol for 30 min. After drying at room

temperature, the treated fibre sheets were removed from the backing substrate and immersed in distilled water overnight, followed by rinsing in distilled water to remove residual salts and then finally dried again.



(a) *(b)*

Figure 2 (a) Schematics of needleless electrospinning setup and (b) a spinning electrode.

Table 1 Spinning parameters of an electrospinning experiment

Parameters	Parameters level
Silk fibroin concentration (wt%)	6, 8, 10,12, 14
Applied Voltage (kV)	30, 35, 40, 45, 50, 55, 60
Distance between electrodes (mm)	100, 125, 150
Roller angular velocity (rpm)	15
Backing fabric take up speed (mm/min)	10
Air humidity (%)	35 - 40
Temperature (^{O}C)	20 - 25

For comparison, a spinning solution from silk powders was prepared as follows: the degummed silk fibres were dissolved in a tertiary solvent system of $CaCl_2$/ C_2H_5OH/H_2O solution (1/2/8 in molar ratio) at 85 ^{O}C for 40 minutes to attain silk fibroin solution. The ratio of silk fibre over dissolving solution was 1:10 (w/w). Then, the silk fibroin solution was dialyzed against deionized water in dialysis tubing cellulose membrane (Sigma, molecular cutoff 12,000-14,000) at room temperature for 3 days. The dialyzed silk solution was filtered and spray dried to obtain silk powders; finally, the dried silk powders were dissolved in formic acid for 4 hours to prepare 12 wt% silk solution for electrospinning.

4.3.3 Preparation of silk fibroin-polycaprolactone blended fibres with needleless electrospinning method

Although silk fibroin nanofibres were successfully fabricated with needleless electrospinning, the obtained fibre sheet is usually fragile. It is brittle in the dry state, which is a disadvantage and would be unsuitable for practical use. However, if the dry state is required and the brittleness is undesirable, mechanical properties of silk fibre sheet can be improved by blending with other synthetic polymers [25]. In these studies, polycaprolactone has been used for blending with silk fibroin owning to its good mechanical properties, non-toxicity and biocompatibility. Various blend ratios of silk fibroin and polycaprolactone could

be investigated for the fabrication and evaluation. The effect of blend ratio of silk fibroin and polycaprolactone in spinning solution is investigated as a function of the properties of the blended solution, the morphology of electrospun fibres and the spinning performance of the process.

For a preparation of spinning solutions, polycaprolactone pellets were dissolved in formic acid to prepare 15 wt% of polycaprolactone solutions. The solutions were continuously stirred with a magnetic stirrer at ambient temperature for 4 hours. Silk fibroin solution was prepared as described in topic 4.3.2. The silk fibroin concentration was fixed at 12 wt%. The SF/PCL blended solutions were prepared by mixing the silk fibroin solution with the polycaprolactone solutions at weight ratios of 9/1, 8/2, 7/3, 6/4 and 5/5 (w/w), respectively. After that, all solutions were magnetically stirred at room temperature. The properties of blended solutions were measured. Subsequently, the blended solution was electrospun at a high voltage of 55 kV. The equipment used for these studies is same as a previous experiment (topic 4.3.2). Electrospinning was carried out at a distance of 100 mm. After spinning, the obtained electrospun fibre sheets were treated with ethanol and characterised as same as the electrospun silk fibroin fibre sheets. Moreover, the physical properties of these blended electrospun fibre scaffolds were characterized when compared to silk fibroin fibre sheets.

4.4 *In vitro* tests of electrospun fibre sheets from silk fibroin and its blend with polycaprolactone

In order to evaluate interaction between the electrospun fibre sheets with living cells; 3T3 mouse fibroblasts, normal human dermal fibroblasts, MG-63 osteoblasts and human umbilical vein endothelial cells were seeded on silk fibroin (SF) and silk fibroin/polycaprolactone (SF/PCL) blended fibre sheets [prepared from SF/PCL blended solutions at weight ratios of 7/3 and 5/5 (w/w)].

- Preparation of scaffolds and cell seeding

The fibre sheets were cut into small disks with a diameter of 6 mm to fit each well of 96-well plate. The specimens were sterilized by immersion in 70% aqueous ethanol solution for 30 minutes followed by double washing in phosphate buffer saline (PBS, Lonza). 3T3 mouse fibroblasts, normal human dermal fibroblasts and MG-63 osteoblasts were seeded on the fibre sheets placed in 96-well plate at density of $5x10^3$ per well plate. During the experiment, a medium was changed 3 times per week. On the other hand, human umbilical vein endthelial cells were seeded on the scaffolds placed in 96-well plate at density of $1x10^4$ per well plate and a medium was changed 3 times during the 7 days experiment.

- Cell adhesion and proliferation analysis

To test cell adhesion and proliferation, MTT test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide was used for measurement of cell viability during the cultivation time. The morphologies of cellular on the fibre sheets were analysed by fluorescence microscopy and scanning electron microscope (SEM). The results after 1 day are depicted in result section for cellular adhesion evaluation. The results at the end of the experiment are presented for proliferation rate assessment.

4.5 Immobilisation of laccase on polycaprolactone/silk fibroin blended fibre sheets

- *Preparation of polycaprolactone/silk fibroin nanofibre sheets*

For a preparation of blended solution, PCL pellets were dissolved in formic acid to prepare 20 wt% of polycaprolactone solution. The blended solution was prepared by mixing the polycaprolactone solution with the silk fibroin solutions (12 wt%) at weight ratios of 8/2 (w/w). After that, the solution was magnetically stirred at room temperature overnight. Subsequently, the electrospinning was performed by using Nanospider™ NS1WS500U (Elmarco, Czech Republic). The solution was electrospun at a high voltage of -10/50 kV.

- *Enzyme immobilisation on polycaprolactone/silk fibroin nanofibre sheets.*

Laccase from *Trametes versicolor* was immobilised on the blended nanofibre sheets by covalent attachment method. In an immobilisation process, the blended fibre sheet was cut into small pieces. Each of them weighed 5 mg. These samples were washed with ethanol and distilled water and after that, modified via combinations of glutaraldehyde and bovine serum albumin. After modification, the fibre sheets were thoroughly washed with distilled water. After that, the washed samples were placed in 100 mM McIlvaine buffer with pH 3.0 to measure the activity of the laccase immobilised on the fibre sheets [26-27]. The parameters for immobilisation are shown in Table 2.

Table 2 Parameters for immobilisation process

Modification method	**Enzyme solution**	**Time/ Temperature**
GA (2 h, 20 ^{O}C, milli-Q, 12.5 %v/v)- BSA (14 h, 20 ^{O}C, milli-Q, 1 mg/ml)- GA (2 h, 20 ^{O}C, milli-Q, 12.5 %v/v)	0.5 ml laccase (2 mg/ml) 20 mM McIlvaine buffer pH 3.0	20 hours 4 ^{O}C

Remark: GA, BSA and Milli-Q refer to glutaraldehyde, bovine serum albumin and ultrapure water

- *Determination of degradation of endocrine disrupting chemicals (EDCs) by immobilised laccase on PCL/SF blended fibre sheets*

The fibre sheets with immobilised laccase were tested for the degradation of micropollutant mixture. Bisphenol A (BPA) and 17α-ethinyl estradiol (EE2) were selected as an endocrine disrupting chemical for these studies. The micropollutant mixture consisted of 50 μM bisphenol A and 50 μM 17α-ethinylestradiol (EE2). Stock solutions with a concentration of 500 μM of these two chemicals were prepared separately by dissolving in methanol. These two solutions were mixed together and diluted with ultrapure water. All samples were placed into glass vials with 3 ml of micropollutant mixture and these vials were constantly shaken in a water bath at 37 ^{O}C. Blank samples that were prepared alike to the actual samples but in their case the immobilised laccase was inhibited by 10% sodium azide. Additional vials contained certain amount of free laccase that approximately corresponded to the units of laccase immobilised on the samples. In selected time intervals, the supernatant from all vials was collected and measured by HPLC. The sampling consisted of 70 μl of the supernatant diluted by 140 μl of methanol and 1.5 μl of 2.8% sodium azide, which was added to stop the degradation in case some of the laccase was collected within the supernatant. After the first use, all samples were removed from the reaction mixture, washed with ultrapure water and stored in ultrapure at 4 ^{O}C until they were used for the next trial.

5. Summary of the results achieved

5.1 Effect of calcium chloride on dissolution behavior of silk fibroin and morphology of silk fibroin electrospun fibres

Figure 3 exhibits the difference dissolution of silk fibroin in formic acid with different amounts of calcium chloride. It was observed that silk fibroin insoluble in formic acid, but soluble in a mixture of formic acid and calcium chloride. This shows that calcium chloride can improve the solubility of silk fibroin in formic acid. It is suggested that calcium chloride has a chaotropic property that disrupts stabilizing intramolecular forces such as hydrogen bonds, van der Waals forces and hydrophobic interactions in protein structures by shielding charges and preventing the stabilization of salt bridges. Hydrogen bonds are stronger in nonpolar media. Calcium chloride that increases the chemical polarity of the solvent can also destabilize hydrogen bonding in silk fibroin structure and ion-dipole interactions between the salts and hydrogen bonding species, which are more favorable than normal hydrogen bonds. It will make hydrophobic proteins more soluble in the solvent [21, 28]. From the results show that silk fibroin could completely dissolve in formic acid when the weight ratios 1:0.25 (w/w) of silk fibroin to calcium chloride was used.

(a) without $CaCl_2$ (b) 1:0.15 (c) 1:0.20 (d) 1:0.25

Figure 3 Photographs of dissolution of silk fibroin in formic acid with different amounts of calcium chloride; [SF:$CaCl_2$ (w/w)]

The SEM micrographs and diameter distribution of the silk fibroin electrospun fibres prepared from silk solutions with different amounts of calcium chloride are shown in Figure 4. The results show that there is a significant increase in the average fibre diameter with an increase in the amount of calcium chloride. Average fibre diameter was increased to a range of 360 nm to 720 nm. It is assumed that adding a large quantity of calcium chloride can cause a change in the evaporation of solvent. Due to calcium chloride has an ability to attract moisture from the air and surroundings, then the ability to absorb water of the silk fibroin solution was increased when an amount of salt was increased. As the result, the solution can absorb more ambient water during electrospinning. The absorption of water does not allow to complete the drying process during the time of flight of the solution jet and this phenomenon could cause a slowing in evaporation of the solvent, which may cause an increase in fibre diameter and produce congealed mats instead of unwoven fibres [29-30]. Therefore, the weight ratio of 1:0.25 and 1:0.30 (w/w) of silk fibre to calcium chloride seem to be a suitable ratio for a preparation of silk fibroin solution, which used for an electrospinning process. Eventually, the weight ratio of 1:0.25 (w/w) was preferred and used throughout the remaining experiment.

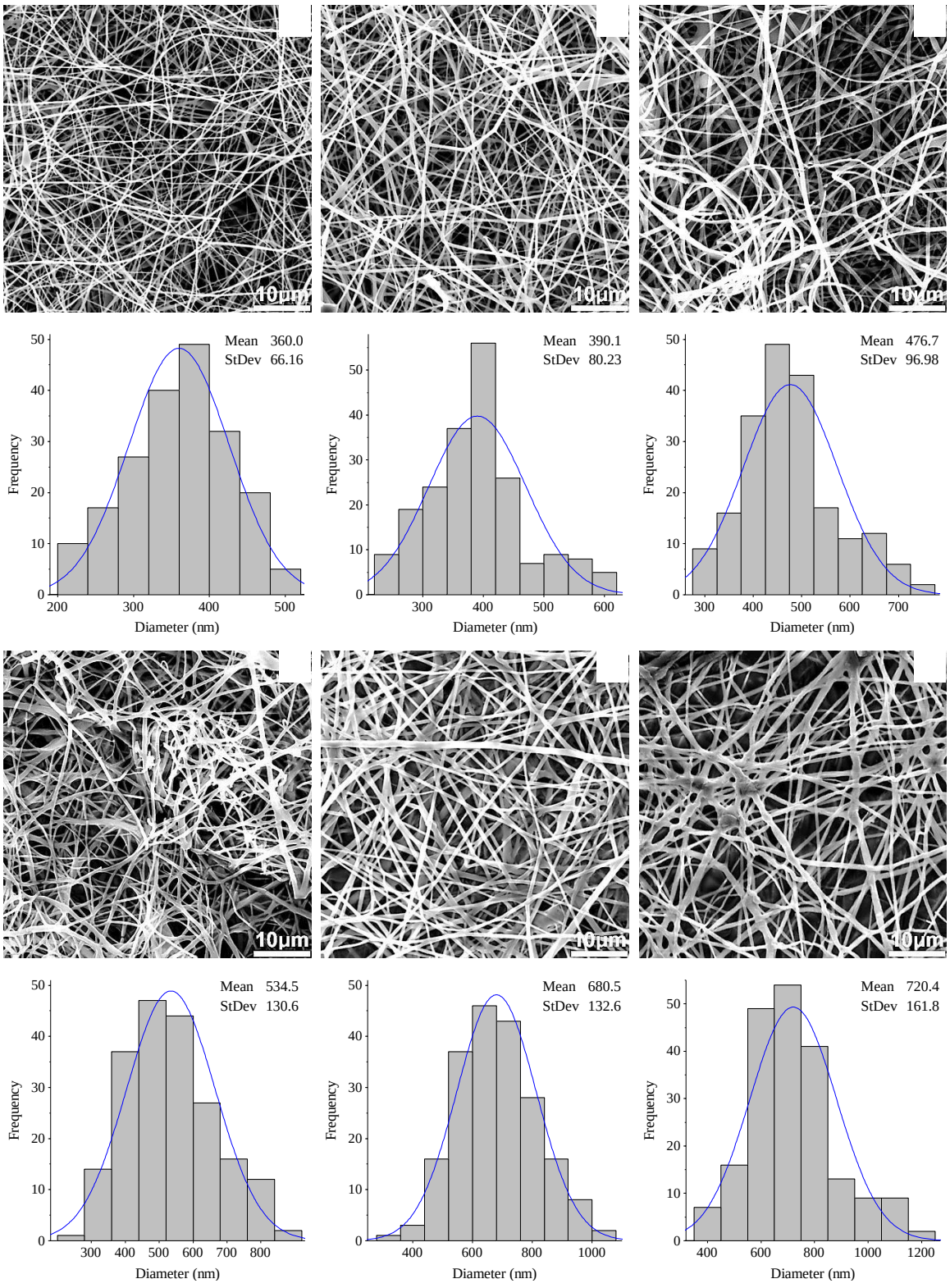


Figure 4 SEM micrographs and diameter distribution of electrospun fibres prepared from silk fibroin 8 wt% with different amounts of $CaCl_2$. a) 1:0.25; b) 1:0.30; c) 1:0.35; d) 1:0.40; e) 1:0.45; f) 1:0.50 (SEM magnification 5 kx).

5.2 Effect of parameters on needleless electrospinning of silk fibroin

- Effect of silk fibroin concentration

Considered the effect of concentration of spinning solution on fibre morphology with the applied voltage of 60 kV, when the silk fibroin concentration increased from 6 wt% to 14 wt%. The effect of silk concentration on the morphological appearance and diameter of the electrospun fibres was investigated by SEM, as shown in Figure 5. It was found that an increase in the concentration of silk fibroin solution produces a significant effect on the average fibre diameter and the uniform diameter distribution of the obtained electrospun fibre. The results show that under the same electrospinning conditions, the fibre diameter and non-uniform fibre diameter distribution of the obtained electrospun fibres increased with an increase in the silk fibroin concentration, demonstrating the important role of the concentration of the spinning solution in fibre formation during the electrospinning process. When the concentration increased from 6 wt% to 14 wt%, the average fibre diameter increased from 191 nm to 1500 nm, respectively.

The concentration of the polymer solution reflects the number of entanglements of polymer chains in the solution, which, in turn, affect the viscosity of the solution. An increase in the concentration of the silk solution will result in greater polymer chain entanglement in the solution. Thus, the viscosity of the solution also increases. At higher concentrations, the diameter of the fibre is greater. In addition, the interaction between the solution and the charges on the jet determine the distribution of the fibre diameters obtained. This is probably due to the number of jets that form during electrospinning. Multiple jets may form from the main electrospinning jet, which is stable enough to yield fibres of smaller diameter at certain concentrations, thereby generating fibres with various diameters [31-32].

