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Short communication

Electrospinning of hyaluronic acid nanofibers from aqueous ammonium solutions

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ABSTRACT

For several reasons, the electrospinning of nanofibrous mats comprised purely of biopolymers, such as hyaluronic acid (HA) has been difficult to achieve. Most notably, due to its polyelectrolytic nature, very low polymer concentrations exhibit very high solution viscosities. Thus, it is challenging to obtain the critical chain entanglement concentration necessary for biopolymer electrospinning to ensue. While the successful electrospinning of HA fibers from a sodium hydroxide:dimethylformamide (NaOH:DMF) system has been reported, the diameter of these fibers was well above 100 nm. Moreover, questions regarding the degradation of HA within the solvent system arose. These factors supported our ongoing research into determining an improved solvent system. In this study, the use of a less basic (pH 11) aqueous ammonium hydroxide (NH_4OH) solvent system, NH_4OH:DMF, allowed for the fabrication of HA mats having an average fiber diameter of 39 ± 12 nm. Importantly, while using this solvent system, no degradation effects were observed and the continuous electrospinning of pure HA fibers was possible.
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1. Introduction

Hyaluronic acid (HA) is a major glycosaminoglycan found in the extracellular matrix of many soft tissues in higher animals. It is a linear natural polysaccharide composed of repeating disaccharide units of β-1-4-D-glucuronic acid and β-1-3-*N*-acetyl-D-glucosamine. The complete biocompatibility of HA has led to extensive research into its use in biomedical applications, including, ophthalmology, drug delivery, dermatology, tissue scaffolding, and medical implants (Huskisson & Donnelly, 1999; Jia & Kiick, 2009; Kim, Chung, & Park, 2008; Nesti et al., 2008; Yoo, Lee, Yoon, & Park, 2005).

Numerous tissue engineering applications – substrates for tissue regeneration, wound dressing scaffolds, and artificial blood vessels – would benefit from the increased surface area-to-volume ratios and range of pore sizes that electrospun nanofibrous mats have to offer. Electrospinning is a simple and inexpensive method for producing nanoscale non-woven polymer mats which exhibit these desirable intrinsic structure–property relationships. It is for this reason that the production of pure biopolymer nanofibrous mats has been of renewed interest in recent years (Schiffman & Schauer, 2008).

However, complications arise when working with charged biopolymer solutions due to their long-range electrostatic interactions and the presence of counter ions (Schiffman & Schauer, 2008). As a result of these interactions, HA forms highly viscous solutions at low polymer concentrations, which severely hinders its electrospinnability. Reaching the critical chain entanglement concentration is one requirement for fiber formation as it ensures that a critical amount of polymer chains will overlap and topologically constrain each other's motion (McKee, Wilkes, Colby, & Long, 2004). To reach this critical point before the onset of high solution viscosity, literature has previously blended HA with uncharged carrier polymers including collagen (Hsu, Hung, Liou, & Shen, 2010), gelatin (Li, He, Han, et al., 2006; Li, He, Zheng, & Han, 2006), and zein (Yao, Li, & Song, 2007). Poly(ethylene oxide) (PEO) was mixed with a thiolated HA derivative, 3,3′-dithiobis(propanoic dihydrazide) (HA-DTPH) to create crosslinked nanofibrous mats (Ji, Ghosh, Li, et al., 2006; Ji, Ghosh, Shu, et al., 2006). To overcome the issue of high solution viscosity and electrospin HA without a carrier polymer, Um et al. (Um, Fang, Hsiao, Okamoto, & Chu, 2004), have demonstrated the use of an altered electrospinning setup, which featured air assisted blowing and elevated temperatures.

Electrospinning of pure HA dissolved in dimethylformamide (DMF):water (H_2O) (Li, He, Han, et al., 2006) solutions has been demonstrated with the aid of elevated temperatures and an ethanol coagulation bath. Recently, a tri-component by weight system featuring 25:50:25 H_2O:formic acid (FA):DMF was reported by Liu et al. (2011). However, since the pH of this solution is approximately 2–3, the protonation of the HA carboxylate can change the properties of the biopolyelectrolyte thus reducing the charged groups. A third system featuring sodium hydroxide (NaOH) and DMF (Kim, Chung, & Park, 2008) has also been reported in the literature. At the time of our work, the H_2O:FA:DMF system was not yet

