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ORIGINAL PAPER

Electrospinning cellulosic nanofibers for biomedical applications: structure and in vitro biocompatibility

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Abstract Electrospinning of cellulose acetate (CA) was studied in relation to factors of solvent composition, polymer concentration, and flow rate to elucidate how the processing parameters impact electrospun CA structure. Fibrous cellulose-based mats were produced from electrospinning cellulose acetate (CA, Mn = 30,000, DS = 2.45) in acetone, acetone/isopropanol (2:1), and acetone/dimethylacetamide (DMAc) (2:1) solutions. The effect of CA concentration and flow rate was evaluated in acetone/DMAc (2:1) solution. The morphology of electrospun CA mats was impacted by solvent system, polymer concentration, and solution flow rate. Fibers produced from acetone and the mixture of acetone/isopropanol (2:1) exhibited a ribbon structure, while acetone/DMAc (2:1) system produced the common cylindrical fiber shape. It was determined that the electrospinning of 17 % CA solution in acetone/DMAc (2:1, w/w) produced fibers with an average fiber diameter in the submicron range and the lowest size distribution among the solvents tested. The solution flow rate had a power law relationship of 0.26 with the CA fiber size for 17 % CA in acetone/DMAc (2:1). Solvent composition and flow rate also impacted the stability of the network structure of the electrospun fibers. Only samples from acetone/DMAc (2:1) at solution flow rates equal or higher than 1 mL/h produced fibrous meshes that were able to preserve their original network structure after deacetylation. These samples after regeneration showed no residual DMAc and exhibited no cytotoxic effects on mammalian cells.

Keywords Cellulose · Electrospinning · Biomaterials · Biocompatibility · Flow rate

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Introduction

Electrospinning is a novel method for producing ultrafine fibers from polymeric solutions with diameters from the submicron down to the nanometer scales (Son et al. 2004; Tungprapa et al. 2007). These materials have found increasing applications in the biomedical field for drug delivery (Cui et al. 2006) and tissue engineering (Li et al. 2002). Cellulose is emerging as an extremely relevant biomedical material due to its biocompatibility and performance

(Dugan et al. 2010; Helenius et al. 2006). Fiber formation through electrospinning is based on the uniaxial stretching of a viscoelastic solution due to electrostatic forces that cause a fine cone at the tip of a droplet. When the electrical force at the interface of the polymer liquid solution overcomes the surface tension, a charged jet is ejected and the solvent evaporates as the polymer fibers are deposited on the collector. Equipment set-up for this process is relatively simple; laboratory-based electrospinning only requires a syringe to hold a polymer solution, a pump to control the solution volumetric flow rate, a voltage supplier (in the kV range), two electrodes to generate an electrical potential difference, and a fiber collector. As such, a variety of solvent-cellulosic combinations are being explored to produce electrospun materials (Freire et al. 2011; Frey 2008; Kim et al. 2005; Quan et al. 2010).

By varying the parameters in the electrospinning process, it is possible to control fiber diameter, surface morphology, shape, and orientation. Fiber size and fiber morphology are related to the polymer composition and impacted by a variety of electrospinning parameters (Andrady 2007). Key in controlling fiber size is the concentration of the polymer in solution, as fiber diameter scales with solution viscosity (Andrady 2007; Fong et al. 1999; Huang et al. 2003; Munir et al. 2009; Thompson et al. 2007; Veleirinho et al. 2008). Also, solvent choice greatly influences fiber size along with solution conductivity. Processing parameters specific to electrospinning such as flow rate of the polymer solution and electric field strength, are also important factors that impact the fiber size (Andrady 2007; Fong et al. 1999; Huang et al. 2003; Munir et al. 2009).

Interest in electrospun fibers is due to the attractive characteristics such as high surface area to mass ratio, high degree of porosity, and flexibility in surface functionality (McCullen et al. 2010; Lim et al. 2011; Rebollar et al. 2011; Tungprapa et al. 2007). Hence, fibers produced by electrospinning have a variety of uses such as filters, electronics, catalyst supports, affinity membranes, sensors, reinforced nanocomposites, drug delivery systems, and scaffolds for tissue engineering. These two latter areas have seen additional interest in cellulosic and other polysaccharide systems as their performance can easily be justified relative to the cost of production (compared to synthetic systems).

Cellulose is the most abundant renewable polymer on earth and naturally found as a fibrous structure. This polysaccharide is made up of glucose units linked together by β (1–4) glycosidic bonds and has strong intra- and intermolecular hydrogen bonds (Klemm et al. 2005). As a result, cellulose exhibits high tensile modulus and is insoluble in many organic and inorganic solvents. Furthermore, electrospinning of cellulose is a challenge due to its restricted solubility requiring ionic liquids and other highly polar solvent systems for dissolution that have low volatility. An alternative approach is to use a derivatized cellulose that has expanded range of solubility. Cellulose acetate (CA) is a commercial cellulose derivative that has been used as an alternative to the direct spinning of cellulose. CA can be easily dissolved in several solvents (acetone, acetic acid, chloroform, dimethylacetamide (DMAc), dimethylformamide (DMF), methanol, pyridine, etc.) and then regenerated maintaining the fiber form. CA nanofibers can be converted to cellulose using alkali catalyzed saponification reactions in limited swelling solvents.

Several studies have explored electrospinning CA in different solvents; a list of seventeen different solvent systems used for electrospinning CA is shown in Table 1. Additionally, Table 2 highlights fiber size as a function of solvent system, CA concentration, CA molecular weight, environment, and collection materials (Table 2). However, the effect of the flow rate on the morphology, fiber size, and fiber size distribution of the electrospun fibers has not been discussed in detail and is scarcely mentioned in the majority of published works. This current study, builds from several studies regarding the parameters of CA electrospinning to quantify fiber size and distributions based on image processing of scanning electron micrographs (Liu and Hsieh 2002; Han et al. 2008; Tungprapa et al. 2007; Liu and Tang 2006; Son et al. 2004; Han and Gouma 2006). The goal of this study is to determine viable solvents and the electrospinning parameters to produce CA nanofibers that can be converted into cellulose with an adequate morphology, fibers size, and fiber size distribution to perform as scaffolds for tissue engineering applications. Related to this biomedical application, regenerated cellulose is evaluated for its potential cytotoxicity. Overall, the study illustrates options for producing cellulose acetate nanofibers with industrially relevant solvents, while demonstrating how certain