In this study, the concentration of the silk solution played an important role in the spinnability of the needleless system. At low concentrations of the spinning solution, nonfibrous formations were produced instead of nanofibres with beads. It is possible that Taylor cones are created in needleless electrospinning by picking up the spinning solution covering the surrounding spinning electrode [5, 14]. Generally, in spinning solutions with a low concentration, the viscosity of the solution is also low. Such solutions cannot be loaded on the surface of the spinning electrode because of their lack of viscosity. When Taylor cones do not form on the surface of an electrode, the electrospinning process results in nonfibrous formations [24, 31]. It was observed that no fibres were formed when silk fibroin concentration less than 5 wt% for this spinning condition. When the concentration of the silk solution was increased to 6 wt%, nanofibres were formed. Although, silk fibroin solution with the concentration of 6 wt% can spin into nanofibres with the needleless system, the spinning solution still has a low viscosity. There some droplets were observed on the obtained fibre sheet. Further increase in the concentration of silk fibroin up to 8 wt% results in continues nanofibres and droplets disappeared. This was due to the fact there were sufficient molecular chain entanglements in the polymer solution to prevent the breakup of the electrically driven jet and to allow the electrostatic stresses to further elongate the jet to form fibres. Therefore, the silk fibroin solution should have a concentration at least 8 wt% to produce continuous silk fibres with a nanometer diameter under the experimental conditions of this study. Concentrations of silk fibroin in the range of 8 wt% to 12 wt% seem to be a suitable concentration for a preparation of nanofibre sheets with needleless electrospinning.

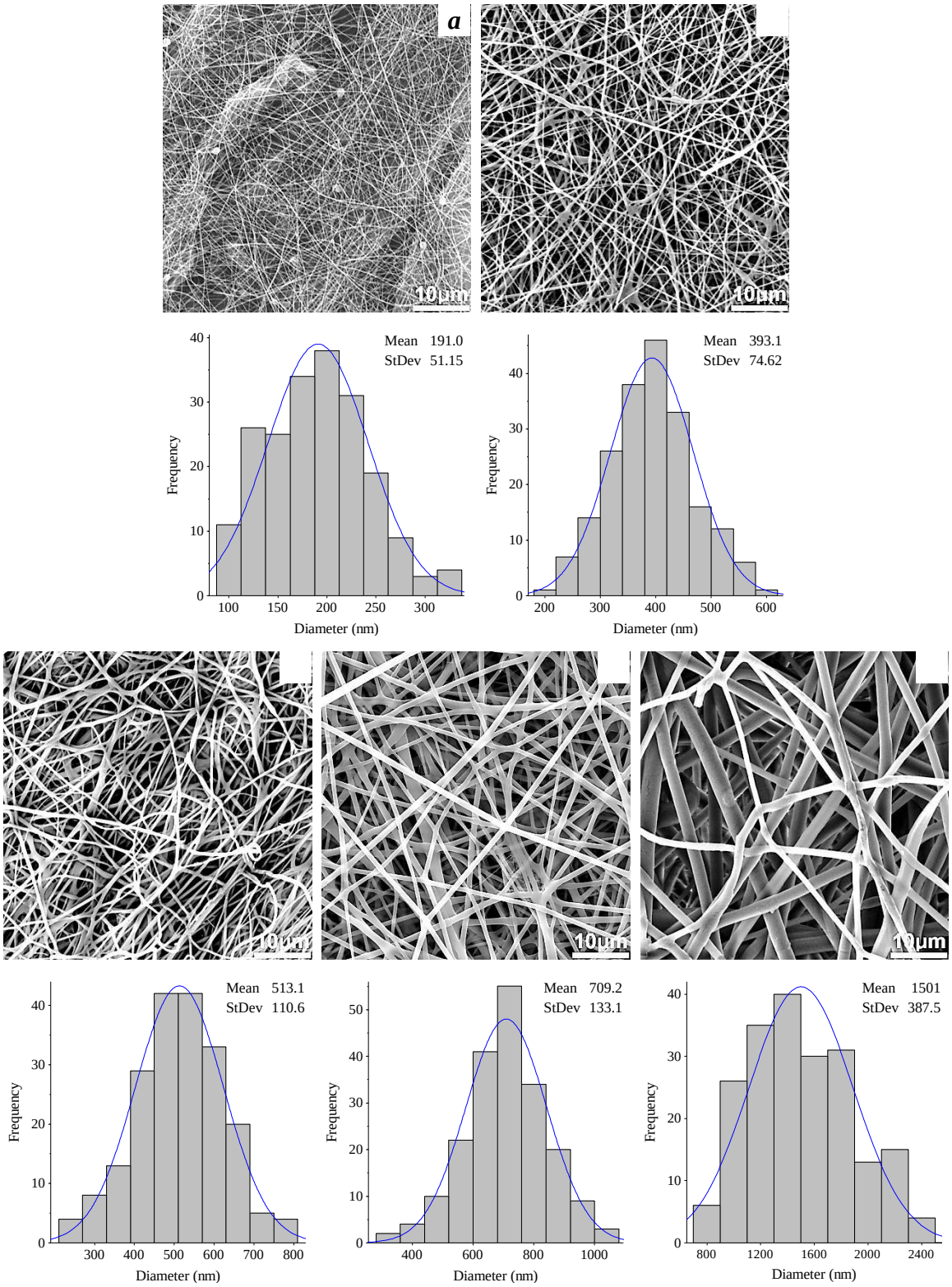


Figure 5 SEM micrographs and diameter distribution of electrospun fibres produced by needleless electrospinning with silk fibroin solution at various concentrations. (a) 6 wt%, (b) 8 wt%, (c) 10 wt%, (d) 12 wt%, (e) 14 wt% (magnification 5 kx).

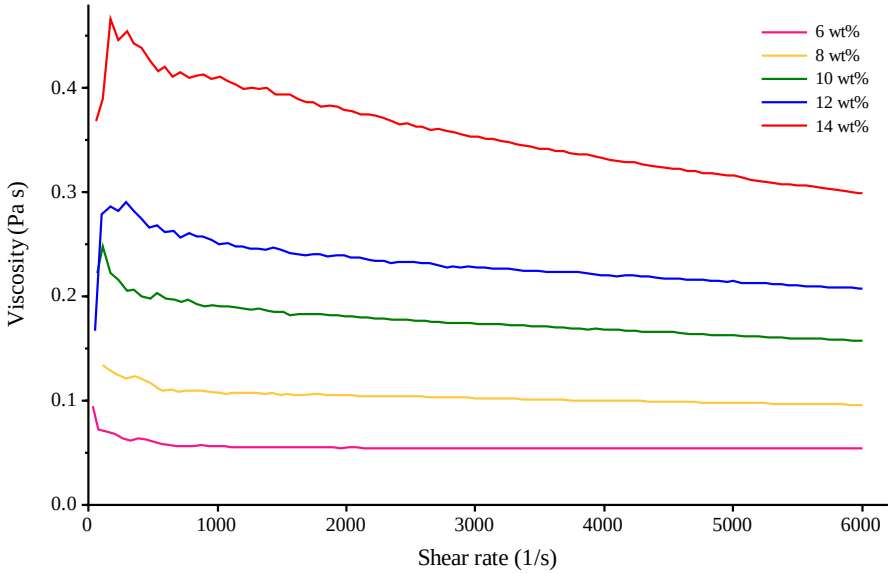


Figure 6 Rheological behaviour of spinning solutions prepared from different concentrations of silk fibroin.

For comparison, silk electrospun fibres prepared by using silk powder from the ternary solvent system of $CaCl_2/C_2H_5OH/H_2O$, was also electrospun by the needleless system. Under the same operating conditions, the diameters of the silk electrospun fibres obtained from the ternary solvent system were smaller and the diameter distribution was narrower than those obtained from the solvent system composed of formic acid and calcium chloride (see Fig.7). However, the disadvantage of the ternary solvent system is time-consuming and complicated preparation process.

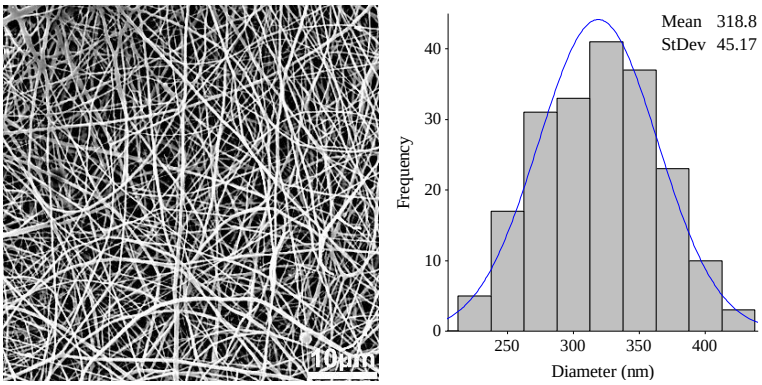


Figure 7 SEM micrograph and diameter distribution of electrospun fibres produced by needleless electrospinning with 12 wt% silk powder from the ternary solvent system (SEM magnification 5 kx).

In addition to affecting the fibre morphology and spinnability, the concentration of the spinning solution also influenced the spinning performance. Under the same processing parameters, with the increase in the silk fibroin concentration, the spinning performance increased constantly. The spinning performance of the electrospinning process increased from 0.224 g/min/m to 2.051 g/min/m when the concentration increased from 6 wt% to 14 wt%. The reason for the increased spinning performance of the process at the increased silk fibroin concentration was the high solution viscosity, which facilitated jet/filament formation.

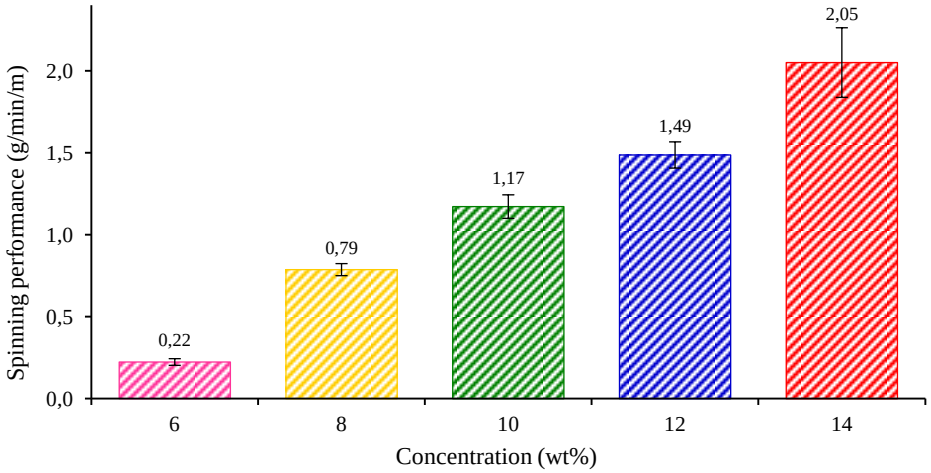


Figure 8 Effect of silk fibroin concentration on spinning performance of the process.

SEM micrographs of silk fibroin electrospun fibres (12 wt%) after treatment with ethanol are shown in Figure 9. Compared with non-treated electrospun fibres, morphologies of all treated fibre sheets were significantly changed. The diameters of treated electrospun fibres increased in comparison with non-treated relevant electrospun fibres. This was probably due to the swelling of the silk fibroin electrospun fibres by ethanol during treatment. The diameters of silk fibroin electrospun fibres after treatment with ethanol was slightly increased. On the other hand, In addition, the non-treated electrospun fibres sheet was found to shrink and dissolve after being soaked in deionized water. As shown in Figure 9 (d), the nanofibres were obviously swollen and bonded with each other. However, the treated nanofibrous scaffolds after being soaked in deionized water for 2 days still maintained good morphologies.

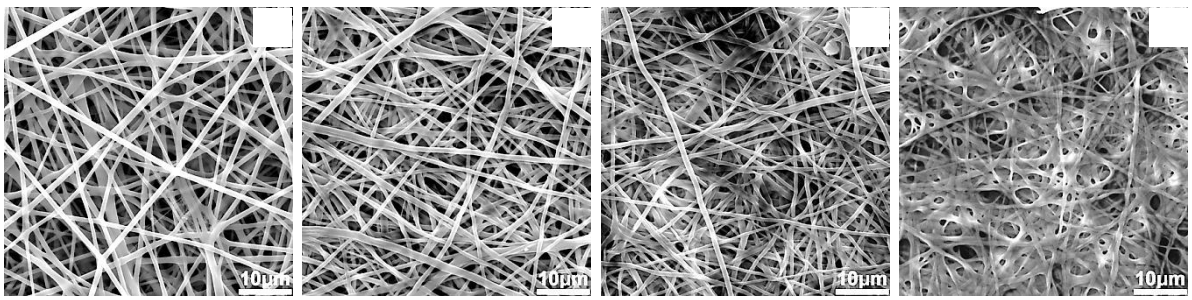


Figure 9 SEM micrographs of silk fibroin electrospun fibres (a) non-treated fibres, (b) electrospun fibres after treatment with ethanol, (c) electrospun fibres after treatment with ethanol and soaking in distilled water for 2 days, (f) non-treated fibres after soaked in water, (SEM magnification 5 kx).

- Effect of applied voltage

The applied voltage is a very important parameter with regard to the formation of jets in electrospinning systems because a high voltage is used to create an electrically charged jet of a polymer solution [10, 13]. For evaluating the effect of the applied voltage on the electrospinning process, silk fibroin solutions with the concentration 8 wt% and 12 wt% were electrospun at a voltage between 30 kV and 60 kV. SEM micrographs of the obtained fibre and their diameter distributions at the different applied voltages are shown in Figure 10 and 11, respectively.

In this electrospinning system, when the silk fibroin solution was charged with an electric voltage higher than 26 kV, a number of jets were generated from the surface of the spinning electrode. The results show that increasing the applied voltage produced an effect on the fibre diameter, but the concentration of silk fibroin solution apparently has more effect on the fibre diameter than the applied voltage. With an increase in the applied voltage from 30 kV to 60 kV, the average fibre diameter was decreased from 487 nm to 393 nm for 8 wt% of silk solution and from 940 nm to 710 nm for 12 wt% of silk fibroin solution, respectively.

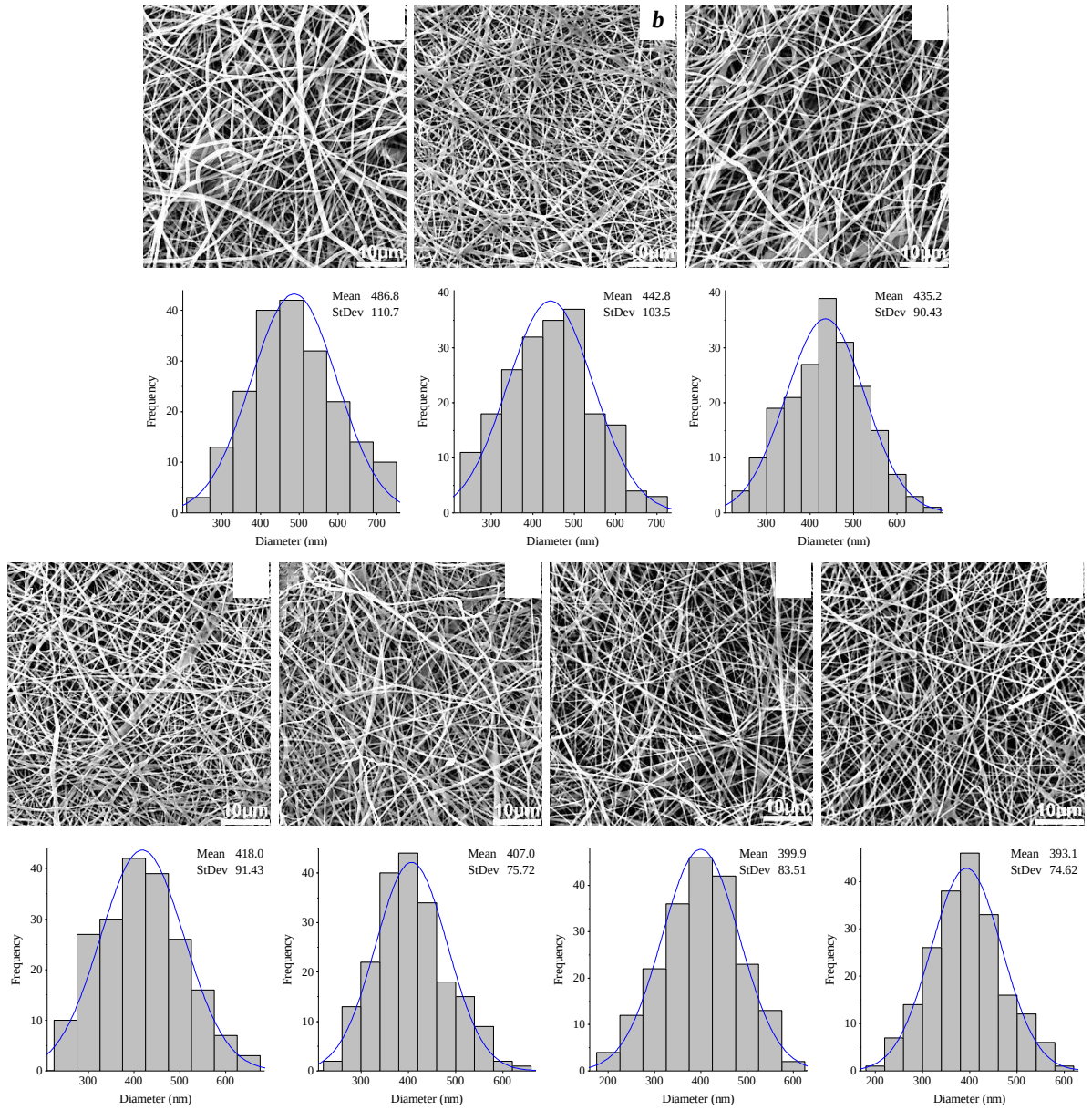


Figure 10 SEM micrographs and diameter distribution of electrospun fibres prepared by needleless electrospinning from silk fibroin 8 wt% at various applied voltage (a) 30 kV, (b) 35 kV, (c) 40 kV, (d) 45 kV, (e) 50 kV, (f) 55 kV, (g) 60 kV (SEM magnification 5 kx).