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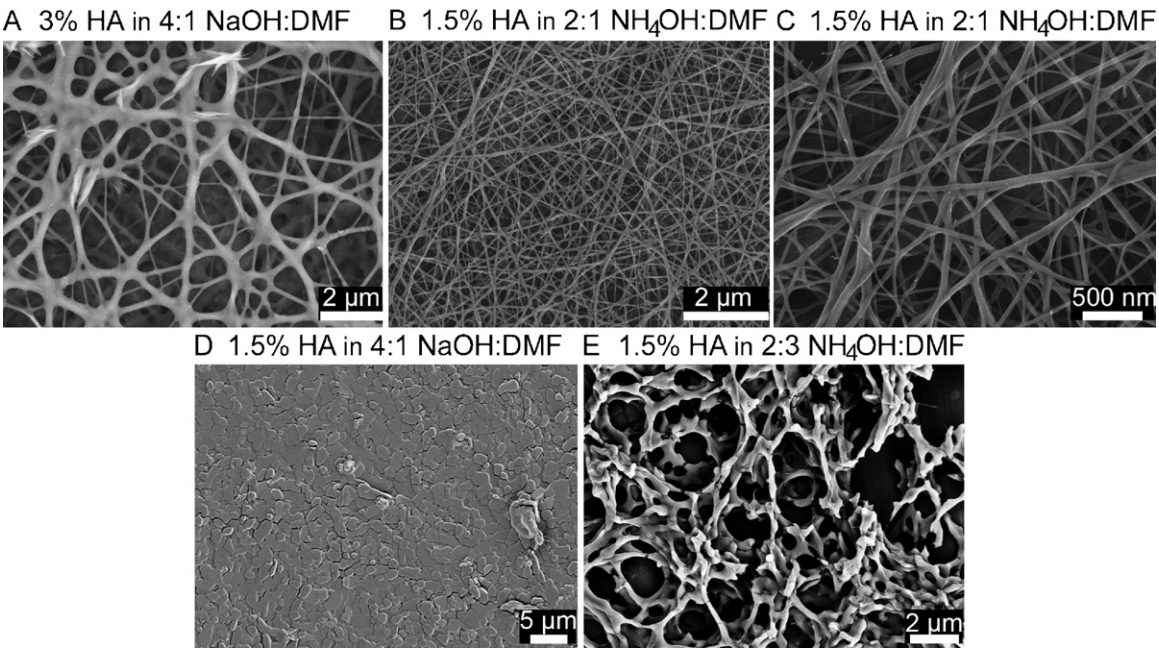

Fig. 1. SEM micrographs displaying the morphology of fibrous mats electrospun from (A) 3% HA in dissolved in a 4:1 NaOH:DMF solution, (B and C) 1.5% HA dissolved in a 2:1 NH_4OH:DMF solution, (D) 1.5% HA dissolved in a 4:1 NaOH:DMF solution, and (E) 1.5% HA dissolved in a 2:3 NH_4OH:DMF solution. Solutions containing NaOH were spun within 5 min of mixing to avoid degradation effects.

published, and thus, our attention was put towards reproducing HA fiber mats electrospun from DMF:H_2O and NaOH:DMF solutions using an unmodified electrospinning apparatus. Despite our efforts, results were only possible using the strongly basic solvent system of 4:1 NaOH:DMF (Fig. 1A). The fibers produced exhibited average diameters greater than 100 nm, consistent with Kim et al. However, 30 min after preparing the HA solution in the 4:1 NaOH:DMF solvent system, electrospinning became hindered. Maleki, Kjoniksen, and Nystrom (2008), have shown that HA decreases in viscosity at the extreme ends of the pH spectrum, which they have attributed to the degradation of HA. We observed a viscosity change in our solutions after 30 min, which we hypothesize resulted from the degradation of HA in the highly basic, pH 13, 4:1 NaOH:DMF solution.

In this study, we investigate the electrospinning of pure hyaluronic acid (HA) nanofibrous mats utilizing a solvent system of aqueous ammonium hydroxide (25% NH_4OH) and DMF. This solution has a pH of 11 versus that of 13 in the 4:1 NaOH:DMF system. This effort is driven by the desire to eliminate any solvent induced degradation effects on the biopolymer to further HA nanofibrous mats applicability for biomedical applications.

2. Methods and materials

2.1. Materials

All compounds were used as received. Hyaluronic acid (HA, cosmetic grade, Mw = 2,000,000 Da) was purchased from Dali Chemical Co. (China). Pure sodium hydroxide (NaOH, 97+ ACS) and dimethylformamide (DMF, 99.8% ACS) were purchased from Sigma–Aldrich (St Louis, MO). Ammonium hydroxide (NH_4OH, 25% in water) was purchased from Fluka (Switzerland).