Table 1 Solvents employed in the electrospinning of cellulose acetate

Solvent composition	Fiber formation[a]	References
Acetone	Discrepancy	Liu and Hsieh (2002), Tungprapa et al. (2007), Son et al. (2004), Han and Gouma (2006)
Acetone/water	Smooth fibers	Son et al. (2004), Song et al. (2012)
Acetone/DMAc	Smooth fibers	Liu and Hsieh (2002), Liu and Tang (2006), Tungprapa et al. (2007)
Acetic acid	Few fibers	Liu and Hsieh (2002)
Acetic acid/acetone	Beaded fibers	Liu and Hsieh (2002)
Acetic acid/water	Smooth fibers	Han et al. (2008)
Chloroform	Beads	Tungprapa et al. (2007)
Chloroform/methanol	Smooth fibers	Tungprapa et al. (2007)
Dichloromethanol	Beads	Tungprapa et al. (2007)
Dichloromethanol/methanol	Smooth fibers	Tungprapa et al. (2007)
Dimethylformamide (DMF)	Beads	Tungprapa et al. (2007)
Acetone/DMF/trifluoroethylene	Smooth fibers	Ma et al. (2005), Ma and Ramakrishna (2008)
DMAc	No fibers	Liu and Hsieh (2002)
Formic acid	Beads	Tungprapa et al. (2007)
Methanol	Beads	Tungprapa et al. (2007)
Methylene chloride/ethanol	Smooth fibers	Han et al. (2005)
Pyridine	Beads	Tungprapa et al. (2007)

[a] Solvent is not the only parameter that will impact fiber formation

combinations enhance the ability to retain structure after regeneration required for select tissue engineering applications.

Materials and methods

Materials

Acetone (99.9 %, Sigma Aldrich), isopropanol (99.9 %, Burdick and Jackson), dimethylacetamide (DMAc, 99.9 %, Sigma Aldrich), and vacuumed-dried cellulose acetate (CA, Mn = 30,000, 39.8 % acetyl groups, Sigma Aldrich) were employed without additional purification.

Electrospinning process

CA was dissolved in acetone, acetone/isopropanol (2:1 w/w), and acetone/DMAc (2:1 w/w) under constant stirring at room temperature until the solution was transparent. The solutions were left standing without stirring for a minimum of 10 min to release air bubbles. Each CA solution was placed in a 10 mL disposable syringe and fed by a syringe pump (Harvard Apparatus) through a stainless steel needle with an inner diameter of 0.643 mm (Howard Electronic Instruments Inc.). The needle was connected to a high voltage power supply (Spellman's CZE1000R) to the positive lead. A voltage of 25 kV was applied between the needle and the grounded collector, in a horizontal collection set-up. The throw distance between needle tip and collector was 25 cm. The static collector consisted of 10 cm × 10 cm steel mesh covered with aluminum foil. The experiments were carried out at approximately 20 °C and relative humidity (RH) of 50–60 %. Solutions of CA (17 % wt/wt) in acetone, acetone/isopropanol (2:1), and acetone/DMAc (2:1) were electrospun at a flow rate of 1 mL/h. CA concentrations of 10, 13, 15, 17, 18, and 19 % (wt/wt) were tested for acetone/DMAc (2:1) solvent system at 1 mL/h. CA solution of 17 % in acetone/DMAc (2:1) at flow rates of 0.025, 0.1, 0.25, 0.5, 1, and 3 mL/h were electrospun as well. Morphological and microstructural features of the samples were investigated by field-emission scanning electron microscopy (SEM), LEO 1550 (Zeiss). The analysis

Table 2 Fiber size and morphology of electrospun cellulose acetate

Authors	CA	CA concentration (%)	Solvents	Flow rate	Voltage/Throw distance	Needle ID (mm)	Collector	Average fiber size	Relevant findings
Liu and Hsieh (2002)	Mn = 30,000	15, 20 (w/w)	Acetone		5–10 kV		Al foil	–	Few fibers
	DS = 2.45		Acetone/ DMAc						
		10	(2:1)	Not specified	10–15 kV/ 6–10 cm	Not specified	Al foil		No fibers
		12.5			12; 15 kV/ 10 cm		Al foil, paper		Small beads
		15			10; 15 kV/ 6–10 cm		Al foil, paper, wáter	100 nm–1 μm	Fibrous mat
		20			12; 15 kV/ 10 cm		Al foil, paper		Fibrous mat
		25			12; 15 kV/5 cm		Al foil		Few fibers
Son et al. (2004)	Mn = 30,000	10–15 (w/w)	Acetone/ water	5–20 mL/h	8–12 kV/ 6–12 cm	0.84	Stainless steel sheet	2.3	Fibrous mat
	DS = 2.45		Acetone	5–20 mL/h	8–12 kV/ 6–12 cm		Stainless steel sheet		
		9							Spherical beads
		13							Spindle beads
		17						2.25	Fibers
		21						3.23	Fibers
Han et al. (2005)	Mw = not specified		Methylene chloride/ ethanol						
	DS = 3		(100/0)						Porous fibers
			(90/10)					50–100 nm	Porous fibers
		5 (w/w)	(85/15)	1 mL/h	15 kV/10 cm	0.495	Not specified	200–500 nm	Smooth fibers
			(80/20)						Smooth fibers
Han and Gouma (2006)	Mw = 29,000	7.5 (w/v)	Acetone	1.8 mL/h	19 kV	Not specified	Al foil		Dense beaded mat
	Acetyl 40 %	17.5	Acetone	9.6 mL/h	7 kV		Al foil	1.2	Lose fiber mat

Table 2 continued

Authors	CA	CA concentration (%)	Solvents	Flow rate	Voltage/Throw distance	Needle ID (mm)	Collector	Average fiber size	Relevant findings
Liu and Tang (2006)	DS = 2.45 Mw	(w/w)	Acetone/ DMAc					Size distribution	
	30,000	15	(1:2)	Not specified		0.55		90–790 nm	Large spindle beads
	35,000	15			8 kV/15.24 cm	and	Al foil	90–430 nm	Few conical beads
	40,000	15				0.84		90–550 nm	Fibrous mat
	50,000	15, 10							No fibers
Tungprapa et al. (2007)	Mw = 30,000	5 (w/v)	Acetone		15 kV	0.584	Al sheet		Short and beaded fibers
	DS = 2.45		Acetone/ DMAc						Beaded fibers
		12	(2:1)	Not specified				0.14 ± 0.31*	Fibrous mat
		14			12 kV/15 cm			0.23 ± 0.25	Fibrous mat
		16						0.26 ± 0.20	Fibrous mat
		18						0.33 ± 0.34	Fibrous mat
		20						0.37 ± 0.28	
Han et al. (2008)	Mn = 30,000	17 (w/w)	Acetic acid/ water	3 mL/h	25 kV/10 cm	0.84	Stainless steel sheet	180–1,280 nm	Fibrous mat (ribbon shaped fibers)
	DS = 2.45		(3:1)						
Song et al. (2012)	Mn = 30,000	17 (w/v)	Acetic acid/ water	5 mL/h	15 kV/10 cm	0.394	Al sheet	5–8 μm	Fibrous mat (ribbon shaped fibers)
	DS = 2.45		(85:15)						

was carried out with samples coated in gold–palladium with thickness of 35–40 Å. The fiber diameter and size distribution of the samples were evaluated from 200 representative fibers of the samples via image analysis software (NIS-Elements BR 3.1). For the samples that had ribbon like geometries, the width of the ribbon was determined from the image. Thermogravimetric analysis was performed on a Perkin Elmer TGA-7 in a nitrogen atmosphere.