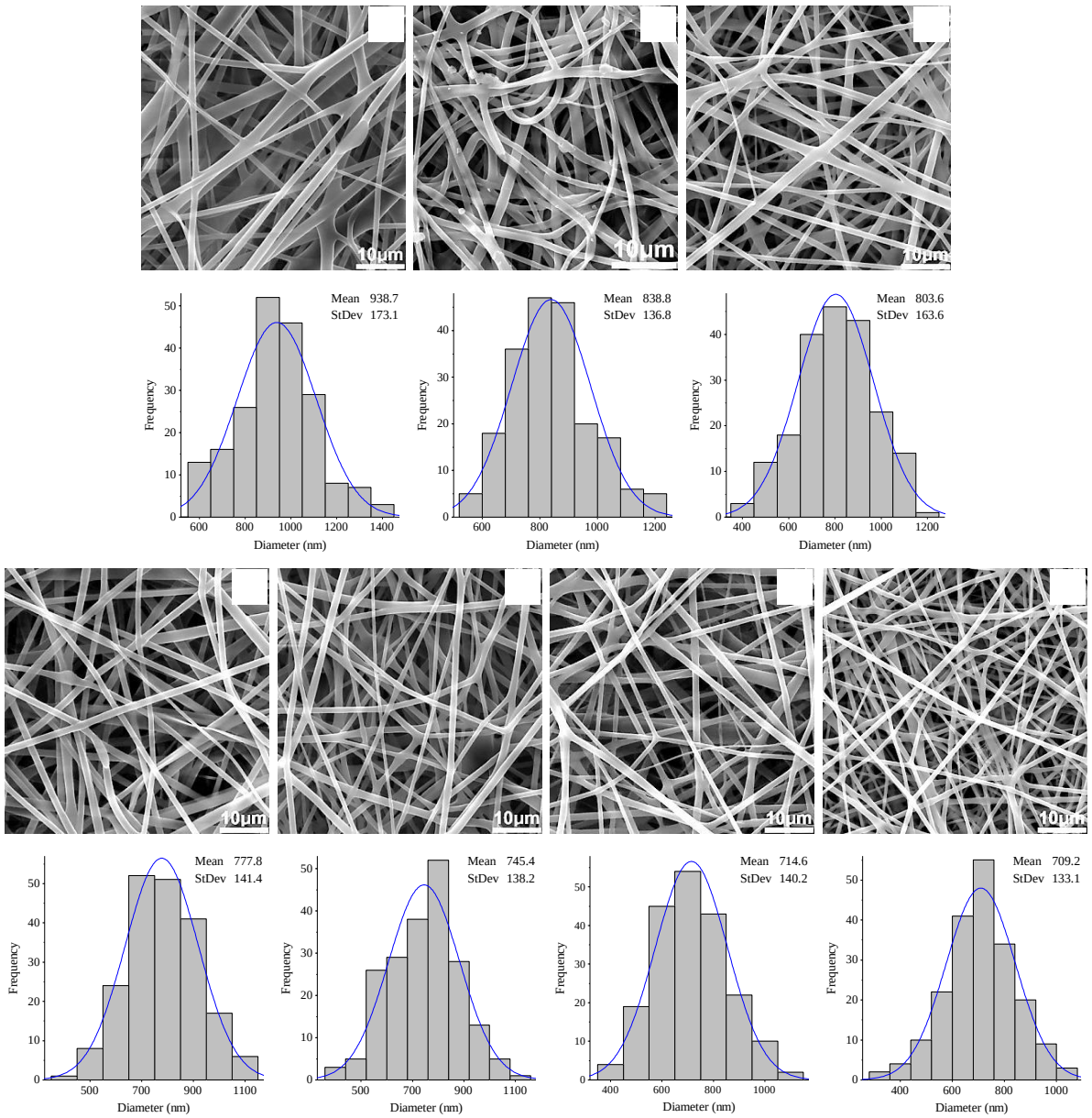


Figure 11 SEM micrographs and diameter distribution of electrospun fibres prepared by needleless electrospinning from silk fibroin 12 wt% at various applied voltage (a) 30 kV, (b) 35 kV, (c) 40 kV, (d) 45 kV, (e) 50 kV, (f) 55 kV and (g) 60 kV (SEM magnification 5 kx).

However, the spinning performance of electrospinning process was influenced by the applied voltage and polymer concentration. The spinning performance of the process changed from 0.038 g/min/m to 0.787 g/min/m with silk fibroin concentration of 8 wt% and from 0.246 g/min/m to 1.487 g/min/m with silk fibroin concentration of 12 wt%, when the applied voltage was increased from 30 kV to 60 kV. As the electric field is the main driving force initiating the formation of Taylor cones and jets from the surface of the solution, increasing the electric voltage increases the electrostatic force on the polymer jet, which favours more elongation of the jet and the formation of smaller fibres. On the other hand, the electric field also functions to overcome the frictional forces that act within the moving polymer solution and to accelerate filament movement toward the collector

electrode. It is easier to generate solution jets at higher applied voltage in a polymer solution charged by a stronger electric field because a larger amount of solution is removed from the surface of the solution, thereby improving the spinning performance of the process [7, 10, 12].

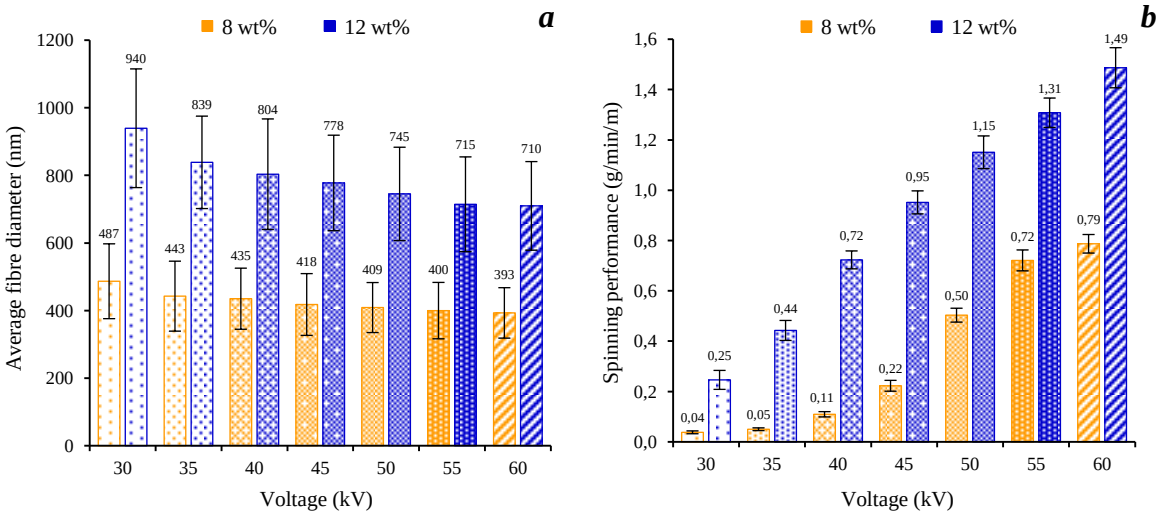


Figure 12 Effect of applied voltage on (a) average fibre diameter and (b) spinning performance of the process.

- Effect of distance between electrodes

To study the effect of spinning distance on the morphology of the obtained electrospun fibres and the spinning performance of the process. Spinning solutions with a concentration of 8 wt% and 12 wt% were electrospun at a high voltage of 60 kV. Electrospinning was carried out at a distance of 100 mm, 125 mm and 150 mm. SEM micrographs of the resulting fibres and their distributions at the different spinning distance are shown in Figure 13 and 14, respectively.

The results show that an increase in a spinning distance had a less significant effect on the average fibre diameter but produces an influence on the spinning performance of electrospinning process. An increase in the distance from 100 mm to 150 mm, the average fibre diameter was decreased from 393 nm to 343 nm with silk fibroin concentration of 8 wt% and from 710 nm to 647 nm with silk fibroin concentration of 12 wt%. The performance of electrospinning process changed from 0.787 g/min/m to 0.295 g/min/m with silk fibroin concentration of 8 wt% and from 1.487 g/min/m to 0.695 g/min/m with silk fibroin concentration of 12 wt% when the distance was increased.

The distance between the spinneret and the collector is a key factor in determining the morphology of fibres and the spinning performance that are produced. It is suggested that increasing the distance has the same effect as decreasing the applied voltage and this will cause a decrease in the field strength. Since the electric field is the main driving force to initiate the formation of jets from the surface of the solution, decreasing the electric voltage will decrease the electrostatic force on the polymer jet, which results in a decrease of the spinning performance of the process. In other circumstances, increasing the distance results in a decrease in the average fibre diameter.

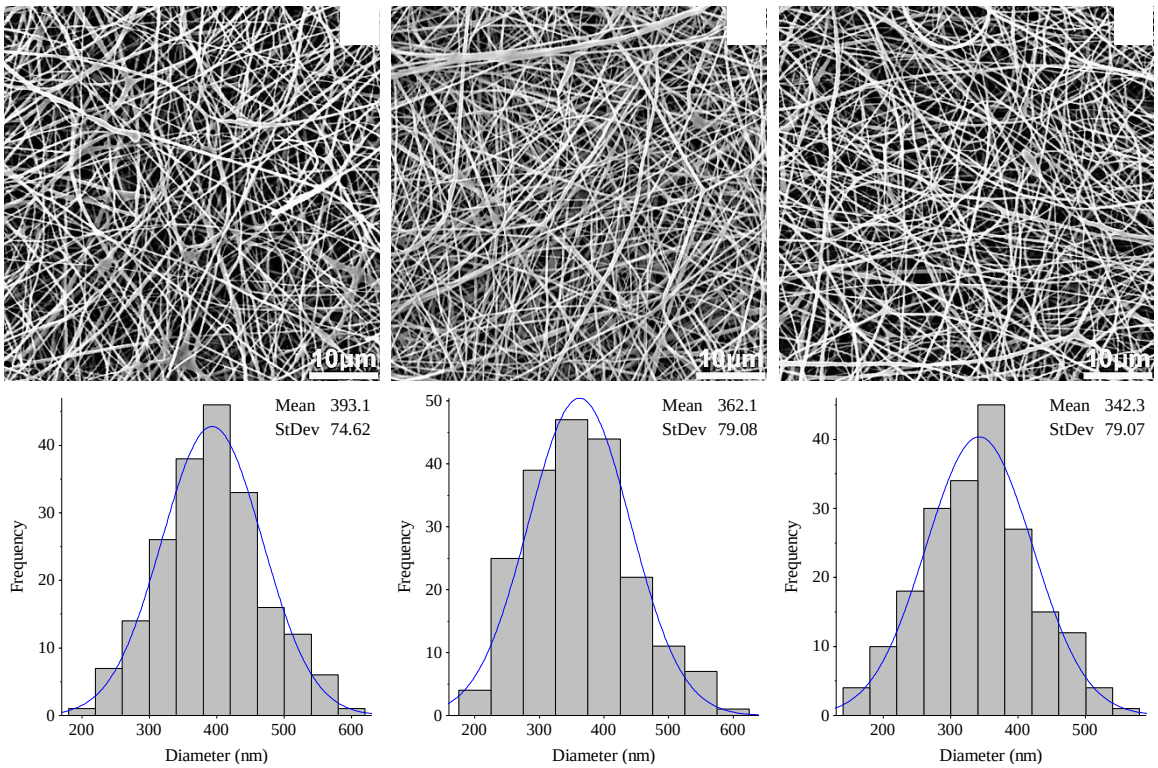


Figure 13 SEM micrographs and diameter distribution of electrospun fibres prepared by needleless electrospinning from silk fibroin 8 wt% at different spinning distance. (a) 100 mm; b) 125 mm; c) 150 mm (SEM magnification 5 kx).

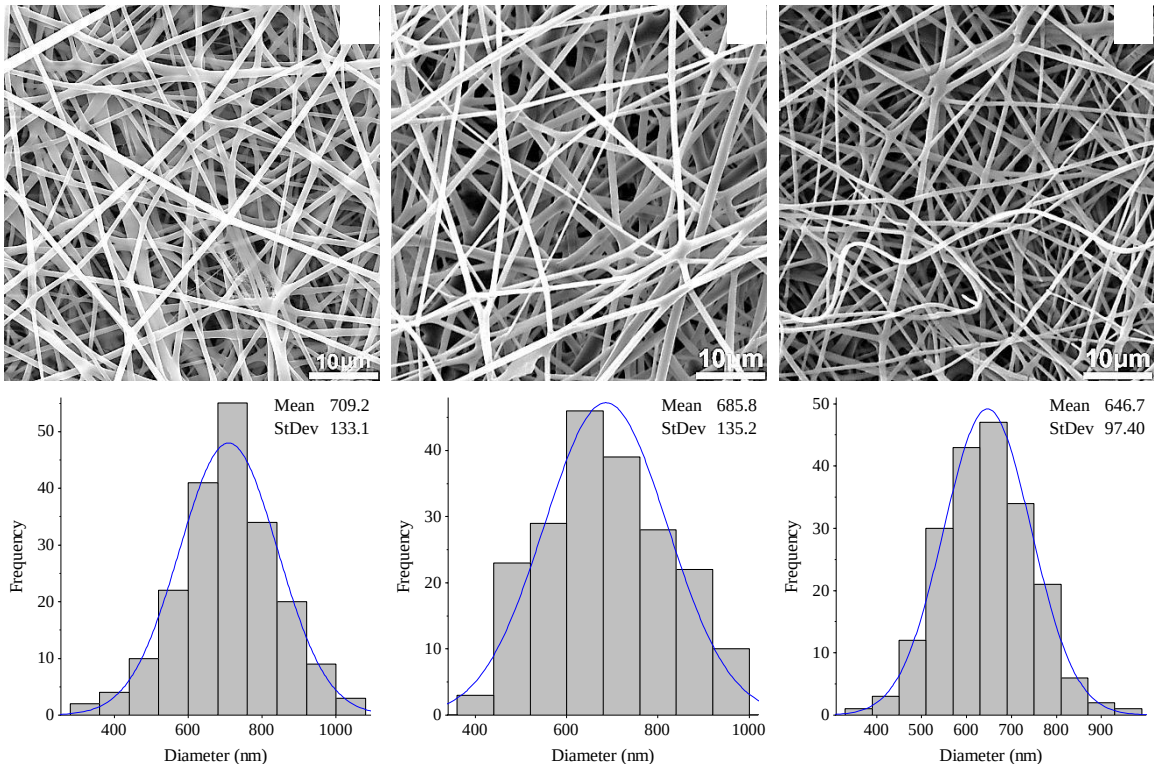


Figure 14 SEM micrographs and diameter distribution of electrospun fibres prepared by needleless electrospinning from silk fibroin 12 wt% at different spinning distance. (a) 100 mm; b) 125 mm; c) 150 mm (SEM magnification 5 kx).

As mentioned earlier, jet elongation and thinning only happens while the jet is in flight and still a fluid. This elongation occurs owing to charge repulsion between ions in the solution combined with a net pull towards the collector. While in flight, the polymer solution solidifies as the solvent is evaporated from the surface, forming polymer fibres. Therefore, increasing the spinning distance will increase the time for thinning to occur and provided the polymer is not yet solid, the fibre diameter will be reduced [29].