2.2. Hyaluronic acid mat fabrication

2.2.1. Solution preparation

HA solutions of 3% (w/v) in a 4:1 mixture of (0.5 M) NaOH:DMF, as well as, 1.5% HA in a 2:1 solution of NH_4OH:DMF were prepared. Solutions containing NaOH and NH_4OH were mixed using an Arma-Rotator A-1 (Bethesda, MD) until completely dissolved, colorless solutions were obtained, which took 5 min and 24 h, respectively. HA solutions corresponding to 0.2%, 0.4%, 0.6%, 0.8% and 1.0% in both solvents, as well as in ultrapure water (H_2O) (Millipore QPAK system) were prepared for conductivity measurements.

2.2.2. Solution conductivity

Solution conductivity measurements were taken using a CON 510 conductivity meter (Oakton, Vernon Hills, IL). The meter was calibrated using a 1413 μS standard solution (potassium chloride, Fluka) prior to each use. Measurements were taken in triplicate.

2.2.3. Electrospinning

A previously described electrospinning apparatus was utilized (Schiffman & Schauer, 2007). Briefly, a 5 mL Luer-Lok Tip syringe (Becton Dickinson & Co, Franklin Lakes, NJ) was loaded with approximately 4 mL of solution and a Precision Glide 21-gauge needle (Becton Dickinson & Co, Franklin Lakes, NJ) was attached. By use of an alligator clip, the positive electrode of a high-voltage supply (Gamma High Voltage Research Inc., Ormond Beach, FL) was directly connected to the needle. The syringe was then placed on an advancement pump (Harvard Apparatus, Plymouth Meeting, PA), which was at a fixed distance from the negative electrode that was clipped to a copper plate wrapped in aluminum foil. For 4:1 NaOH:DMF solutions, a separation distance of 5 cm was used while for 2:1 NH_4OH:DMF, the distance was fixed at 6 cm. An applied voltage and pump advancement speed of approximately, 10 kV and 15 μL/min for 4:1 NaOH:DMF, and 20 kV and 0.01 μL/min for 2:1 NH_4OH:DMF systems were utilized. The temperature (°C) and relative humidity during electrospinning were monitored by a digital thermohygrometer (Fisher Scientific, Pittsburgh, PA).

2.3. Characterization of electrospun hyaluronic acid mats

2.3.1. Microscopy

Micrographs of electrospun fiber mats were acquired using a Zeiss Supra 50/VP field emission scanning electron microscope

(SEM). A Denton vacuum desk II sputtering machine was utilized to coat the samples for 10 s with platinum–palladium. Average fiber diameter was determined using ImageJ 1.41 software (National Institutes of Health, Bethesda, MD) by measuring the diameter of 50 random fibers from at least three different micrograph images.

2.3.2. Chemical analysis

Fibers were collected from the aluminum foil and analyzed using a Smith's IlluminatIR attenuated total reflectance Fourier transform infrared spectroscopy (FTIR). The ATR objective was composed of diamond with a numerical aperture of 0.71. The software was set to perform 64 scans within the range of 4000–500 cm^{-1} at a resolution of 4 cm^{-1}.

X-ray photoelectron spectroscopy (XPS) was performed using a PHI 5000 VersaProbeTM Scanning Multi-Technique system (monochromatic Al Kα X-rays with 1486.6 eV). The (25 kV) X-ray source had a beam diameter of 100 μm and power of 25 W. The spectra were recorded in fixed analyzer transmission (FAT) mode with a pass energy of 117.40 eV, a step size of 1 eV for survey scans, and a pass energy 23.5 eV (step size 0.2 eV). For high resolution scans, a pass energy of 11.75 eV (step size 0.1 eV) was employed with a spectral resolution of 0.6 eV. Static charge and localized positive charge created by the X-ray beam were eliminated using a low energy Ar+ ion beam and a low energy electron beam, respectively. Binding energies were charge corrected to 284.8 eV for the adventitious C1s peak. A Gaussian/Lorentzian peak shape was assumed for the curve-fitting process performed using CASA or MultiPak.