Regeneration of cellulose

Electrospun cellulose acetate was regenerated to cellulose by using alkaline saponification in ethanol based on previous reports (Rodriguez et al. 2011; Liu and Hsieh 2002). Samples were soaked in a Petri dish containing 0.05 M NaOH for 24 h and subsequently rinsed with deionized water.

In vitro cytotoxicity tests

The cytotoxic effect of electrospun cellulose fibers was evaluated in vitro on a culture of mouse fibroblast cells (L-929). Cytotoxicity was assessed by direct incubation of cellulose fiber extracts with the cultured cells (MEM elution test) and by diffusion of electrospun cellulose leachable substances over a monolayer of cells (agar overlay test). The assessment was carried out by Nelson laboratories (Salt Lake City, UT, USA) in conformity with procedures established by United States Pharmacopeia and National Formulary (USP 87) and the ANSI/AAMI/ISO 10993-5 standards.

Results

Fiber geometry and size

Acetone, acetone/isopropanol (2:1, w/w), and acetone/dimethylacetamide (2:1, w/w) were selected as solvent systems to dissolve CA. These specific solvent systems were chosen based on the literature (Table 1) and the fact that acetone is a commercially used solvent in many applications. Mixing DMAc or isopropanol impacts the vapor pressure of the systems, providing a possible range of responses for fiber spinning. Each solution was prepared at a concentration of 17 % (w/w), the selected flow rate was 1 mL/h, and the applied electrostatic voltage during electrospinning experiments was 25 kV. Continuous electrospun CA fibers were obtained from all the solvent systems tested (Fig. 1). No previous research reported the capability of acetone/isopropanol solvent system to produce electrospun CA fibers (see Table 2). There is controversy in the formation of electrospun fibers from pure acetone solution. Liu and Hsieh (Liu and Hsieh 2002) (15 and 20 % wt) and Tungprapa et al. (Tungprapa et al. 2007) (5 %, w/v) reported that it is not possible to produce continuous CA fibers from pure acetone. However, Han and Gouma (Han and Gouma 2006) and Son et al. (Son et al. 2004) described electrospun CA fibers from solutions of concentrations of 17.5 % (w/v), 17 %, and 20 % (w/w), respectively. The disagreement was due to the fact that cellulose acetate in acetone easily obstructs the tip of the needle during electrospinning because of the fast evaporation of acetone (Son et al. 2004; Tungprapa et al. 2007). Son et al. reported that the fibers created were produced previous to clogging of the needle tip (Son et al. 2004). In the present work, the tip was continuously cleaned during the electrospinning process by removing build-up with an insulating spatula. In this way, it was further possible to create electrospun CA meshes of 10 cm × 10 cm with various thicknesses after 1, 2, or even 3 h of electrospinning process. This cleaning procedure was also required during the electrospinning of CA dissolved in the solvent mixture of acetone/isopropanol due to similar issues.

Fibers produced from acetone and the mixture of acetone/isopropanol (2:1) exhibited a flat ribbon structure (Fig. 1a, b) in contrast to the acetone/DMAc (2:1) system that produced a cylindrical fiber shape (Fig. 1c). It has been established that fibers with ribbon structure contain a high molecular orientation, which is the result of the rapid evaporation of the solvent forming a skin structure that later collapses (Koombhongse et al. 2001). When using acetone (BP = 56 °C) or acetone/isopropanol (BP_{mix} = 64.8 °C) mixture, a skin is formed on the surface of the droplet. This skin is uniaxially stretched at the jet forming the ribbon like structure when the rest of the solvent evaporates.

With respect to the fiber size, acetone produced the highest average fiber size in width (2.77 μm, see Fig. 1a) followed by the mixture acetone/isopropanol (2:1) with an average fiber width of 2.47 μm (Fig. 1b). The value of the fibers obtained from acetone solution is close to the value reported by (Son et al. 2004). With