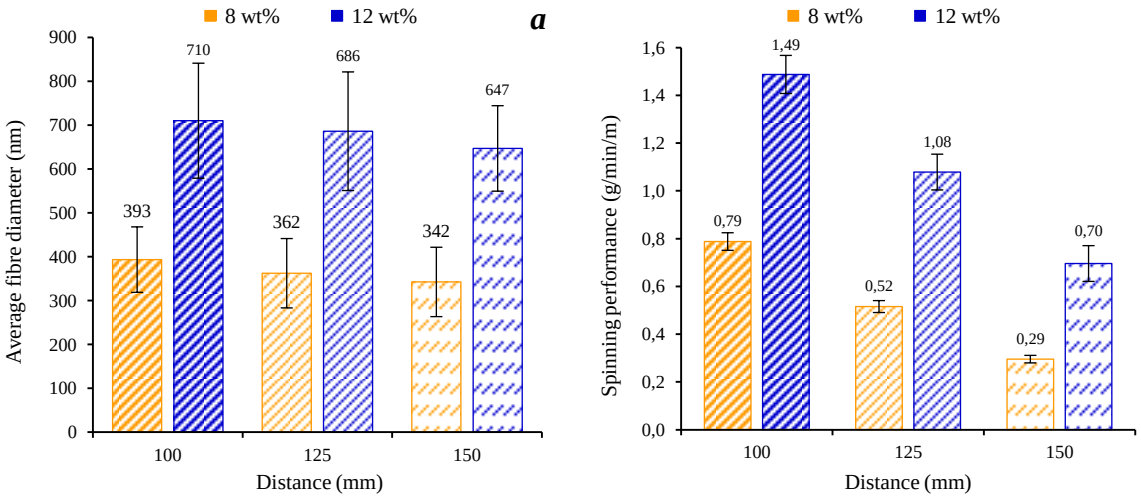


Figure 15 Effect of spinning distance on (a) average fibre diameter and (b) spinning performance of the process

5.3 Effect of polycaprolactone on needleless electrospinning of silk fibroin

- Effect of blend ratio of silk fibroin and polycaprolactone on properties of spinning solution

The properties of silk fibroin/polycaprolactone blended solution including conductivity, surface tension and viscosity at various weight ratios are shown in Figure 16 and 17, respectively. The results show that the variation of the weight ratio of polycaprolactone in the blended solution had a significant effect on properties of the solutions. When silk fibroin solution (12 wt%) blends with a solution of 15 wt% polycaprolactone, it is obviously clear that conductivity and viscosity of the blended solution decreased with increasing weight ratio of polycaprolactone, while surface tension of the blended solution was increased. It seems that conductivity and surface tension of the blended solutions are affected by a variation of polycaprolactone content in the solution. In an electrospinning process, spinning parameters of the process were the same for all the solution. It can assume that conductivity and surface tension of the blended solutions are responsible for significant differences in a fibre morphology and a spinning performance of the process.

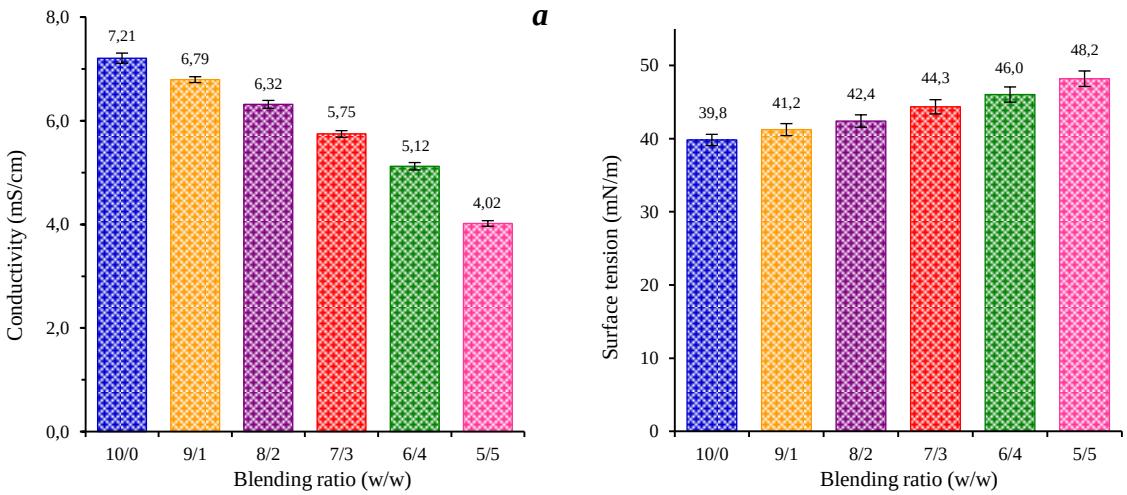


Figure 16 Effect of blend ratio of silk fibroin and polycaprolactone on (a) conductivity and (b) surface tension [blending ratio 10/0 (w/w) means pure silk fibroin solution].

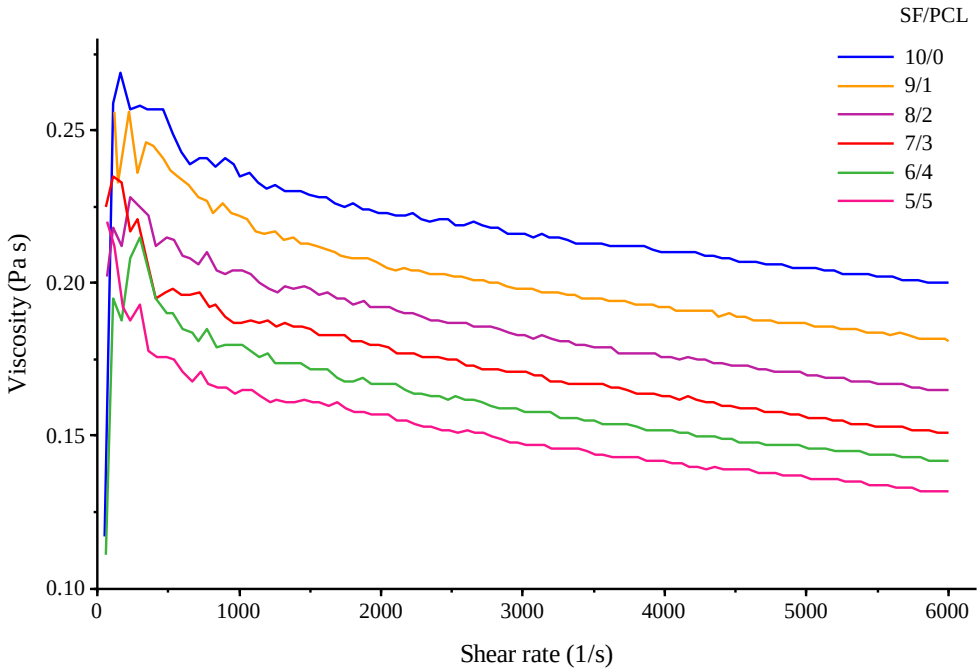


Figure 17 Rheological behaviour of silk fibroin and polycaprolactone blended solutions at various weight ratios

- Morphology of silk fibroin/polycaprolactone blended electrospun fibres

SEM micrographs and diameter distribution of the electrospun fibre composed of silk fibroin and polycaprolactone at various weight ratios of polycaprolactone solution are shown in Figure 18. The results show that under the same concentration of silk solution and electrospinning conditions, the fibre diameter and diameter distribution of the obtained electrospun fibres decreased in accordance with an increase in the weight ratio of polycaprolactone in a spinning solution. When the weight ratio of polycaprolactone in the blended solution increased from 9/1 to 5/5, the average fibre diameter decreased from 691 nm to 340 nm, respectively.

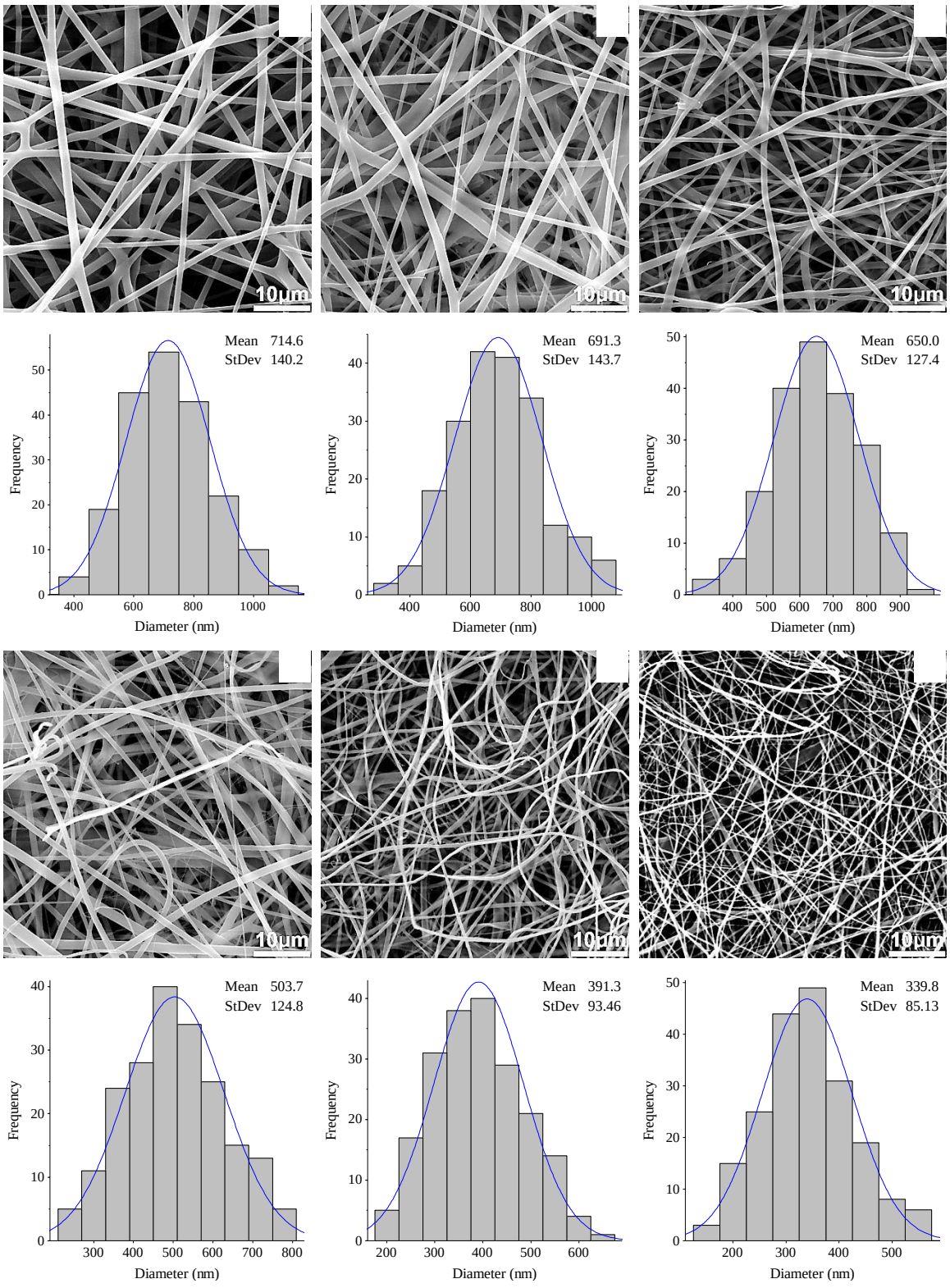


Figure 18 SEM micrographs and diameter distribution of electrospun fibres produced by needleless electrospinning with SF 12 wt% and PCL 15 wt% at various weight ratios. a) 10/0, b) 9/1, c) 8/2, d) 7/3, e) 6/4, f) 5/5, (SEM magnification 5 kx).

In particular, pure silk fibroin electrospun fibres had larger diameters and showed greater variation in fibre diameter when compared to silk fibroin/polycaprolactone electrospun fibres, which is attributed to differences in properties of spinning solution.

This was presumably due to the dielectric constant of the polycaprolactone solution in this solvent system. Generally, the dielectric constant of a solvent has a significant influence on electrospinning; a solution with a greater dielectric property reduces the diameter of the resultant electrospun fibre and improves uniformity. The bending instability of the electrospinning jet also increases with higher dielectric constant. This may also facilitate the reduction of the fibre diameter due to the increased jet path [32]. In addition, Luo at el [33] described the influence of dielectric constant of solvent systems on an electrospinning of polycaprolactone solutions. They observed that the dielectric constant of the solvent showed a dominant influence on the diameters of the polycaprolactone electrospun fibres. When dissolved polycaprolactone in the solvent with dielectric constants was ~19 and above (at 20 ^{O}C), diameters of polycaprolactone electrospun fibre in the nanometer range were achieved.

In these experiments, polycaprolactone was dissolved in formic acid, a solvent with high dielectric constant (ε ~58 at 20 ^{O}C), also resulted in a reduction in electrospun fibre diameter. Therefore, when silk fibroin solution was blended with polycaprolactone solution in this solvent system, the blended solution will have a greater dielectric constant than pure silk fibroin solution (it can assume that pure silk fibroin solution has a lower dielectric constant than polycaprolactone solution due to a presence of calcium chloride in silk fibroin solution). As a result, the fibre diameter of obtained fibres tends to decrease as the polycaprolactone content in the blended solution increases. In addition to affecting the diameter of electrospun fibres, the weight ratio of polycaprolactone in the blended solution also influenced the spinning performance. Under the same processing parameters, with the increase in the weight ratio of polycaprolactone in the blended solution, the spinning performance decreased (Fig. 19). When the weight ratio of polycaprolactone in the blended solution increased from 9/1 to 5/5, the spinning performance decreased from 1.104 g/min/m to 0.552 g/min/m, respectively. From the results, it is possible that the addition of polycaprolactone causes an increase in surface tension and a reduction in conductivity of the blended solution. Electrospinning involves stretching of the solution caused by repulsion of the charges at its surface. A reduction in conductivity tends to decrease the charge density at the surface of the jets, which it results in a lower bending instability. As a result, the deposition area of the electrospun fibres is decreased and will also decrease the throughput of electrospinning process [34].

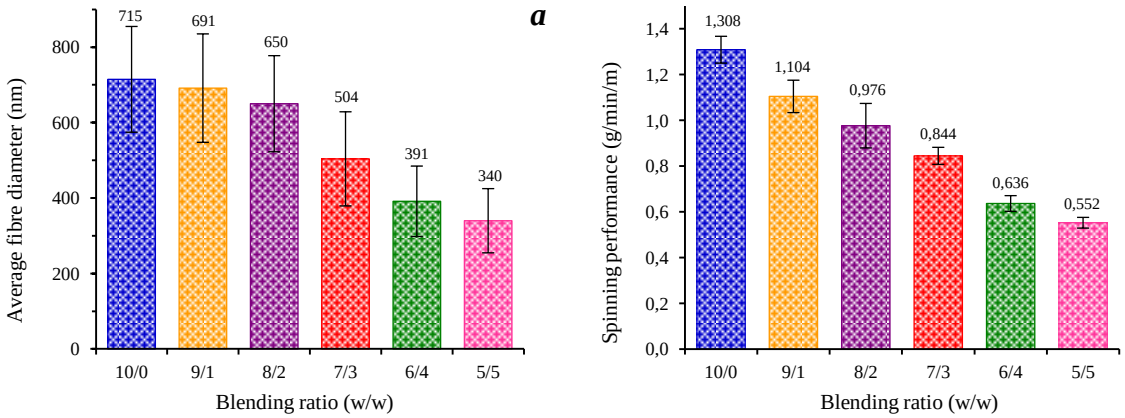


Figure 19 Effect of blend ratio of silk fibroin and polycaprolactone on (a) average fibre diameter and (b) spinning performance of the process.

- Effect of blend ratio of silk fibroin and polycaprolactone on physical properties of the obtained elctrospun fibres

Tensile properties of silk fibroin and silk fibroin/polycaprolactone blended fibre sheets are shown in Figure 20. From the results, silk fibroin electrospun fibre sheet showed poor tensile properties (typical brittle fracture), the average elongation at break was 5.49%, and the average tensile strength was 5.5 N. In the case of silk fibroin/polycaprolactone blended fibre sheets, the tensile properties were significantly different, depending on the blend ratios of silk fibroin and polycaprolactone in the solution. Considered the effect of the blend ratios on tensile properties of the blended fibre sheets, when the weight ratio of blended solutions (SF/PCL) changed from 9/1 to 5/5, the average elongation at break increased from 7.29% to 19.51%, respectively. This indicated that the elasticity of silk fibroin electrospun fibres sheet was improved by blending with polycaprolactone.

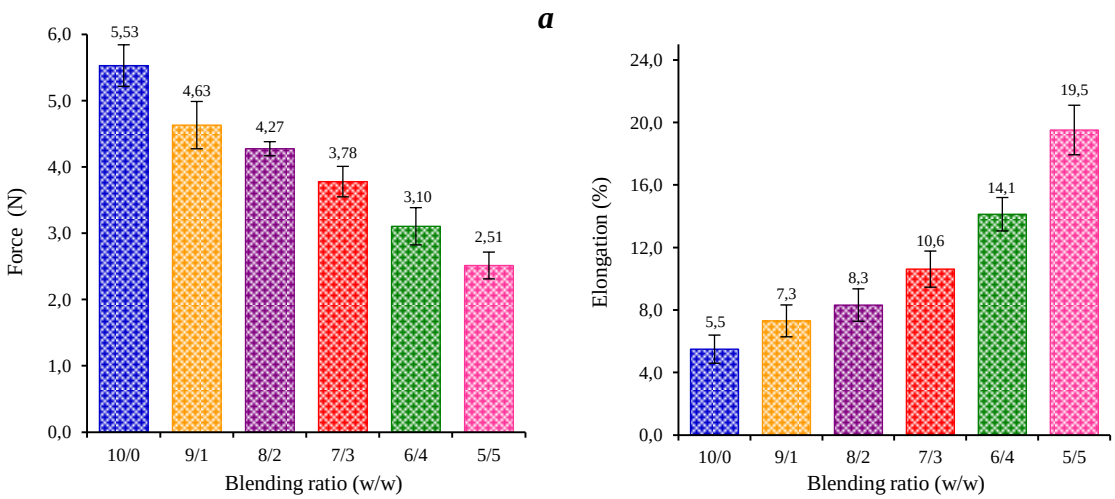


Figure 20 Effect of blend ratio of silk fibroin and polycaprolactone on (a) tensile strength and (b) elongation at break of the electrospun fibre sheets.