3. Results and discussion

3.1. Characteristics of electrospun hyaluronic acid mats

SEM micrographs taken at various magnifications, Fig. 1B and C, micrographs display that fibers were successfully electrospun from 1.5% hyaluronic acid (HA) dissolved in a 2:1 ratio of NH_4OH:DMF solution. Consistent, randomly oriented continuous fibers are produced utilizing this new solvent system. No beading is observed. When attempts were made using the same concentration (1.5%) HA in the previously published (Kim, Chung, & Park, 2008) solvent system, 4:1 NaOH:DMF, fiber formation was unsuccessful (Fig. 1D). Doubling the polymer concentration in the same solvent system yielded fibers featuring a branched morphology (Fig. 1E). To avoid branching, the balance between electrical forces and surface tension must be maintained (Schiffman & Schauer, 2007). As noted previously, degradation of the HA might have occurred when it was dissolved in the pH 13 solution of 4:1 NaOH:DMF. This might result in molecular weight fluctuations within the electrospinning dope that could consequently affect the morphology of the fibers.

HA mats produced from solutions of 2:1 NH_4OH:DMF exhibited an average diameter of 39 ± 12 nm (Fig. 2). These fibers had a narrow distribution of diameters, all of which were smaller than the larger diameter fibers 224 ± 81 nm produced from the 4:1 NaOH:DMF system. This value is within one standard deviation from the previously reported (Kim et al., 2008) NaOH based fibers, 198 ± 45 nm. These fibers were fabricated from a 10% HA solution and increasing the polymer concentration has been reported to increase the fiber diameter.

We investigated the electrospinnability of a less basic NaOH:DMF solution by adjusting the pH to 11 using 1 N HCl. At the same biopolymer concentration previously spun, 3% HA, the dope became extremely viscous in the pH adjusted solvent system and could not be electrospun. A lower concentration (1.5%) of HA in the pH 11 NaOH:DMF solution also was too viscous to be electrospun. Solutions comprised of 3% HA in 2:1 NH_4OH:DMF, pH 11, failed to produce any fibers; the solution gelled almost immediately upon

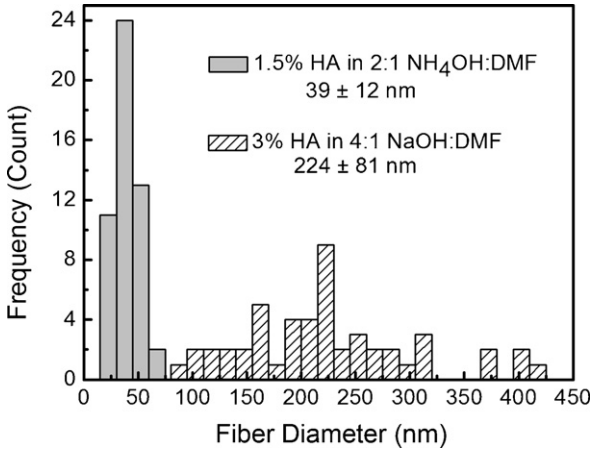

Fig. 2. Fiber diameter distribution for mats electrospun from (left) 1.5% HA dissolved in 2:1 NH_4OH:DMF and (right) 3% HA dissolved in 4:1 NaOH:DMF as determined using ImageJ software on SEM micrographs. The average fiber diameter and standard deviation of 50 random fiber diameters measured are also given.

the addition of the polymer. In addition to the 2:1 ratio of NH_4OH to DMF, Fig. 1E indicates that the electrospinning of a 2:3 solvent ratio with 1.5% HA was successful. These fibers appear less cylindrical and have a higher average diameter (269 ± 83 nm) than the fibers spun from the 2:1 ratio. This increase results from the higher proportionality of the less volatile and non-solvent DMF within the binary system.

3.2. Role of solution conductivity on the electrospinnability of hyaluronic acid mats

Fig. 3 displays solution conductivity measurements for various concentrations of HA dissolved in 2:1 NH_4OH:DMF, 4:1 NaOH:DMF, and ultrapure water (H_2O). Solutions prepared using the 4:1 NaOH:DMF solvent system exhibited the highest conductivity, which might be attributed to the high proportion of NaOH in the solution and its dissociation into Na^+ and OH^- ions. Here, the solvent appears to play the dominant role as solution conductivity remains constant over the polymer concentration range explored. HA dissolved in 2:1 NH_4OH:DMF and H_2O display a slightly increasing trend in conductivity and measurements of the same magnitude. These results echo previous findings (Schiffman