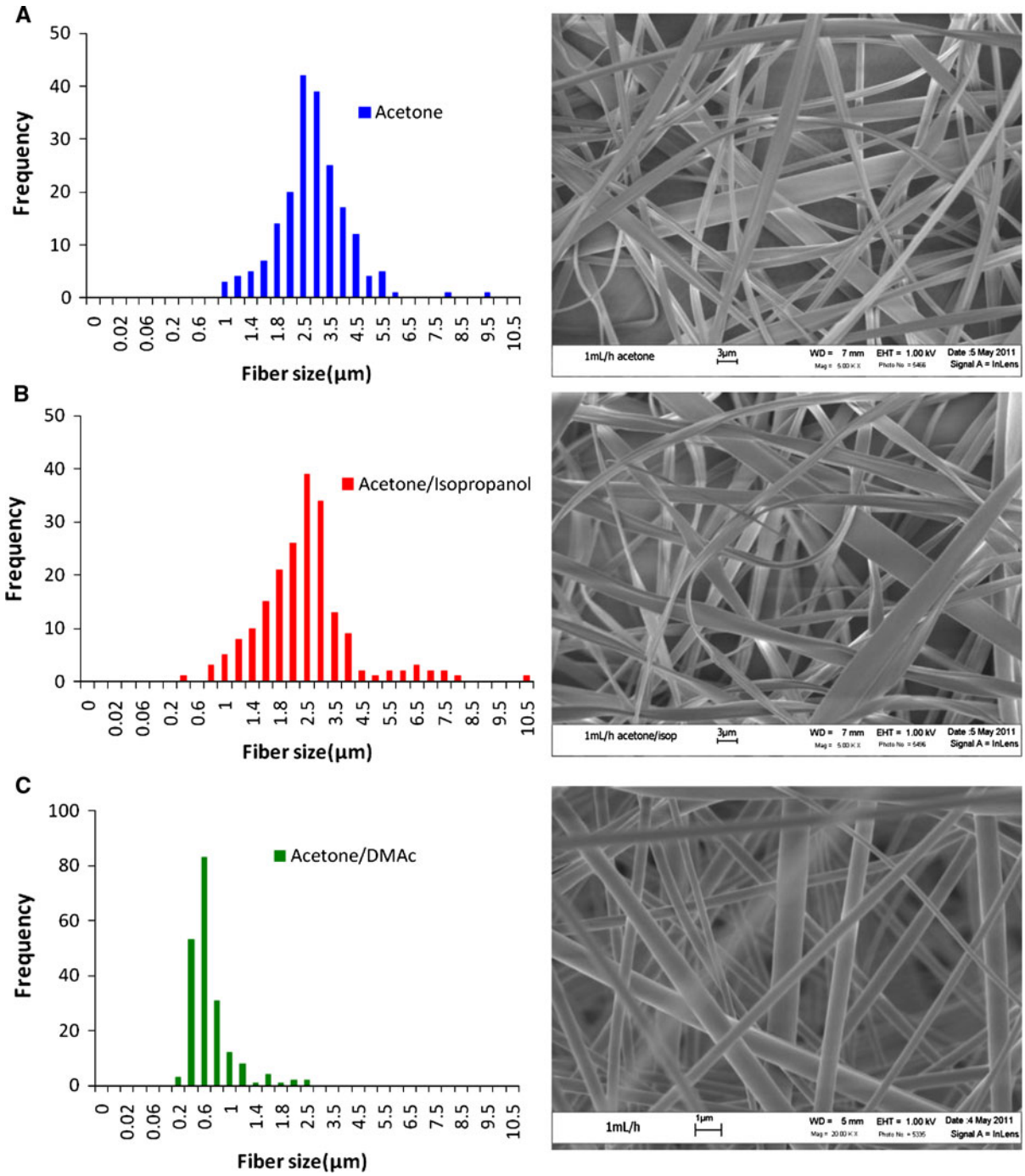

Fig. 1 Effect of solvent composition on the fiber size distribution and morphology of fibers produced from 17 % cellulose acetate (w/w) in **a** acetone, **b** acetone/isopropanol (2:1), and **c** acetone/DMAc (2:1). Fiber size refers to width of ribbon for **a** and **b** and the diameter of the fiber for **c**

the parameters used in this study, only the mixture acetone/DMAc (2:1) was able to generate CA fibers with an average fiber diameter in the submicron range (Fig. 1c), with a range of values from 147 nm to 2.1 µm. These values are comparable to the fibers described by Liu and Hsieh for the same solvent

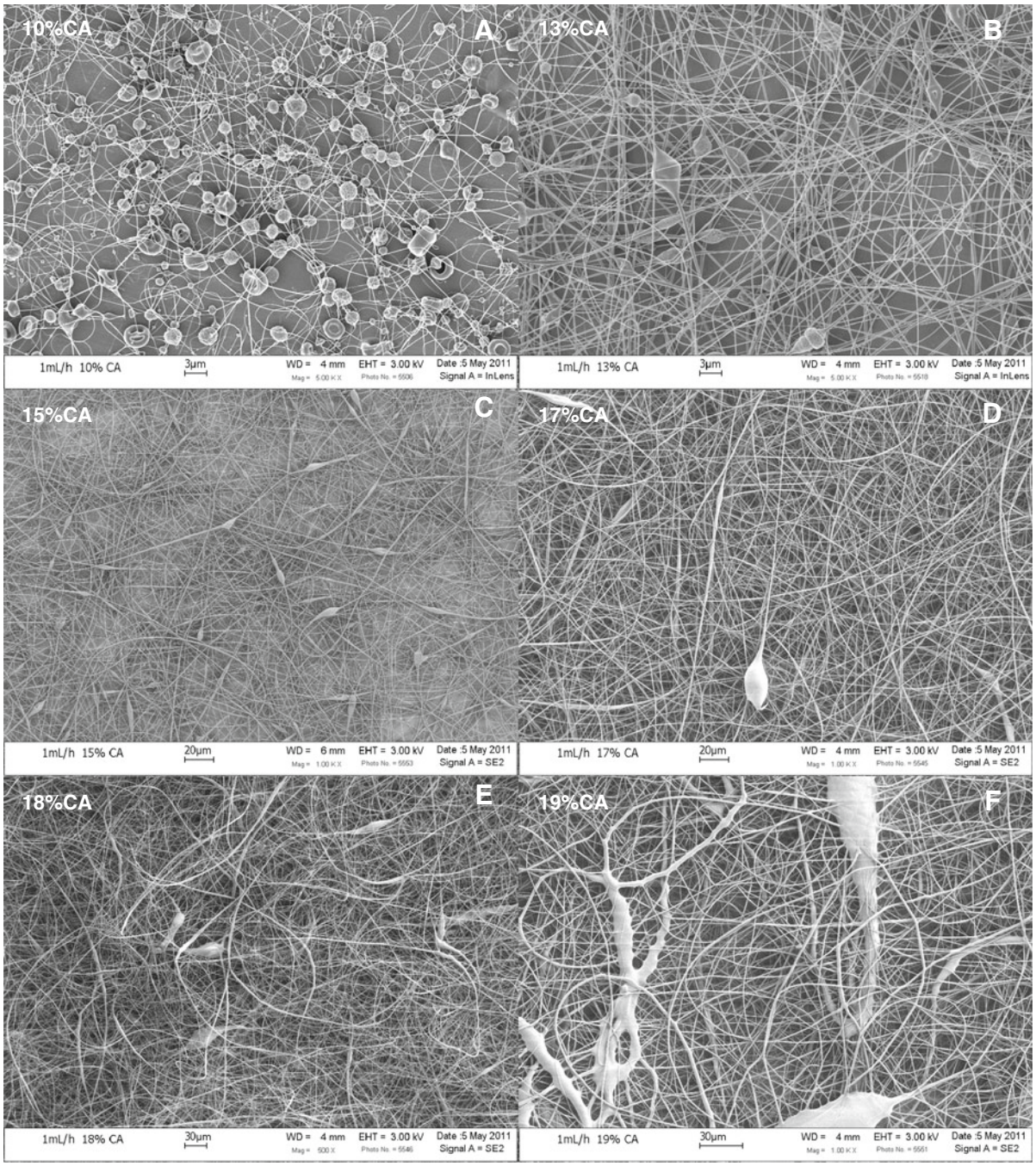

Fig. 2 Effect of cellulose acetate concentration in acetone/DMAc (2:1) on the structure of the electrospun CA fibers

composition (Liu and Hsieh 2002). The mixture of acetone/DMAc (2:1) also produced the lowest distribution in size of the fibers, with approximately 84 % of the fibers between 400 and 800 nm as illustrated in Fig. 1c.

For the purpose of optimizing the morphology, fiber size, and fiber size distribution of CA fibers, additional experiments were carried out relative to polymer concentration and flow rate using the acetone/DMAc (2:1) solvent system. In those experiments, it was

found that the CA concentration greatly impacted the morphology of the electrospun fibers (Fig. 2) (Fong et al. 1999; Huang et al. 2003; Han et al. 2005; Han and Gouma 2006). A low concentration of CA produced a high amount of beaded fibers, whereas an increase in concentration to a specific value reduced the amount of beads. This result originated mainly due to the lack of polymer entanglements that resulted in the instability of the solution jet (Fong et al. 1999; Liu and Hsieh 2002; Huang et al. 2003; Son et al. 2004; Han et al. 2008). Additionally, the shape of the beads changed from spherical to spindle-like as the concentration increased from 10 to 15 % and the beads size increased as well. These results were also documented by Fong for electrospun poly(ethylene oxide) (Fong et al. 1999) and previously observed for CA (Son et al. 2004; Liu and Tang 2006). As shown in Fig. 2d at a concentration of 17 %, there were limited numbers of beads formed during spinning. After increasing the concentration from 17 to 18 % the beads were present again, though the size of the beads were larger and the amount were reduced relative to the beads formed at 15 %. There were large clusters observed in samples at 19 % concentration. These clusters appeared to be the result of the fusion of several fibers (Fig. 2f). Therefore, as revealed in Fig. 2d, 17 % of CA is considered the optimal concentration for the formation of ultrafine CA fibers. This result is consistent with Son et al. observations (2004).