The water contact angle of silk fibroin and silk fibroin/polycaprolactone fibre sheets with different weight ratios was shown in Figure 21. The pure silk fibroin fibre sheet showed a water contact angle around 42.1^{O}, indicating that the silk fibres sheet possessed good hydrophilicity. When the weight ratio of blended solution (SF/PCL) changed from 9/1 to 5/5, the water contact angles of the fibre sheets increased from 51.3^{O} to 81.7^{O}, respectively. Even though, silk fibroin is mostly composed of hydrophobic amino acids (glycine and alanine) and serine, its structural characteristic provides some hydrophilicity, resulting in a lower contact angle as compared to the blended fibre sheet and pure polycaprolactone (119^{O}). However, silk fibroin/polycaprolactone blended fibre sheets still have good compatibility with living cells.

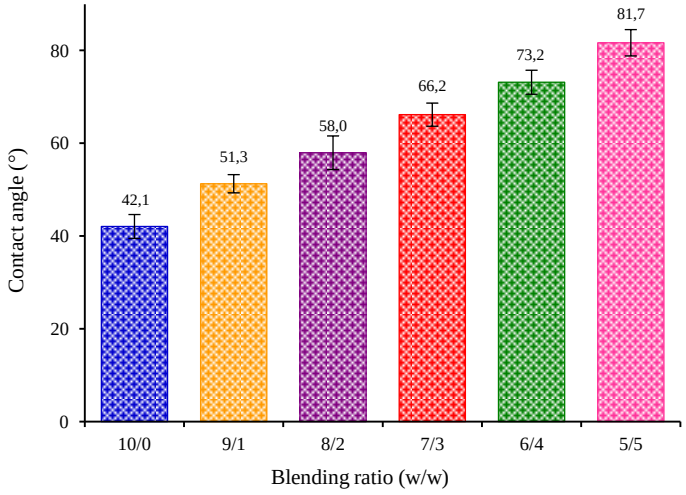


Figure 21 Effect of blend ratios of silk fibroin and polycaprolactone on water contact angle of the blended electrospun fibre sheets.

5.4 *In vitro* test results of electrospun fibre sheets from silk fibroin and its blend with polycaprolactone

- In vitro tests with 3T3 mouse fibroblasts

Cell viability seeded on silk fibroin and silk fibroin/polycaprolactone blended fibre sheets was measured by MTT test during the time of cultivation in days 1, 3, 7 14 and 21. From the results are shown in Figure 22, it can see that all tested materials support fibroblast proliferation during 3 weeks of cultivation. Cells adhered to the scaffold and proliferated through the surface. The proliferation rate increased after 7 days of cultivation where the absorbance increased. The highest proliferation rate was found between days 7 and 14. Then it was slowed down probably because of the fact that cells had covered almost all the surface of tested scaffolds.

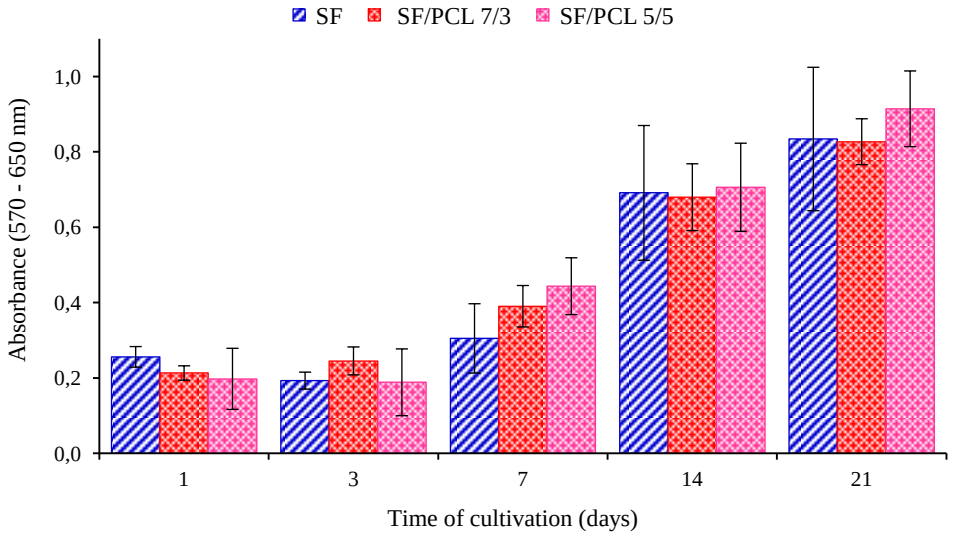


Figure 22 Cell viability measured by MTT test after cultivation with 3T3 mouse fibroblasts.

After a period of incubation, the fibre sheets were fixed and analyzed by fluorescence microscope. Red cell nuclei were stained by propidium iodide. The results are shown in Figure 23. It can see that during the time of cultivation, cells adhered and

proliferated well on the surface of the fibre sheets in all tested materials. It showed a very good biocompatibility of pure silk fibroin and silk fibroin/ polycaprolactone blended fibre sheets.

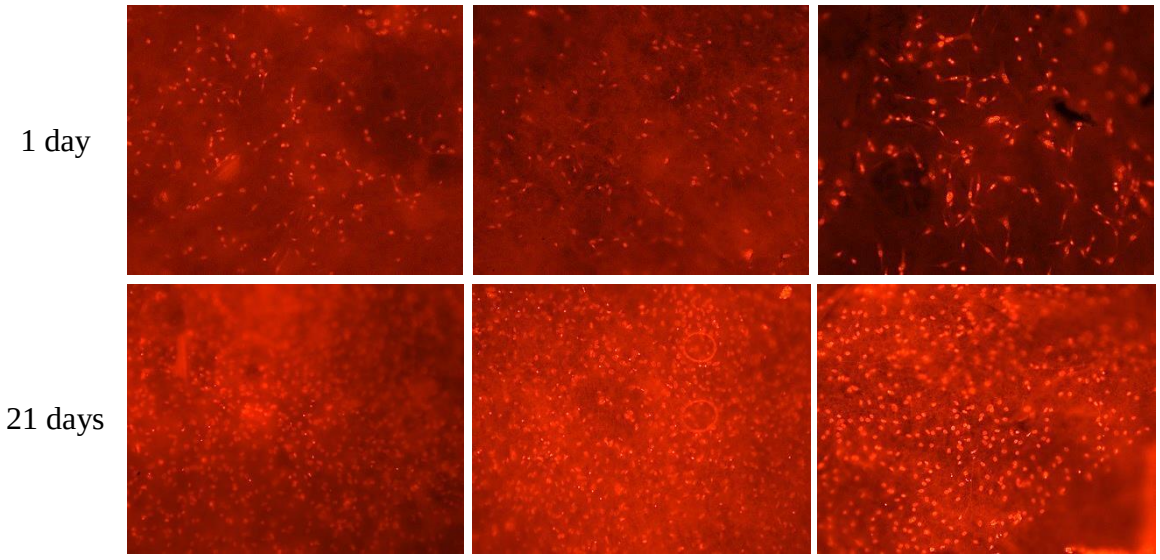


Figure 23 Fluorescence microscopy pictures of 3T3 mouse fibroblasts stained with propidium iodide during cell culture (magnification 100 x).

For electron microscopic analysis, after a period of cultivation the fibre sheets were fixed and dehydrated and analyzed by SEM microscope. The cells adhered on the surface of the scaffolds is depicted in Figure 24. The results are in agreement with fluorescence microscopy results. Cells proliferated through the surface of the fibre sheets and in the end of the experiment (after 21 days of cell culture), cells almost completely covered all the surface of the tested specimens. Finally, all tested materials showed very good properties in terms of proliferation rate.

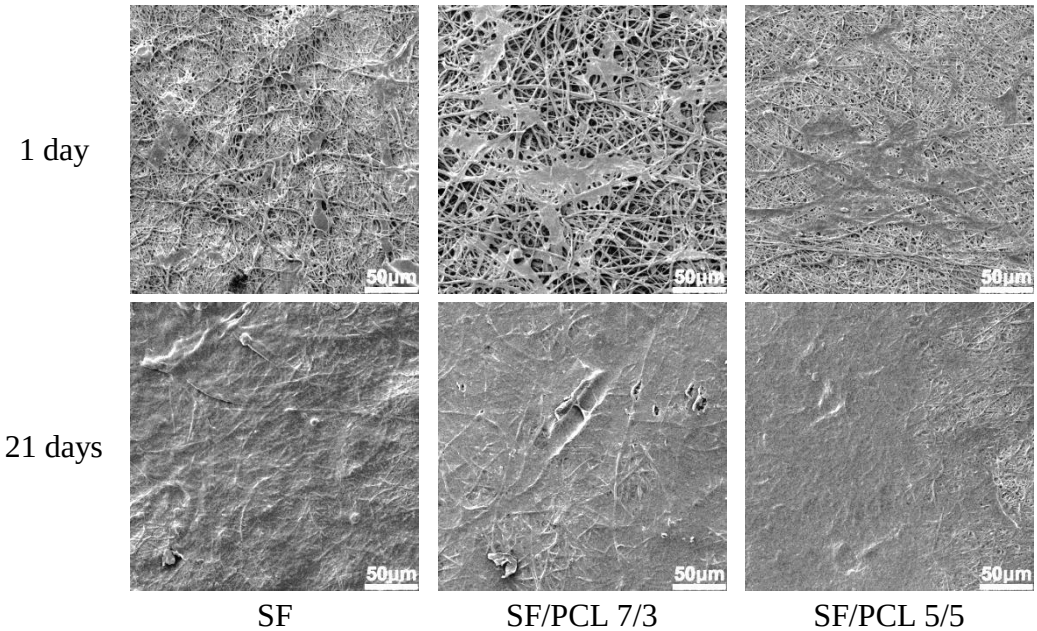


Figure 24 SEM micrographs of the fibre sheets after cell cultured with 3T3 mouse fibroblasts (SEM magnification 1 kx).

- ***In vitro tests with normal human dermal fibroblasts (NHDF)***

Cell proliferation was measured by MTT test during the time of cultivation in days 1, 3, 7 and 14. The results are shown in Figure 25, it can see that all tested samples support fibroblast proliferation during 2 weeks of cultivation. Cells adhered to the fibre sheets and proliferated through the surface. From the third day of an incubation period, the fibre sheet composed of silk fibroin/polycaprolactone in the ratio of 7/3 (w/w) showed the highest cell viability in comparison to the other tested materials. Pure silk fibroin and combination of silk fibroin and polycaprolactone in the ratio of 5/5 (w/w) showed similar cell proliferation rate during 2 weeks incubation period.

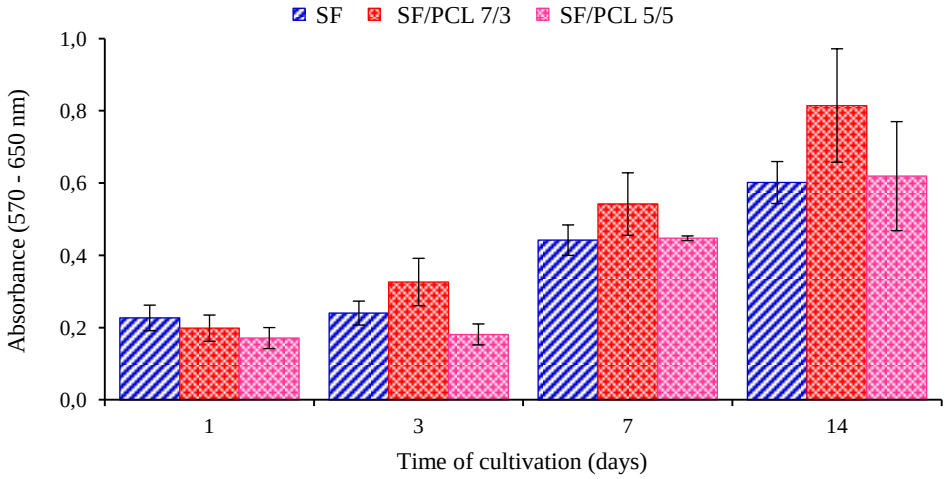


Figure 25 Cell viability measured by MTT test after cultivation with normal human dermal fibroblasts.

From the results are shown in Figure 26, it can see that during the time of cultivation, cells adhered and proliferated well on the surface of the fibre sheet in all tested materials, but silk fibroin has a high level of autofluorescence. Therefore, it is difficult to distinguish between red cell nuclei and stained fibres when pure silk fibroin or combination of silk fibroin and polycaprolactone is used.

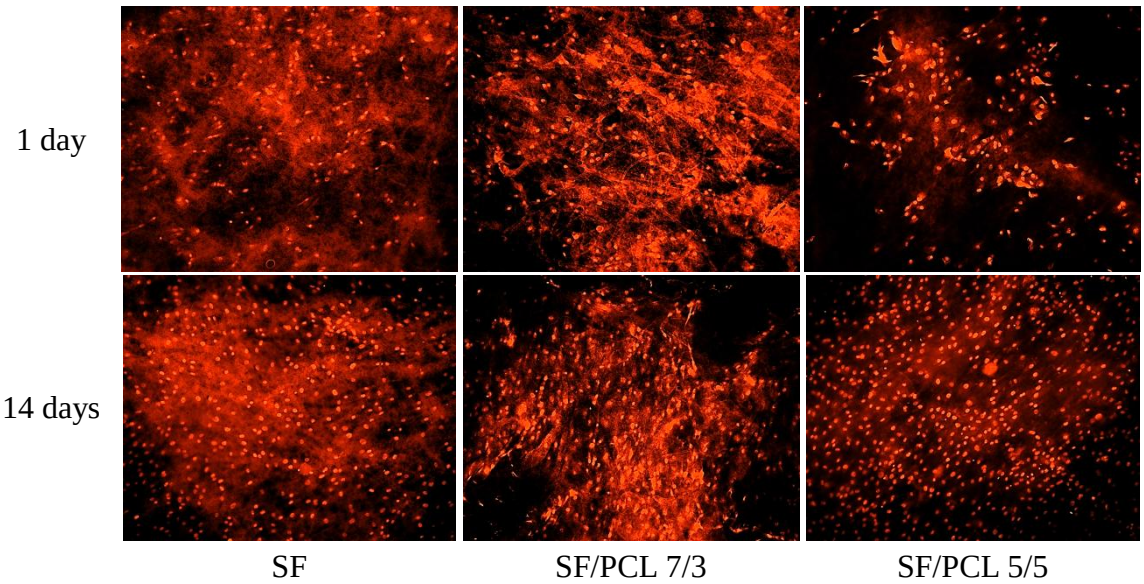


Figure 26 Fluorescence microscopy pictures of normal human dermal fibroblasts stained with propidium iodide during cell culture (magnification 100 x).

For electron microscopic analysis, the cells adhered on the surface of the fibre sheets that is depicted in Figure 27. The cells are well dispersed onto nanofibrous layers and proliferated through the surface of the fibre sheets, and at the end of the experiment cells covered almost all the surface of the fibre sheet, especially on the fibre sheet made from the composition of 7/3 (SF/PCL). This outcome is in agreement with MTT results where this layer showed the highest value of cell viability. All tested materials showed very good properties for normal human dermal fibroblast spreading through their surface.