Controlled by the syringe pump, the flow rate of the solution impacted bead formation (Fig. 3), fiber size (Fig. 4), and size distribution (Fig. 5). There was formation of beads and large clusters of CA through the initial polymer/solvent composition when the strength of the electric field was held constant. The formation of beads and clusters was observed for the highest flow rate tested (Fig. 4a). That result is similar to that observed during electrospinning when CA concentration was above 17 % w/w (see Fig. 2f). It appears that the electric field was not great enough to produce smooth fibers, as there was too much material at the syringe tip. Accordingly, the high flow rate (or high concentration) caused an increase in viscosity and/or surface tension due to a local accumulation of the polymer at the tip of the needle as evaporation of the solvent occurred. The clusters were reduced and disappeared by decreasing the flow rate as shown in Fig. 3b–f). The flow rate had a positive effect on the size of the CA fibers, i.e. a decrease of fiber size coincided with the decreased flow rate. Empirical observations supported that the smallest diameters occurred during the lowest flow rates (Han and Gouma 2006; Thompson et al. 2007; Munir et al. 2009). Currently, there is only one study that describes the effect of the flow rate on CA fiber size and is concerned with the acetone solvent system (Han and Gouma 2006). Although the relationship between these variables follow the conventional tendency, the reported values are inconsistent (much smaller average fiber size) with those obtained by Son et al. (Son et al. 2004) and the present work for the same solvent system at similar CA concentration (see Table 1).

In the present study, the average CA fiber size produced from acetone/DMAc solvent mixture did not follow a linear correlation with the flow rate (Fig. 4). The expected phenomenon is that jet radius and flow rate (Q) have a non-linear relationship, radius of the jet is proportional to $Q^{2/3}$ (Munir et al. 2009). A power law dependency of 0.26 was found among fiber size and flow rate (Fig. 4). Munir et al. also indicated a power law dependency between flow rate and fiber size for polyvinyl pyrrolidone (Munir et al. 2009). In addition, the distribution of the fiber size also changed as a function of the flow rate (Fig. 5). An optimum value in the fiber size distribution was found at a flow rate of 0.5 mL/h with approximately 80 % of the fibers had a size between 400 and 600 nm (Fig. 5). While the lower fiber size was obtained at 0.025 mL/h, this system included the highest fiber size distribution from 8 nm to 1.28 μm (Fig. 5).

As described in previous studies, jet/fiber diameter depends on the flow rate (Munir et al. 2009; Thompson et al. 2007). There are empirical equations that correlate the jet radius and the flow rate. For instance, Oswald-deWaele law has been used to describe polymer fluids (Munir et al. 2009). The law's model for the prediction of the jet radius is:

$$h_r = 2\lfloor \varepsilon_O \gamma \rfloor^{1/3} \left(\frac{Q}{I}\right)^{2/3} \quad (1)$$

where h_r is the jet radius, ε_o the permittivity of a vacuum (A^2s^4/kgm^3), γ the surface tension (N/m), Q the flow rate (L/min), and I the electric current (A).

The terminal jet diameter was predicted using a model of a charged fluid jet in an electric field under conditions applicable to whipping instability (Munir et al. 2009). The equation is as follows:

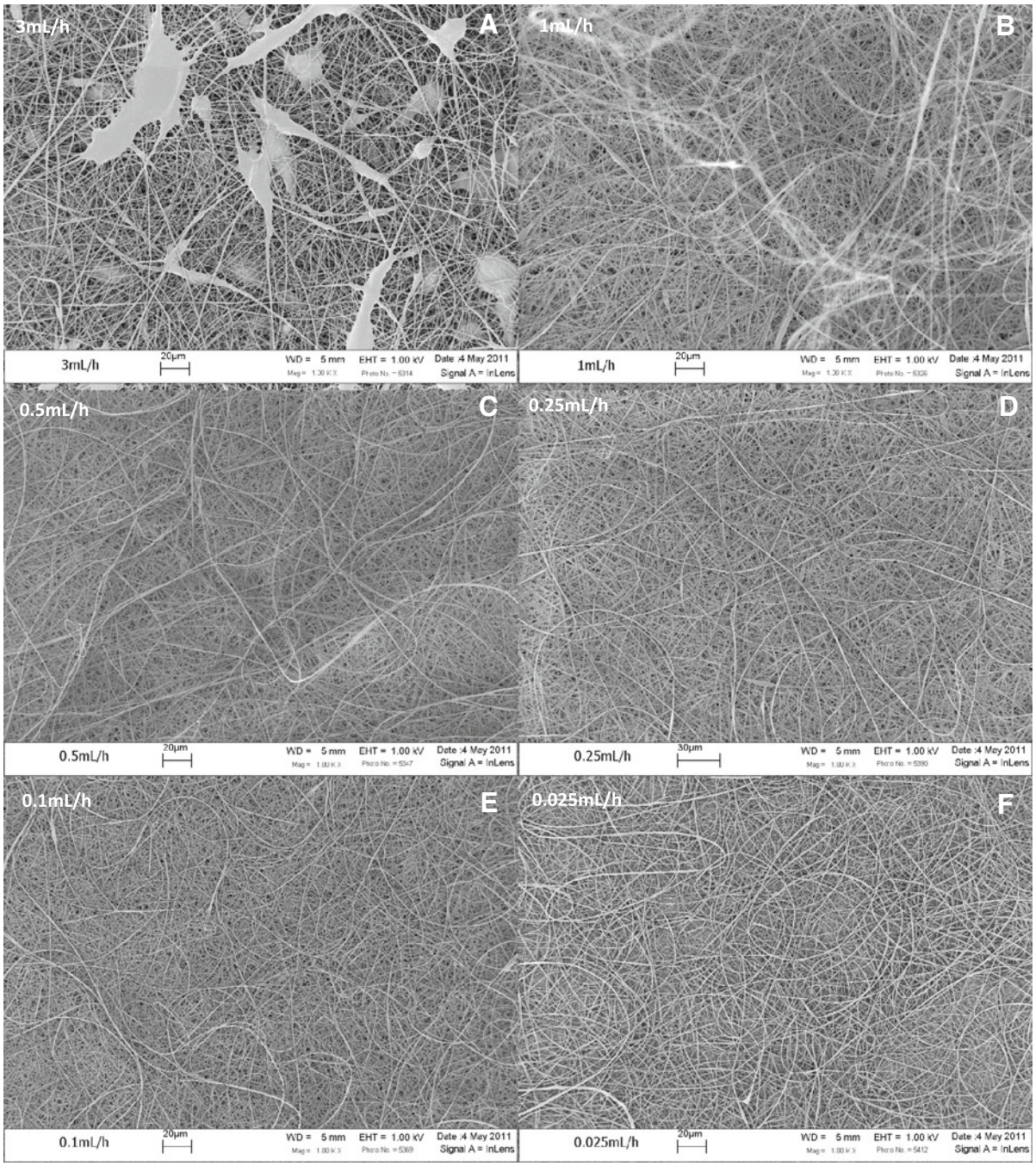

Fig. 3 Effect of flow rate on bead formation for acetone/DMAc (2:1) solvent system

$$h_t = \left[\gamma\varepsilon\frac{2}{\pi(2\ln(-3))}\frac{Q^2}{I^2}\right]^{1/3} \quad (2)$$

where h_t is the terminal diameter of the jet and $\chi \approx R/h$, R is the radius of curvature.

Equations 1 and 2 show that jet/fiber diameter not only depends on the solution properties, but also the processing variables such as flow rate and current. Nevertheless, the relation between the jet radius and dried fiber diameter is not simple as it is impacted by

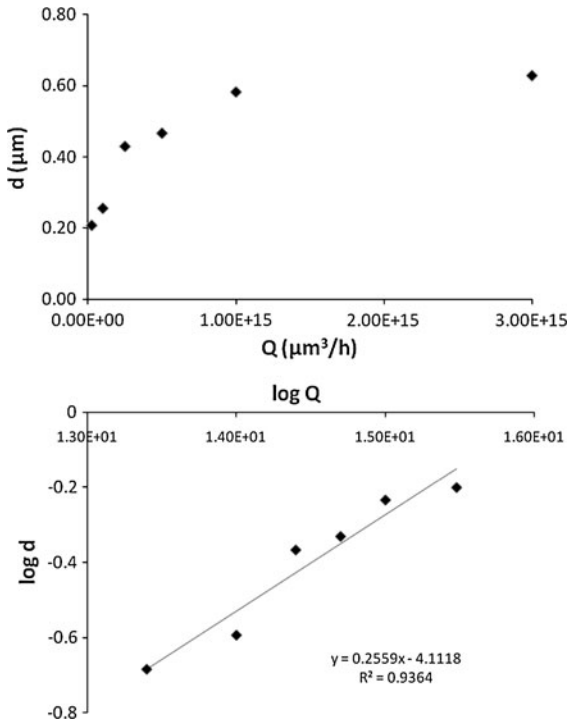

Fig. 4 Average fiber size diameter as a function of flow rate (Q) for 17 % w/w cellulose acetate in acetone/DMAc

factors such as solvent evaporation (Munir et al. 2009).

Processing parameters impacting regeneration

Another important criterion in the selection of the conditions to electrospin CA was the performance of the electrospun fibrous meshes after deacetylation treatment; since the ultimate purpose of the electrospun fibers was to create nanofibrous cellulose scaffolds from them. Consequently, electrospun meshes fabricated at 3, 1, and 0.5 mL/h were deacetylated with alkaline ethanol based on previous protocols (Rodriguez et al. 2011). After regeneration it was observed that the nanofibers produced at 0.5 mL/h were not able to preserve their original network structure; the fibers moved and aligned after deacetylation treatment (see Fig. 6a, b). The alignment may be due to the wet tissue stretching as it was pulled from the solutions. Additionally, the material at low flow rates was difficult to handle when placed in water, which is a major drawback when performing tissue engineering experiments like cell seeding. In contrast, the nanofibrous meshes produced at higher flow rates (3 and 1 mL/h) were able to retain their physical structure, almost intact, after regeneration treatment and when exposed to a mineralization bath, as corroborated by SEM examination (Rodriguez et al. 2011). Similar regeneration experiments were carried out for the meshes produced from acetone and acetone/isopropanol solutions. Structural changes to these materials were more severe than in the low flow rate for acetone/DMAc system; the fibers were completely separated after the regeneration process (Fig. 7a, b). Previous studies noted that when using a low volatility solvent, for instance DMAc (BP = 165 °C), a certain amount of solvent remains producing wet fibers. Because of the remaining solvent, adhesion between fibers via polymer diffusion occurs at intersecting fibers, creating a strong interconnected porous structure (Veleirinho et al. 2008; Han et al. 2005; Baji et al. 2010). This phenomenon explains the difference in the physical stability of the structure after the deacetylation process for the electrospun CA from acetone versus the electrospun CA from acetone/DMAc. The result also suggests that the flow rate impacts the ability of physical crosslinking to occur. Fibers formed at a high flow rate (especially when DMAc is present) have more solvent residual adhering to the fibers, compared to low flow rates. The effect of the high flow rate to bind fiber is evident in Fig. 3a. The optimum condition to electrospin CA based on the morphology and fiber size and its distribution was observed at flow rate of 0.5 mL/h, the practical flow rate selected was 1 mL/h to keep the mesh structure intact. The existence of DMAc within the electrospun mesh after electrospinning process is corroborated by TGA analysis (vide infra).

The thermogravimetric analysis (TGA) of electrospun CA and regenerated cellulose (24 h treatment) materials were analyzed by TGA with the purpose of determining the existence of trapped solvents, particularly dimethylacetamide (DMAc) due to its toxicity, from the electrospinning process (Fig. 8). The thermal decomposition of electrospun CA and regenerated cellulose consisted of a series of steps. The first step of weight loss, from room temperature to 110 °C, is related to the evaporation of absorbed water since the materials are hygroscopic; a 3 % decrease in weight occurred below 110 °C, which corresponds to the water content. This weight loss is followed by the evaporation of DMAc at 170 °C. For the CA material a