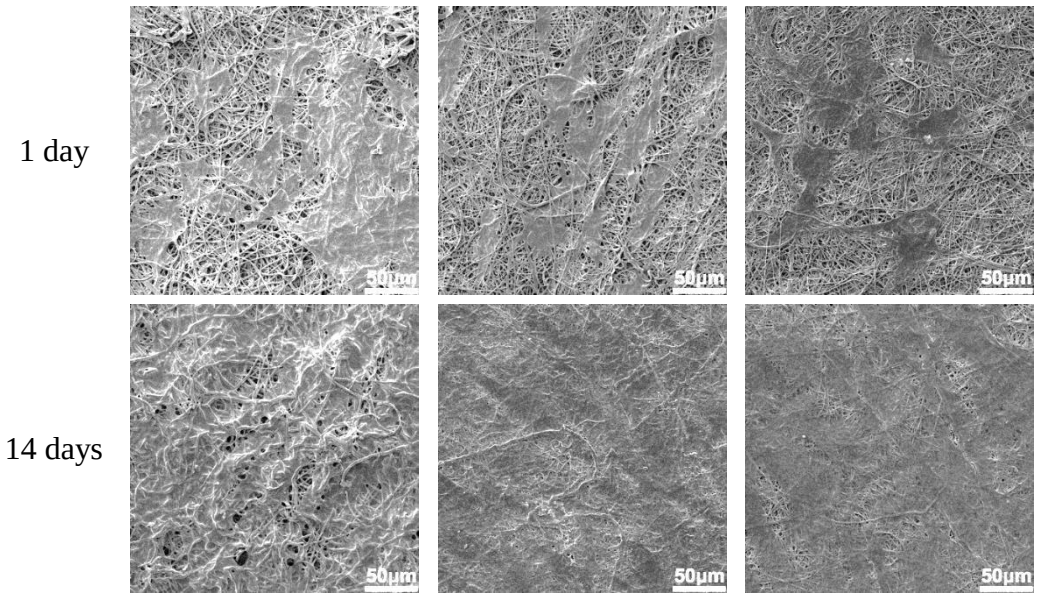


Figure 27 SEM micrographs of the fibre sheets after cells cultured with normal human dermal fibroblasts (SEM magnification 1 kx).

- ***In vitro tests with MG 63 osteoblasts***

Cell viability seeded on the fibre sheets was measured by MTT test during the time of cultivation. The results are shown in Figure 28, it can see that all tested materials support osteoblast proliferation during 2 weeks of cultivation. Cells adhered to the fibre sheet and proliferated through the surface. Silk fibroin electrospun fibre sheet showed the highest proliferation rate at the end of cultivation. This phenomenon could explain silk is a natural polymer that contains amino acid sequences that promote cell adhesion and proliferation.

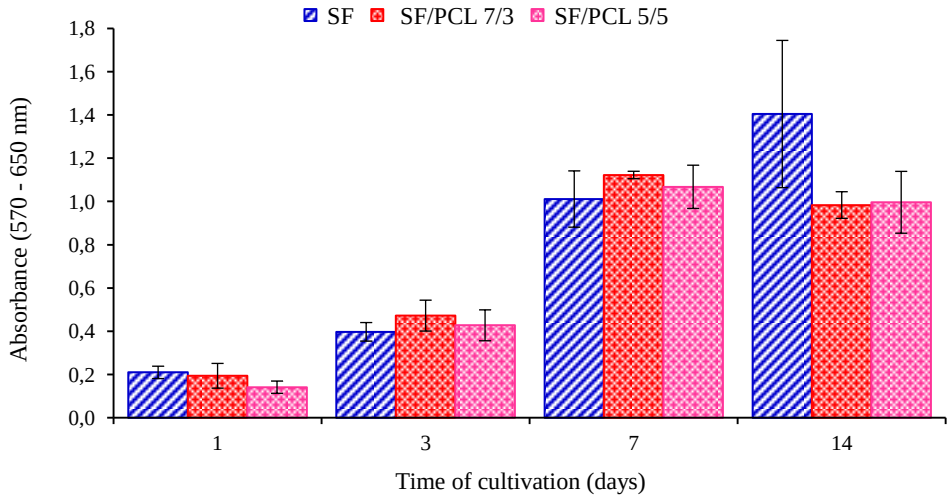


Figure 28 Cell viability measured by MTT test after cultivation with MG 63 osteoblasts.

From the results are shown in Figure 29. It can see that during the time of cultivation, cells adhered and proliferated well on the surface of the fibre sheets in all tested materials. Fluorescence microscopy pictures confirmed MTT test results that obtained by the previous experiment. It showed a very good biocompatibility of silk fibroin and silk fibroin/polycaprolactone blended fibre sheets with MG 63 osteoblasts.

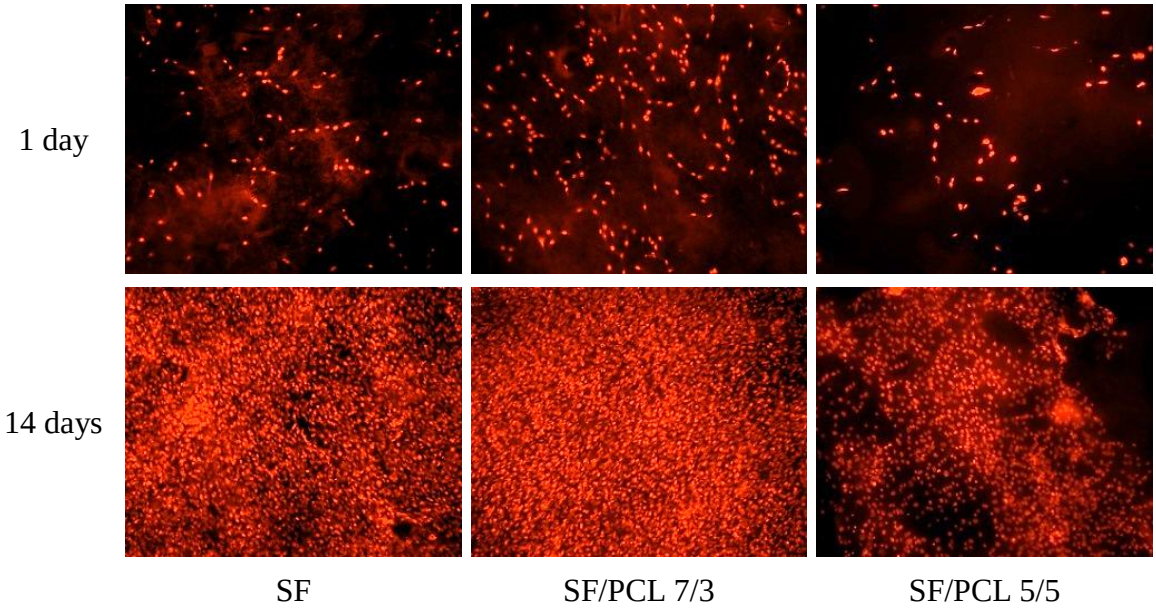


Figure 29 Fluorescence microscopy pictures of MG-63 osteoblasts stained with propidium iodide during cell culture (magnification 100 x).

SEM micrographs from scanning electron microscopy are in agreement with fluorescence microscopy as well as MTT test. As shown in Figure 30, all tested materials showed good adhesion evaluated 1 day after seeding of osteoblasts onto the fibre sheets. During the 2 weeks of the experiment, the cells proliferate through the surface of the fibre sheets and cover most of the fibre sheets surface. Cells in silk fibroin electrospun fibre sheets have different morphology. In the other fibre sheets, cells create a confluent monolayer. But in silk fibroin fibre sheets, single cells can be distinguished. It is possible that not only a single layer is created. *In vitro* tests show very good biocompatibility of all tested

nanofibrous material with MG-63 osteoblasts. The most promising one seems to be the pure silk fibroin. The results were confirmed by viability measurement with MTT test and fluorescence microscopy as well as scanning electron microscopy. From the results can conclude that silk fibroin or in combination with polycaprolactone is a good material for bone tissue engineering scaffolds.

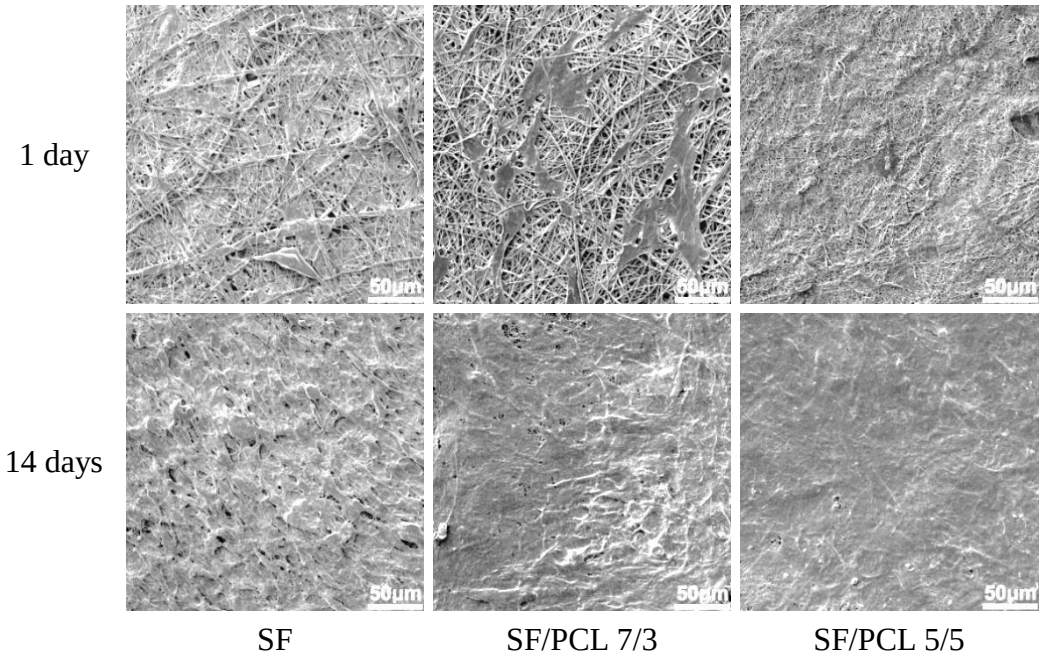


Figure 30 SEM micrographs of the fibre sheets after cells cultured with MG-63 osteoblasts (SEM magnification 1 kx).

- In vitro tests with human umbilical vein endothelial cells (HUVEC)

Cell proliferation was measured during the time of cultivation in days 1, 4 and 7 by MTT test. From the results, as shown in Figure 31, it can see that silk fibroin fibre sheets do not support endothelial cell proliferation during 7 days of cultivation. Cells adhered to the scaffold within the first day but then the proliferation rate was low. Where even lower viability than the first day was measured. After 7 days of incubation period, a slight increase in cell viability was observed.

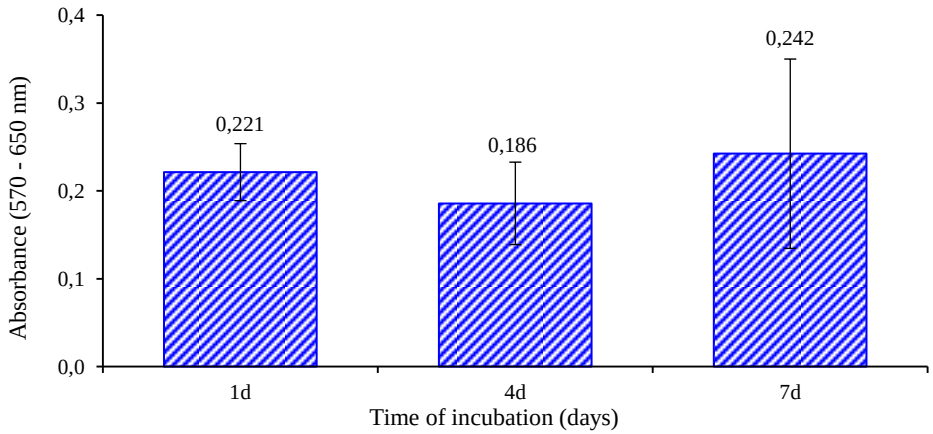
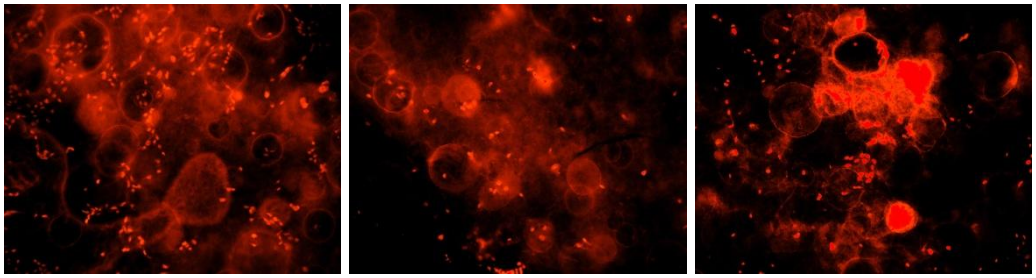


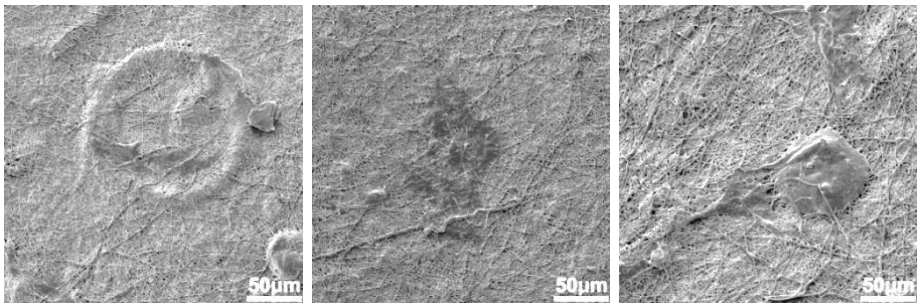
Figure 31 Cell viability measured by MTT test after cultivation with human umbilical vein endothelial cells.

Previous results obtained by MTT test were confirmed by a microscopic technique that the silk fibroin scaffolds do not support endothelial cell proliferation (Fig. 32 and Fig. 33). After 7 days of cultivation, it can still observe just adhered single cells.



1 day 4 days 7 days

Figure 32 Fluorescence microscopy pictures of human umbilical vein endothelial cells stained with propidium iodide during cell culture (magnification 100 x).



1 day 4 days 7 days

Figure 33 SEM micrographs of the silk electrospun fibre sheets after cell cultured with human umbilical vein endothelial cells (SEM magnification 1 kx).

5.5 Degradation of endocrine disrupting chemicals (EDCs) by immobilised laccase on PCL/SF blended fibre sheets

From the obtained result, the polycaprolactone/silk fibroin blended fibre sheet showed to be a promising material for enzyme immobilisation due to its sufficient mechanical stabilities and affinity to the enzyme. After long immobilisation periods, it tended to degrade and partially break down into small filaments, especially when stored at low pH. In a case of several samples, the loading exceeded 100 U/g of the support, which was a very promising result.

The degradation of bisphenol A and 17α-ethinyl estradiol by the immobilised laccase on PCL/SF blended fibre sheets are shown in Figure 34 and 35, respectively. The immobilised laccase blended fibre sheets were able to degrade around 80% of bisphenol A and 97% of 17α-ethinyl estradiol after 4 hours, which was a better result, compared to the free laccase with 0.07 U. The experiment was repeated the next day after an incubation in ultrapure water at 4 ^{O}C overnight. Even during the second usage, they consumed 75% of bisphenol A and 86% of 17α-ethinyl estradiol. After the second usage, the blended fibre sheets showed marks of mechanical damage during the long time process. Their edges were gradually losing small filaments so it was impossible to recover the original mass of the samples for the second measurement.

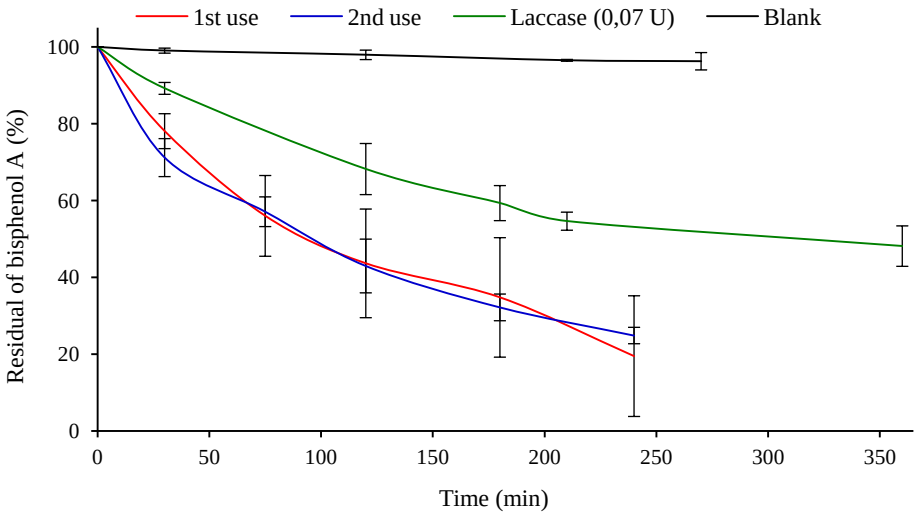


Figure 34 Degradation of bisphenol A by the immobilised laccase on PCL/SF blendeds fibre sheets.