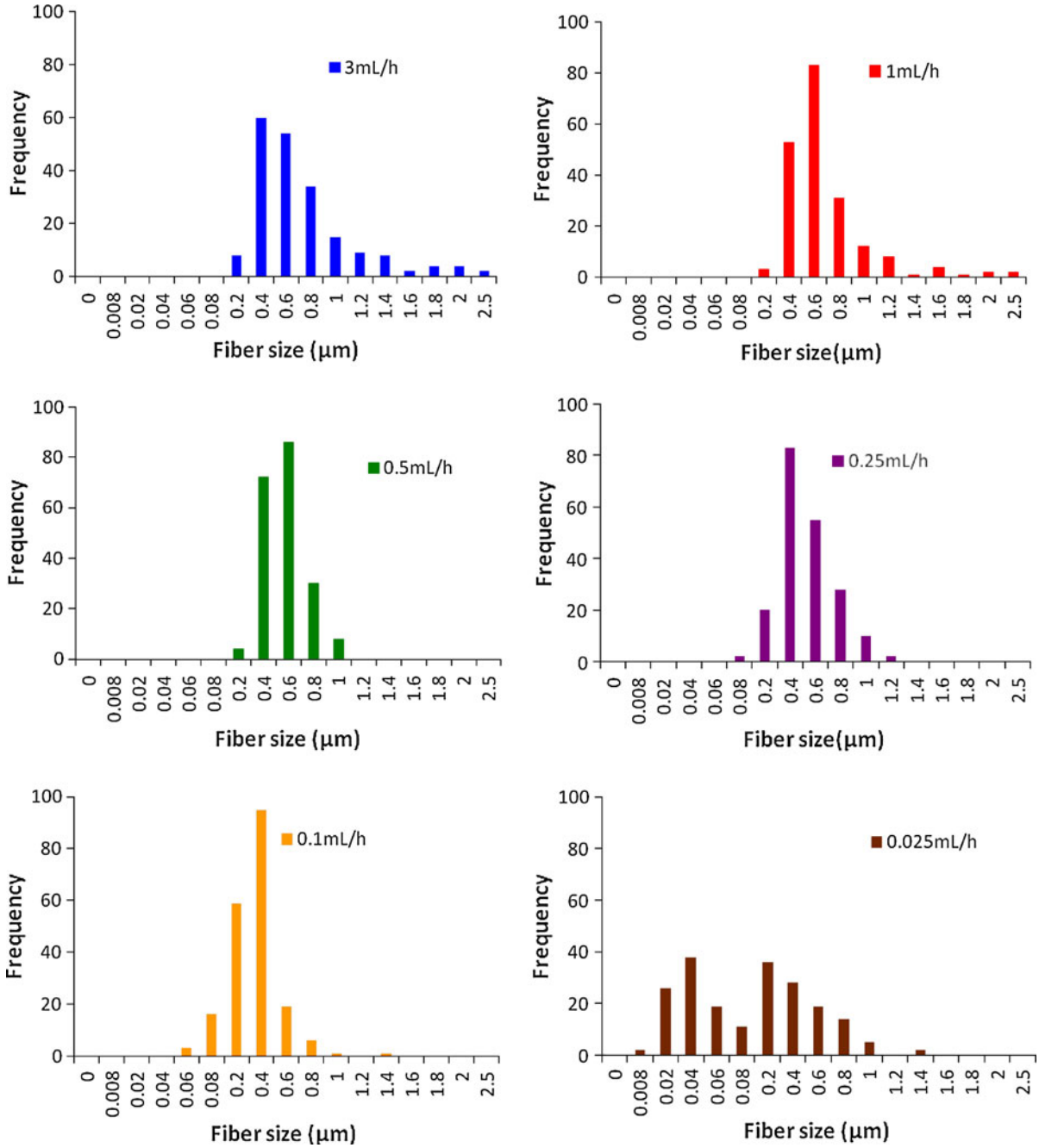

Fig. 5 Effect of flow rate for the solvent system acetone/DMAc on the fiber size distribution

5.8 % decrease occurred from 110 to 170 °C. In contrast to electrospun CA, the regenerated cellulose materials did not exhibit any loss in weight between 110 and 250 °C (Fig. 8), which indicates no detectable levels of DMAc within the electrospun membrane after regeneration treatment. Since cellulose is an established biocompatible material (Miyamoto et al. 1989; Helenius et al. 2006), this finding promotes the use of electrospun regenerated cellulose membranes as biomaterial due to the fact that there is no other substance apart from water and cellulose. However, an additional treatment, vacuum drying or dialysis, is

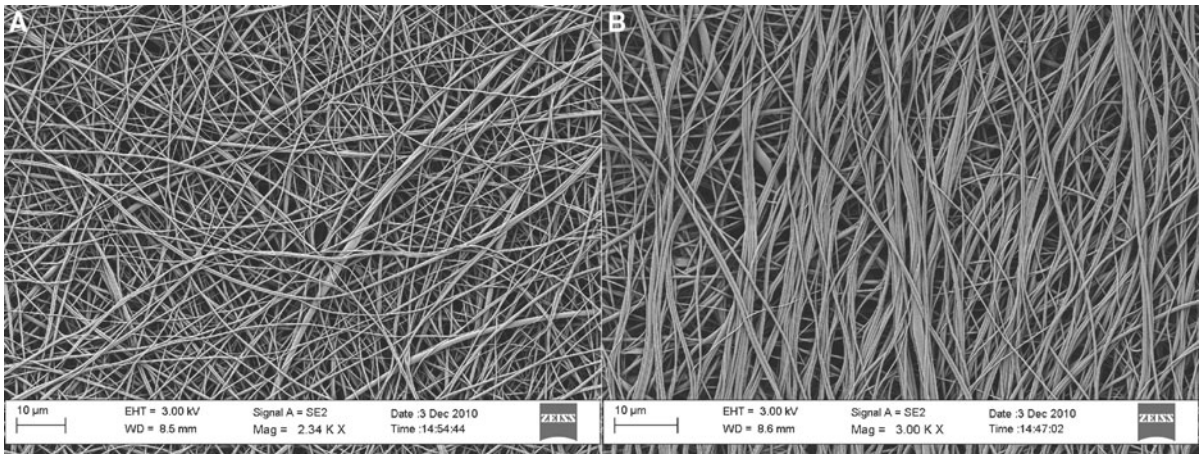

Fig. 6 Electrospun fibrous meshes produced at 0.5 mL/h from acetone/DMAc solution. **a** Cellulose acetate mesh before deacetylation treatment, **b** regenerated cellulose fibrous mesh after treatment with 0.05 M NaOH ethanol solution

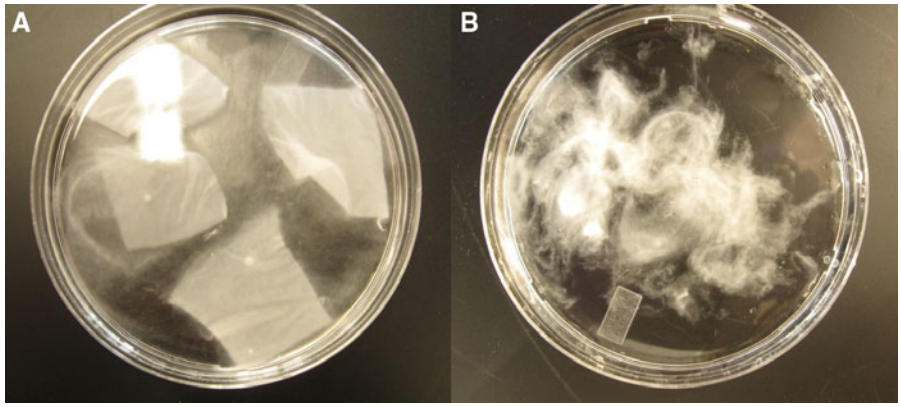

Fig. 7 Morphology of the electrospun fibers produced from 17 % cellulose acetate in acetone solution. **a** During deacetylation treatment, and **b** after deacetylation and rinsed in DI water

necessary for the electrospun CA materials prior to contact with any mammalian tissues. The high surface area samples should be handled with appropriate personal safety gear prior to removal of residual DMAc.

In vitro toxicity tests

A qualitative analysis of the morphology of the cells exposed to the electrospun cellulose fibers extracts was obtained in the minimal essential media (MEM) elution test (Table 3). The electrospun cellulose fiber extracts were compared with polypropylene (negative), latex (positive), and media controls as established by ANSI/AAMI/ISO 10993-5 standards. Microscopic observation revealed that cells exposed to the electrospun cellulose fiber extracts did not present formation of intra-cytoplasmatic granules, breakdown of cells (lysis), or reduction of cell growth in any of the cellulose samples. Therefore, the extracts of electrospun cellulose fibers did not produce any cytotoxic effect on mammalian cells in conformity with the criteria established by United States Pharmacopeia and National Formulary (USP 87) and the ANSI/AAMI/ISO 10993-5 standards.

Electrospun cellulose fibers were tested for cytotoxicity on cultured cells by the agar overlay method for 24 h; cells were evaluated microscopically and the "reactivity grade" of electrospun cellulose due to leachable substances was determined by the criteria established in the ANSI/AMI/ISO 10993-5 standard.

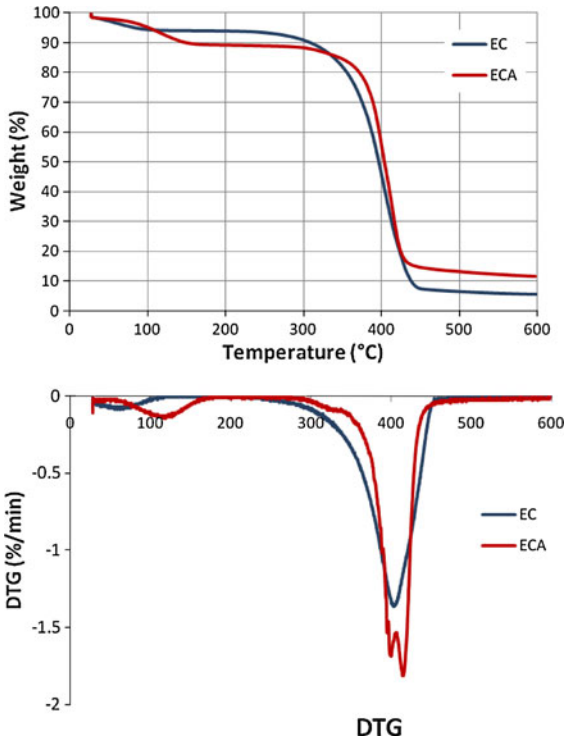

Fig. 8 TGA and derivative TGA curves of electrospun cellulose acetate (*ECA*) and electrospun regenerated cellulose (*EC*) fibers

Electrospun cellulose fibers were evaluated with negative and positive controls (Table 4). There were no degenerate cells around or under any of the three samples in contact with the electrospun cellulose fibers. Consequently, the degree of cytotoxicity of leachable electrospun cellulose fibers was graded as zero. The negative and positive controls displayed the expected behavior, which validates the electrospun cellulose cytotoxic results.

The results from MEM elution and agar overlay tests confirms that there are no cytotoxic extractable or leachable substances presented in electrospun cellulose fibers at levels that can be harmful or pose any adverse effect on mammalian cells. These groups of tests confirm the feasibility of producing cellulose fibers by the electrospinning and deacetylation processes for potential use as substrates for cell growth. There are several studies regarding in vitro cytotoxicity of electrospun cellulose fibers that complement these results. An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl phenyltetrazolium bromide) assay on human fibroblast and human keratinocytes cells was carried out for electrospun cellulose and electrospun cellulose acetate (CA) with 11 and 24 % of acetyl content. The proliferation ability of both types of cells

Table 3 MEM elution results of electrospun cellulose and controls

Controls	Extraction Ratio	Amount tested/extraction solvent amount	Post extraction appearance	Scores				Result (pass/fail)
				1	2	3	Average	
Electrospun cellulose	0.2 g/mL	6.7 g/33.5 mL	Clear	0	0	0	0	Pass
Polypropylene pellets (negative control)	0.2 g/mL	4 g/20 mL	Clear	0	0	0	0	Pass
Media control	N/A	20 mL	Clear	0	0	0	0	Pass
Latex natural rubber (positive control)	0.2 g/mL	4 g/20 mL	Clear	4	4	4	4	Fail

MEM elution test was performed by Nelson laboratories

Table 4 Agar overlay results of electrospun cellulose and controls

Sample	Amount tested	Grades			
		1	2	3	Average
Electrospun cellulose	≥100 mg per well	0	0	0	0
Polypropylene pellets (negative control)	≥100 mm^2 per well	0	0	0	0
Latex natural rubber (positive control)	≥100 mm^2 per well	4	4	4	4

Agar overlay test was performed by Nelson laboratories

was not affected by any of these materials (Supaphol et al. 2012). The viability of human fibroblast cells was also evaluated by a XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide) assay for CA electrospun fibers and CA fibers loaded with gallic acid (Phachamud and Phiriyawirut 2011). The viability of CA fibers was 72.04 ± 5.91 % in contrast to the control (plastic well plates) with 100 % of viable cells. Cell viability decreased with the increase in gallic acid content. Also reported was the cytotoxicity (indirect evaluation) of CA electrospun fiber and CA electrospun fibers loaded with curcumin on normal human dermal fibroblast by a MTT assay (Suwantong et al. 2010). The CA fiber mat provided the best support in attachment and proliferation. All these observations confirm that there are not extractable substances in harmful levels for mammalian cells derived from the electrospinning process of CA and the post regeneration treatment.

Conclusions

Solvent selection along with flow rate strongly influenced the cellulose acetate fiber size, size distribution, and fiber geometry. Dependent upon the conditions and solvents selected, cellulose fibers from single digit micrometers down to single digit nanometers were formed within the electrospun materials. For the acetone/DMAc solvent system selected, both polymer concentration and solution flow rate caused significant changes in average fiber diameter and size distribution with a power law relationship between fiber diameter and flow rate. Furthermore, CA electrospun from highly volatile solvents formed a ribbon-like morphology with widths larger than the acetone/DMAc combination. However, for the acetone/DMAc system, residual DMAc was retained within the electrospun mat. This retention helped to create physical crosslinks between fibers for the electrospun mats allowing the preservation of structure after the regeneration process. This latter observation is extremely important when attempting to create scaffolds for tissue engineering applications. Furthermore, once regenerated, cellulose materials contained no leachable compounds showing no cytotoxicity during in vitro cell tests. This study determined appropriate concentrations, solvent combinations, and flow rates to prepare cellulosic materials for biomedical applications.

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