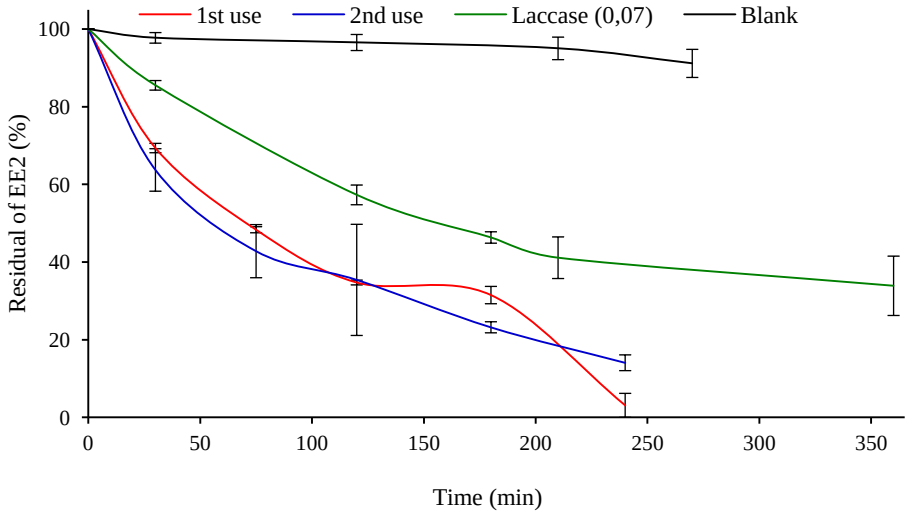


Figure 35 Degradation of 17α-ethinyl estradiol by the immobilised laccase on PCL/SF blended fibre sheets.

The results are shown that a degradation of bisphenol A and 17α-ethinyl estradiol by the immobilised laccase on the blended fibre sheets was faster than degradation by the free laccase. This finding was not expected because the free laccase had an advantage against the immobilised enzyme. This advantage was its solubility in the micropollutant mixture offering the laccase to constantly catalyze the degradation in the whole volume of the liquid. On the other hand; the immobilised laccase was attached to the square matrix with the diameter of only 1 cm and this piece of textile was haphazardly floating in the 3 ml of the liquid. The higher catalytic effectiveness of the modified nanofibres might be explained by an increased stability of the immobilised laccase at 40 ^{O}C and constant shaking of 120 rpm compared to the free enzyme which could have lost its activity at these conditions. In some cases, the degradation curve had a different shape because of a point where the concentration of the micropollutants increased although it generally showed a decreasing tendency. This phenomenon was probably caused by a sampling error.

6. Evaluation of results and new finding

The research studied a fabrication of silk nanofibres with a needleless electrospinning method, concentrating on the effect of the processing parameters on the morphology of the obtained fibres and the spinning performance of the electrospinning process. Biocompatibility of the electrospun fibre sheets was evaluated by *In vitro* testing with living cells.

In these works, a new method for a preparation of a silk fibroin solution by dissolving degummed silk fibre in a mixture of formic acid and calcium chloride is being used instead of a ternary solvent system of $CaCl_2/C_2H_5OH/H_2O$. The use of formic acid-calcium chloride as the solvent for silk fibroin dissolution has an advantage of being simple in an operation when compared to the ternary solvent system. The solvent system consists of formic acid and calcium chloride can directly dissolve silk fibroin at room temperature. The weight ratio of 1:0.25 (w/w) of silk fibres to calcium chloride seems to be a suitable ratio for dissolution of silk fibroin in formic acid.

In an electrospinning process of silk fibroin with formic acid-calcium chloride solvent system, the concentration of the silk solution played an important role in the spinnability of the needleless electrospinning system. The silk electrospun fibres had a diameter ranging from 100 nm to 2400 nm when the concentration of silk fibroin increased from 6 wt% to 14 wt%. Concentrations of silk fibroin in the range of 8 wt% to 12 wt% seem to be a suitable concentration for a preparation of silk fibroin nanofibres with the needleless electrospinning. Furthermore, increasing the concentration of the silk fibroin solution improved the spinning ability and the spinning performance of the electrospinning process. An increase in the applied voltage also enhancing the spinning performance of the process, however, an increase in the applied voltage had a little effect on a reduction of the diameter of silk fibroin electrospun fibres. On the other hand, the variation of spinning distance in the spinning process was affected the spinning performance. The spinning performance of the process was decreased when the spinning distance was increased.

Compared to a needle electrospinning of silk fibroin in the same solvent system, the diameter of the electrospun fibres produced with the needle system was smaller and the fibres had a narrower distribution than those obtained with the needleless system. Furthermore, the applied voltage required to initiate the spinning process from the needleless system was higher than that needed to generate fibres from the needle. However, the spinning performance of the needleless electrospinning was much higher than that of the needle electrospinning. In addition, the difference in a solvent system for silk fibroin dissolution also influenced the diameters of the obtained fibre. Under the same operating conditions, although, the diameters of the silk electrospun fibres obtained from formic acid-calcium chloride solvent system were greater than those obtained from the ternary solvent system, the processing duration of silk electrospun fibres with this solvent system is much shorter than that of the ternary solvent system. Thus, dissolution of silk fibroin in formic acid and calcium chloride could be potentially employed in a preparation of spinning solution for a large-scale production of silk nanofibres with a needleless electrospinning method.

Even though silk fibroin nanofibres were successfully fabricated with the needleless electrospinning, pure silk electrospun fibres sheet is fragile (low elasticity) in the dry state,

which is a disadvantage and would be unsuitable for practical use. Mechanical properties of silk electrospun fibre sheet can improve by blending with synthetic polymers such as polycaprolactone. The blended fibre of silk fibroin and polycaprolactone can also prepare with needleless electrospinning in the same condition and the elasticity of the blended fibre sheet depending on the blend ratios of silk fibroin and polycaprolactone in the solution. An increase in the weight ratio of polycaprolactone in the blended solution increasing the elongation at break of the blended fibres. However, increasing the polycaprolactone content in the blended solution reduced the average fibre diameter and the spinning performance of the process. Moreover, the hydrophilicity of the blended fibre sheet also decreased when increasing the polycaprolactone content, but the blended fibre sheets still have a compatibility with living cells.

The results from *in vitro* tests with living cells show very good biocompatibility of silk fibroin and silk fibroin/polycaprolactone blended fibre sheet. The fibre sheets were able to promote adhesion, spreading and proliferation of 3T3 mouse fibroblasts, normal human dermal fibroblasts and MG-63 osteoblasts. All tested fibre sheets showed good adhesion evaluated 1 day after seeding onto the fibre sheets. During the 2 weeks of the experiment, the cells proliferate through the surface of the fibre sheets and cover most of the fibre sheets surface. On the other hand, pure silk fibroin electrospun fibre sheets do not support human umbilical vein endothelial cells proliferation. Endothelial cells adhered on the fibre sheet within the first day but the proliferation was low. After 7 days of incubation period, a slight increase in cell viability was observed. It can assume that the silk fibroin electrospun fibre sheet and its blends with polycaprolactone are promising materials for the biomedical applications such as wound dressing and bone tissue engineering.

In addition, these works also studied feasibility in enzyme immobilisation on the blended fibre sheet. Laccase from *Trametes versicolor* was immobilised on the blended PCL/SF (8/2) nanofibre sheets by covalent attachment method. The appropriate immobilisation procedure included a modification via glutaraldehyde-bovine serum albumin-glutaraldehyde and the 20 hours for the laccase attachment at temperature 4 ^{O}C and pH 3.0. From the obtained result, the PCL/SF blended fibre sheets showed to be a promising material for enzyme immobilisation for its sufficient mechanical stabilities and affinity to the enzyme. Several samples, the loading exceeded 100 U/g of the support. Moreover, the PCL/SF blended fibre sheets with the immobilised laccase were more efficient in the degradation of endocrine disrupting chemicals (bisphenol A and 17α-ethinyl estradiol) than the approximately same amount of the free laccase. It can suggest that the blended nanofibres could be applied as a component of established water filtering systems for a treatment of effluents coming from facilities known for their high production of endocrine disrupting chemicals in the wastewater.

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8. List of papers published by the author

8.1 Publications in journals

1. N. Sasithorn and L. Martinová, "Effect of Calcium Chloride on Electrospinning of Silk Fibroin Nanofibres", *RMUTP Research Journal: Special Issue 2014*, pp. 62-69, ISSN 1906-0432. Available from: http://journal.rmutp.ac.th/wp-content/uploads/2015/03/008-Textile-Technology-Innovation.pdf

2. N. Sasithorn and L. Martinová, "Preparation of Silk Fibroin/Gelatine Blend Nanofibres by Roller Electrospinning Method", *Advanced Materials Research*, Volume 8499, pp. 45-49, ISBN: 13-978-3-03785-925-4. Available from: doi 10.4028/ www.scientific.net/AMR.849.45

3. N. Sasithorn and L. Martinová, "Needleless Electrospinning of Silk Fibroin/Gelatin Blend Nanofibres", *Applied Mechanics and Materials*, ISSN: 1662-7482, Volume 804 (2015), pp 213-216. Available from: doi:10.4028/www.scientific.net/AMM.804.213

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5. N. Sasithorn, R.Mongkholrattanasit and L. Martinová, "Preparation of Silk Fibroin Nanofibres by Needleless Electrospinning using Formic Acid-Calcium Chloride as the Solvent", *Applied Mechanics and Materials* (Accepted).

6. J. Mašek, D. Lubasová, R. Lukáč , P. Turánek-Knotigová, P. Kulich, J. Plocková, E. Mašková, L. Procházka, Š. Koudelka, N. Sasithorn, J. Gombos, E. Bartheldyová, F. Hubatka, M. Raška, A. D. Miller and J. Turánek, "Multi-layered nanofibrous mucoadhesive films for buccal and sublingual administration of drug-delivery and vaccination nanoparticles - important step towards effective mucosal vaccines", *ACS Nano* (under review)

8.2 Contribution in conference proceeding

1. N. Sasithorn and L. Martinová, "Effect of Calcium Chloride on Electrospinning of Silk Fibroin Nanofibers", the 4th RMUTP International Conference: Textiles & Fashion, 3rd-4th July 2012, Bangkok, Thailand, pp. 51-58, ISBN: 978-974-625-563-9.

2. N. Sasithorn and L. Martinová, "Electrospinning of Silk Fibroin Nanofibres with Polycaprolactone", STRUTEX 2012 : 19th International Conference Structure and Structural Mechanics of Textiles, 3rd-4th December 2012, Liberec, Czech Republic.

3. N. Sasithorn and L. Martinová, "Preparation of Silk Fibroin Electrospun Fibres via Needleless Electrospinning", 1st International Istanbul Textile Congress, 30th May-1st June 2013, Istanbul, Turkey.

4. N. Sasithorn and L. Martinová, "Preparation of Silk Fibroin Electrospun Fibres with Needle and Roller Electrospinning Technique", the Second International Conference on Advanced Materials, Energy and Environments (ICMEE), 8th-9th August 2013, Yokohama, Japan, pp. 43-46, ISSN: 1939-7348 Online.

5. N. Sasithorn, "Fabrication of Silk Fibroin Electrospun Fibres by Roller Electrospinning", Workshop Chata Pod Lipami, 18th-19th September 2013, Czech Republic, ISBN: 978-80-7372-987-5.

6. N. Sasithorn and L. Martinová, "Needleless Electrospinning of Silk Fibroin/Gelatin Blend Nanofibres", the 5th RMUTP International Conference on Science, Technology and Innovation for Sustainable Development, 17th-18th July 2014, Bangkok, Thailand.

7. N. Sasithorn and L. Martinová, "Fabrication of Silk Fibroin-Polycaprolactone Blend Fibres using Needleless Electrospinning", the International Symposium on Fiber Science and Technology (ISF 2014), 28th September-1st October 2014, Tokyo, Japan.

8. N. Sasithorn and L. Martinová, "Needleless Electrospinning of Silk fibroin/ Polycaprolactone Blend Nanofibres", 6th International Conference on Nanomaterials (NANOCON 2014), 5th-7th November 2014, Brno, Czech Republic.

9. N. Sasithorn, R. Mongkholrattanasit and L. Martinová, "Preparation of Silk Fibroin Nanofibres by Needleless Electrospinning using Formic Acid-Calcium Chloride as the Solvent", The 6th RMUTP International Conference on Science, Technology and Innovation for Sustainable Development, 15th-16th July 2015, Bangkok, Thailand.

8.3 Citations

1. "Fabrication of silk nanofibres with needle and roller electrospinning methods" was cited by three documents

- J. Rnjak-Kovacina, L. S. Wray, K. A. Burke, T. Torregrosa, J. M. Golinski, W. Huang, and D.L. Kaplan, "Lyophilized Silk Sponges: A Versatile Biomaterial Platform for Soft Tissue Engineering", *ACS Biomaterials Science & Engineering,* 2015, pp. 260-270, http://dx.doi.org/10.1021/ab500149p

- Z. Luo, Q. Zhang, M. Shi, Y. Zhang, W. Tao, and M. Li, "Effect of Pore Size on the Biodegradation Rate of Silk Fibroin Scaffolds", *Advances in Materials Science and Engineering*, Volume 2015 (2015), http://dx.doi.org/10.1155/2015/315397.

- Y. Zhao, Y. Qiu, H. Wang, Y. Chen, S. Jin and S. Chen, "Preparation of Nanofibers with Renewable Polymers and Their Application in Wound Dressing", *International Journal of Polymer Science,* Volume 2016 (2016), http://dx.doi.org/ 10.1155/2016/4672839.

2. "Effect of calcium chloride on electrospinning of silk fibroin nanofibres" was cited by one document

- B. K. Singh and P. K. Dutta, "Chitin, Chitosan, and Silk Fibroin Electrospun Nanofibrous Scaffolds: A Prospective Approach for Regenerative Medicine" Part of the series Springer Series on *Polymer and Composite Materials* pp. 151-189.

Curriculum Vitae

Name	Nongnut Sasithorn
Date of birth	03 September 1976
Nationality	Thai
Address	
Thailand:	387 Soi Phetkasem 53, Phetkasem Road, Lak song, Bangkhae, Bangkok, 10160, Thailand. Department of Textile Chemistry Technology, Faculty of Industrial Textiles and Fashion Design, Rajamangala University of Technology Phra Nakhon, 517 Nakhonsawan Road, Suan Chitladda, Dusit, Bangkok 10300, Thailand
Czech Republic:	Department of Nonwovens and Nanofibrous Materials, Faculty of Textile Engineering, Technical University of Liberec, Studentská 2, 46117 Liberec, Czech Republic
E-mail address	nongnut.s@rmutp.ac.th

Education

2011 - 2016	Ph.D. candidate at Department of Nonwovens and Nanofibrous Materials, Faculty of Textile Engineering, Technical University of Liberec, Czech Republic
2004 - 2006	Master of Science (M.Sc., Petrochemistry and Polymer Science) Chulalongkorn University, Thailand
2003 -2004	Bachelor of Art (B.A. Mass communication) Ramkhamhaeng University, Thailand
1996 - 1999	Bachelor of Engineering (B.Eng. Textile Chemistry Engineering) Rajamangala Institute of Technology, Thailand (Rajamangala University of Technology Thanyaburi)
1993 - 1996	Diploma of Textile Chemistry, Rajamangala Institute of Technology, Chumporn Khet Udomsak Campus, Thailand

Work Experience

2005 - 2010	Lecturer at Rajamangala University of Technology Phanakorn Faculty of Industrial Textiles and Fashion Design, Department of Textile Chemistry Technology.
2002 - 2004	Lecturer at Rajamangala Institute of Technology, Chumporn Khet Udomsak Campus

Language skills:	Thai (mother tongue), English (good), Japanese (fair)
Computer literacy:	Microsoft Office (Excel, PowerPoint, Publisher, Word) Adobe Photoshop, Adobe Illustrator Minitab (software for statistical evaluation)

Brief description of the current expertise, research and scientific activities

Doctoral studies

Studies	Textile Engineering Textile Technics and Materials Engineering Full time
Exams	- Makrolekulární chemie, 21.11.2011 - Chemické a termické technologie výroby netkaných textilií, 22.03.2012 - Fyzikální principy elektoratického zvlákňování, 21.06.2012 - Vybrané partie z řešení diferenciálních rovnic a jejich soustav, 22.11.2012
SDE	State Doctoral Exam completed on 03.12.2014 with the overall result passed.

Teaching Activities

Teaching	-
Leading Master students	Ing. Milena Maryšková, Enzyme immobilization on microfibrous or nanofibrous materials and their applications in biotechnology, 2015

Research projects

- Preparation of silk nanofibre sheet via electrospinning technique (Student's grant competition TUL in specific University research : Project No. 48010), Project leader, 2013.
- Fabrication of Silk Fibroin Electrospun Fibres and Its Blend by Electrospinning Technique (Student's grant competition TUL in specific University research : Project No. 21034), Project leader, 2014.

ZÁPIS O VYKONÁNÍ STÁTNÍ DOKTORSKÉ ZKOUŠKY (SDZ)

Jméno a příjmení doktorandky:	**Nongnut Sasithorn, M.Sc.**
Datum narození:	**3. 9. 1976**
Doktorský studijní program:	**Textilní inženýrství**
Studijní obor:	**Textilní materiálové inženýrství**
Termín konání SDZ:	**3. 12. 2014**

prospěla **neprospěla**

Komise pro SDZ:		*Podpis*
Předseda:	prof. RNDr. Oldřich Jirsák, CSc.	
Místopředseda:	prof. Ing. Jiří Militký, CSc.	
Členové:	doc. Ing. Antonín Kuta, CSc.	
	doc. Ing. Eva Košťáková, Ph.D.	
	prof. Ing. Ivan Stibor, CSc.	
	prof. Ing. Jaroslav Šesták, DrSc., dr.h.c.	
	prof. Ing. Jakub Wiener, Ph.D.	

V Liberci dne 3. 12. 2014

O průběhu SDZ je veden protokol.

Recommendation of the supervisor

TECHNICKÁ UNIVERZITA V LIBERCI
Ústav pro nanomateriály, pokročilé technologie a inovace

Oddělení nanotechnologií a informatiky iNTEC

Disertační práce: **PRODUCTION OF NON-WOVEN FABRICS BY USING SILK FIBRES VIA ELECTROSPINNING TECHNIQUE**

Autor: **NONGNUT SASITHORN, M.Sc.**

Hodnocení školitele

Disertační práce Nongnut Sasithorn, M.Sc. s názvem Production of Nonwovens using Silk Fibres via Electrospinning Technique" se zabývá izolací silk fibroinu (SF) a přípravou nanovlákenné vrstvy ze SF technikou bez-jehlového elektrostatického zvlákňování za využití originální směsi kyseliny mravenčí a chloridu vápenatého jako rozpouštědla. Navržený rozpouštědlový systém pro SF je vhodný pro kontinuální přípravu nanovlákenné vrstvy na SuperLabu. Autorka podrobně studuje všechny významné materiálové a procesní charakteristiky elektrostatického zvlákňování roztoku SF, popřípadě jeho směsí s jiným polymerem, a jejich vliv na morfologii vznikajících nanovláken. Zabývá se též mechanickými a hydrofilními vlastnostmi nanovláken v závislosti na příměsi dalšího biodegradabilního polymeru, zejména polycaprolactonu (PCL).

The dissertation of Miss Nongnut Sasithorn with the title "Production of Nonwovens using Silk Fibres via Electrospinning Technique" deals with isolation of silk fibroin (SF) and preparation of nanofibre layers from the SF by needleles electrospinning technique using the original mixture of formic acid and calcium chloride as the solvent. Designed solvent system for SF is suitable for the continual preparation of nanofibre layer on SuperLab. The author studied in detail all significant material and process characteristics of the SF electrospinning solution, or mixtures with other polymers, and their effects on the morphology of the produced nanofibres. It also deals with mechanical and hydrophilic properties of nanofibres depending on a mixture of another biodegradable polymer, preferably polycaprolactone (PCL).

Dále byla studována biokompatibilita nanovlákenné vrstvy in vitro pomocí 3T3 myších fibroblastů, lidských kožních buněk a MG 63 osteoblastů. Z testů vyplynulo, že nanovlákna ze směsi SF/PCL jsou velmi slibným materiálem pro biomedicínské aplikace.
Nanovlákenná vrstva ze směsi SF/PCL byla aplikována jako nosič pro imobilizaci enzymu lakáza z dřevokazné houby *Trametes versicolor*.
Takto imobilizovaný enzym vykazuje velmi dobré výsledky při degradaci endokrinních disruptorů jako je bisfenol A a 17α-ethinyl estradiol. Imobilizovaná laktáza na nanovlákenné vrstvě SF/PCL je proto velmi perspektivní systém pro bioremediaci a dočišťování odpadní vody.

Biocompatibility of nanofibre layers was also investigated in vitro using 3T3 mouse fibroblasts, human skin cells and MG 63 osteoblasts. The tests proved that the nanofibres from a mixture of the SF/PCL are a very promising material for biomedical applications.
The SF / PCL nanofibre layer was tested as a carrier for immobilizing the enzyme laccase from the wood-decaying fungi *Trametes versicolor*. The immobilized enzyme showed very good results in the degradation of endocrine disruptors such as bisphenol A and 17α-ethinylestradiol. The laccase immobilization onto PCL/SF blend fibre sheets seems to be a very promising system for bioremediation and wastewater treatment.

Doktorandka přistoupila k řešení disertační práce velmi zodpovědně, o čemž svědčí i rozsáhlá rešerše, čítající cca 90 rozmanitých titulů. Od počátku svého pobytu na TUL projevovala Nongnut Sasithorn hluboký zájem o zvolenou problematiku, ale také nevšední pracovní nasazení a invenci.

The PhD student approached to the solution of the dissertation very seriously, which shows an extensive recherche, numbering about 90 multiple titles. Nongnut Sasithorn showed deep interest in the chosen topic but also hard work and ingenuity from the beginning of her stay at TUL.

Téma elektrostatického zvlákňování silk fibroinu, stejně tak kokony bource morušového, si slečna Nongnut Sasithorn zajistila sama ve spolupráci ve svou mateřskou univerzitou v Thajsku.
Výsledkem její práce je přesně vypracovaná metodika jak z hlediska izolace SF, tak i z hlediska procesu kontinuálního zvlákňování.
Tato disertační práce objasňuje vlivy jednotlivých proměnných na izolaci SF, elektrostatické zvlákňování, stabilizaci proti rozpouštění, morfologii a aplikaci. Kvalitně jsou rozpracovány aplikační směry pro biomedicínu, např. hojivé krytí ran a imobilizaci enzymu.

Miss Nongnut Sasithorn ensured the topic of silk fibroin electrospinning, as well as silkworm cocoons by herself in cooperation with her parent university in Thailand. The result of her thesis is precisely developed methodology of both separation of SF and continual electrospinning process.
This dissertation clarifies the effects of individual variables to the separation of SF, electrospinning, stabilization against dissolution, morphology and application. Guidelines for biomedical applications, e.g. healing wound dressing and enzyme immobilization, are well elaborated in the thesis.

Kandidátka výrazně přispěla k pochopení provázanosti různých materiálových a procesních parametrů při bezjehlovém zvlákňování SF a jeho směsí. Předložila řadu aplikačních možností pro SF a jeho směsi.

Candidate significantly contributed to the understanding of the interdependence of different material and process parameters during the needleles electrospinning of SF and its mixture. She put forward a number of possible applications for SF and its mixtures.

Vysoce hodnotím publikační a konferenční činnost sl. Nongnut Sasithorn, která čítá šest časopiseckých titulů a devět konferenčních příspěvků, které napsala během svého doktorského studia v Liberci. N. Sasithorn vedla v letech 2013-2015 diplomovou práci s názvem
"Imobilizace enzymů v mikrovlákenných a nanovlákenných materiálech a jejich využití v biotechnologiích", která byla oceněna cenou rektora TUL a cenou ČEEP 2014 (Český energetický a ekologická projekt, stavba, inovace).

I highly evaluate publication activities of Mrs. Nongnut Sasithorn including six journal titles and nine international conference papers, which she wrote during her doctoral studies in Liberec. N. Sasithorn supervised a master thesis called "Enzyme immobilization on microfibrous or nanofibrous materials and their application in biotechnology", which was awarded by the rector of TUL and price of ČEEP 2014 (Czech Energy and Ecology Project, Construction and Innovation).

Z výše uvedených důvodů jsem již dříve navrhla práci Nongnut Sasithorn, M.Sc. k přijetí k obhajobě a nyní navrhuji, aby jí byl udělen doktorský titul.

According to above mentioned facts, I have previously suggested submitting the thesis of Nongnut Sasithorn, M.Sc. to the defense and now I also suggest, that Mrs. Nongnut Sasithorn was awarded a doctorate (Ph.D).

Doc. Ing.Lenka Martinová, CSc.

Technická univerzita v Liberci
Ústav pro nanomateriály, pokročilé technologie a inovace

V Liberci 5.5. 2016

Reviews of the opponents

Report on PhD thesis
Nongut Sasithorn: „Production of nonwoven fabrics by using silk fibres *via* electrospinning technique“

The subject of this thesis is related to the utilization of needless electrospinning technique for fabrication of nonwoven sheets from silk fibroin and silk fibroin/polycaprolactone solutions. The topic is very interesting as the silk electrospun fibre sheets and its blends with polycaprolactone are promising materials for biomedical applications. The thesis deals mainly with the spinning process and it comprises two relatively independent parts. The first one is focused on a new solvent system for silk fibroin dissolution and screening different variables in preparation of electrospun nonwoven sheets. The second one deals with the biocompatibility of prepared materials and with their potential utilization in enzyme immobilization.

The chapter Theoretical and literature review, based on up-to-date references, comprises brief information about silk fibre and its characteristics, about fundamentals of electrospinning and state of the art in electrospinning of silk fibroin. This chapter has been written very comprehensibly in a good language. The chapter Experimental describes the used materials, experimental processes and analytical methods. Some queries to this part are as follows:

1. Proper chemical nomenclature is poly(lactic acid), poly(lactic acid-*co*-glycolic acid), abbreviation for gram is g, not gm. – p. 61
2. To specify the used rheometer, the measuring geometry must be specified (coaxial cylinders, cone-plate or plate-plate?) Also specification of measuring spindle should be added. – p.62
3. The description of preparation of the “traditional silk spinning solution” is not fully clear. Why was the composition of $CaCl_2/EtOH/H_2O$ ternary solvent expressed in molar ratio? Ratio 1/2/8 moles represents 111g $CaCl_2$ / 92g EtOH / 144g H_2O. Is this the composition that was used? What does mean the ratio silk:solvent 1:10 (w/v)? It must be specified as $g.cm^{-3}$, $kg.m^{-3}$, $g.L^{-1}$, etc. The concentration 12% (w/w) is not clear as well. Does it mean 12 wt.% (i.e. 12 wt. parts of silk and 88 wt. parts of solvent) or 12 wt. parts of silk on 100 wt. parts of solvent (phs)? – p. 65
4. Similarly, in Table 4.1 a correct caption should be possibly “Effect of added $CaCl_2$ to 8 wt.% silk fibroin/formic acid mixture on some properties of created solutions” instead of “Effect of concentration of $CaCl_2$ on properties of silk fibroin solution 8 wt.%”, because the concentration of $CaCl_2$ was not specified. – p.76
5. The spinning experiments were carried out under different air humidity, cf. 35-40% (p.66), 38% (p.67), and 23.5% (p.70). How does the humidity affect the particular electrospinning process? This variable was not studied but it might be important. – see p. 42

The chapters Results and discussion and Conclusion were well elaborated, unfortunately, the language is not that good as in the previous chapters. The most important achievements of this part were finding the feasible composition of $CaCl_2$/formic acid solution for electrospinning silk and silk/polycaprolactone blends and evaluation of processing variables with respect to properties of electrospun fibre sheets. Author did confirm very good biocompatibility of the fabricated materials as well as their potential application as support for

enzyme immobilization. In summary, the thesis represents a good contribution to improving the knowledge in the field of silk fibroin electrospinning and utilization of the prepared fibrous materials in biomedical applications.

Conclusion

In conclusion, it can be stated that the subject of thesis is highly relevant and that the candidate achieved the aim using modern experimental and analytical methods. By choosing the approach and method of elaboration Ms. Nongut Sasithorn manifested her ability of independent a creative scientific work. By a successful solution of a research problem, she brought new findings and showed a good grasp of scientific methods of work. As Ms. Nongut Sasithorn thus fulfilled the appropriate conditions, I recommend her thesis for defending. After the successful defense, I recommend to confer a scientific degree of Ph.D. upon the candidate.

Pardubice, 2016-03-17

Prof. Jaromír Šňupárek
University of Pardubice
FCHT - Institute of Chemistry and Technology of Macromolecular Materials

FROM: Miroslav Raab, Professor of Macromolecular Technology
SUBJECT: Report on Doctoral Dissertation
"Production of Nonwoven Fabrics by Using Silk Fibers via Electrospinning Technique:
or in Czech:
"Příprava netkaných textilií s obsahem hedvábných vláken získaných metodou elektrostatického zvlákňování"
submitted by
Nongnut Sasithorn, M.Sc.
to the
Technical University of Liberec, Faculty of Textile Engineering
DATE: March 2016
OPINION: Recommended

Technical university of Liberec is internationally acknowledged due to discovery and further development of a special device for the continuous production of nanofibers. The device has been successfully produced by the Elmarco company and received the trade name Nanospider. At the Technical university further research is being conducted to improve the Nanospider technology and find new prospective applications for various types of nanofibers. This dissertation is an important contribution to such efforts.

The comprehensive 142-page Dissertation focused on the preparation of nanofibers from regenerated silk fibroin and its blends with polycaprolactone . The subject area is well selected and topical, as also shown by recently published books . The topic of the dissertation is very interesting scientifically and important both from the point of views of economy and ecology. So, it could show new ways for ecological processing of silk waste.

The Dissertation is well structured. After a short "Introduction" it contains "Theoretical part" together with a review of relevant literature (40 pages). The introductory part is accompanied by a list of references (87 items). This part shows that the author understands well the complex relationships between individual levels of hierarchical structure of silk and is aware of the relevant literature on electrospinning of silk fibroin. Next part of the Dissertation is "Experimental" (16 pages). The starting materials, preparation of spinning solutions and electrospinning processes themselves are described in this part. Moreover, in vitro test of the interaction between prepared webs and living cells are reported. Finally, the part "Results and discussion" summarizes all obtained results and also the useful experience gained during the PhD study. The text is written in understandable English, though the English style would benefit from some more proofreading. Formally, the Dissertation has a very good level and the text is accompanied by well selected and carefully prepared illustrations. I have only two remarks to the theoretical and experimental parts: First, the difference in mechanical strength between native silk and various types of regenerated silk would deserve a comparison and structural explanation. Second, the composition and preparation of spinning solutions would need a clearer description and presentation, perhaps in a form of a table. I recommend to present such a diagrammatical table during the defense.

From the list of references it follows that the candidate appears as co-author in two conference contributions, one publication in a special issue of a Research Journal and one publication in Journal of Nanomaterials. Unfortunately, the corresponding reference (Ref. 51) is incomplete. Definitively, some further original publications would be possible and desirable.

I have some questions to the author that can initiate discussion during and after the defense.

(1) There are two possibilities how to improve mechanical strength of silk fibroin nanofibers, namely: a) controlling supermolecular structure of fibroin and b) blending fibroin with other polymers. Try to compare briefly these two approaches.

(2) How stable are the silk fibroin solutions ?

(3) Which applications of silk fibroin mats are perspective ?

These questions do not deny the original work of the candidate and the very good standard of the presented Dissertation. The aim of the doctoral study, as specified on page 18, has been successfully achieved. Clearly, the obtained results will be useful for the future research at the Faculty of Textile Engineering and show a good scientific level of the whole University. The very positive role of the supervisor, Assoc. Prof. Lenka Martinová, is here obvious. The candidate has proven an expertise in needleless spinning on one hand and a promising class of nanomaterials on the other. Her scientific work is original and independent. Besides, the amount of work is quite substantial. I recommend the Dissertation for the award of PhD. degree by the Technical University of Liberec.

March 23, 2016

Miroslav Raab, PhD.
Professor of Macromolecular Technology

Reviewing report

PhD thesis: Production of Nonwoven Fabrics by Using Silk Fibres via Electrospinning Technique

PhD Candidate: Nongnut Sasithorn, Technical University of Liberec

Reviewer: Dr. Xin Wang, School of Fashion and Textiles, RMIT University, Australia

The research work of the thesis focuses on the scaled up fabrication of nanofibres from silk fibroin either as solely or blended with polycaprolactone, with application potentials as biomaterials and waste water purification. As nanofibre has great application potential and silk products are promising in different industrial areas, this research work has great significance in both academia and industry.

Needleless electrospinning method has been utilised in this research, which is properly fulfilled the purpose of large scale production of nanofibre from silk fibroin. A new solvent system containing calcium chloride and formic acid was invented to properly dissolve silk and a blending electrospinning of silk fibroin and polycaprolactone was applied to fabricate silk fibroin nanofibres with proper mechanical properties. These methods and approaches successfully fulfilled the proposed aims of the study.

The results of the thesis are correct according to the reviewer's knowledge, and the candidate has contributed greatly to the knowledge of fabrication nanofibres from silk fibroin and its application in biomaterials and water treatment.

While the candidate properly wrote the thesis according to scientific standard and critical thinking, it is greatly recommended to have a careful proofreading so as to improve the language and correct typos and grammar.

As there is no publication list of the candidate in the thesis, the reviewer cannot comment in this regard.

Considering the academic merits and significance of the research work in this thesis, I recommend the PhD. Thesis for defence.

Posudek byl zaslán elektronicky bez podpisu.