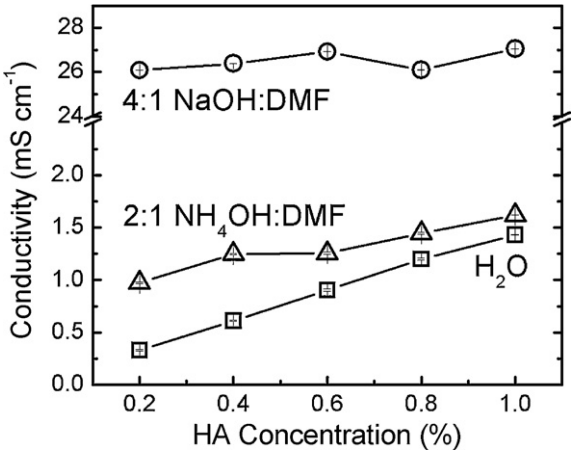

Fig. 3. Conductivity of solutions containing 0.2–1.0% HA in various solvent systems including (top, circles) 4:1 NaOH:DMF, (middle, triangles) 2:1 NH_4OH:DMF, and (bottom, squares) 100% H_2O. Polymer concentration was kept below 1% HA in order to maintain an ideal solution viscosity for measurement acquisition. All measurements were taken in triplicate, error bars indicate one standard deviation.

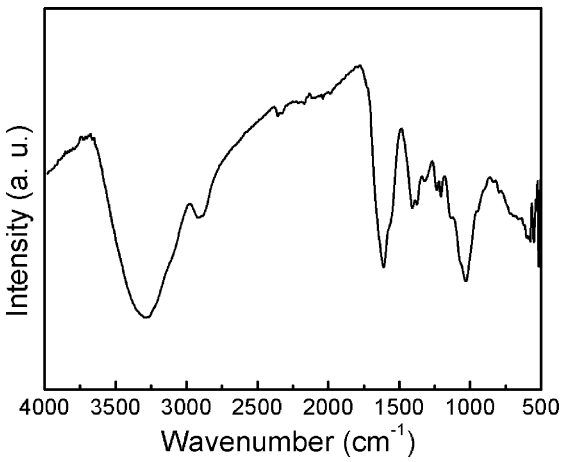

Fig. 4. FTIR transmission spectra of 1.5% HA fibers electrospun from a 2:1 NH_4OH:DMF solution.

& Elimelech, 2011; Schiffman, Blackford, Wegst, & Schauer, 2011) that while solution conductivity may influence the final fiber morphology; it does not play as large of a role in electrospinnabilty as previously thought. We know that while HA can spin in 2:1 NH_4OH:DMF, when dissolved in H_2O, solution viscosity, surface tension along with other factors prohibit it from electrospinning. The ability of the solvent system to disrupt the strong inter- and intra-molecular hydrogen bonds of the biopolymer structure appear to play the stronger role. In this case, changing the chain conformation of HA from a rigid alpha helix structure to the coil conformation would encourage chain flexibility and entanglement.

3.3. Chemical analysis of electrospun hyaluronic acid mats

The FTIR spectra of 1.5% HA fibers electrospun from a 2:1 NH_4OH:DMF solution is shown in Fig. 4. Characteristic peaks for HA are evident at 1375 cm^{-1} for C–O and 1600 cm^{-1} for C=O/C–N. Published spectra for NH_3 and DMF were used to locate potential residual NH_4OH/DMF in the fibers (Holt, Sadoskas, & Pursell, 2004). Theory assumes complete evaporation of the solvent during electrospinning. But previous work has shown the existence of residual solvent (trifluoroacetic acid) in electrospun chitosan nanofibers (Schiffman & Schauer, 2007) due to the ionic interaction of the trifluroacetate ion and the protonated amine. Characteristic peaks for NH_3 located near 1070 cm^{-1} for stretching, 1650 cm^{-1} for bending and 3200 cm^{-1} for wagging were apparent. For DMF within the HA mats, characteristic peaks at 1675 cm^{-1} for C=O and 3000–3300 cm^{-1} for CH_3 are not distinctly evident. These results suggest that the solvent to biopolymer ratio is too low for FTIR detection. Further investigations using XPS indicate that the fibers do not have residual solvent from the electrospinning process. Electrospun HA was compared to HA thin films using the O/N ratio, O1s and N1s at 37.5 and 3.3 at%, respectively (Suh et al., 2005). The ratios indicate that no solvent (DMF and NH_4OH) is present within the collected electrospun HA fibrous mats.

4. Conclusion

Pure HA mats with an average fiber diameter of 39 ± 12 nm have been successfully electrospun using a new solvent system that features aqueous NH_4OH. We suspect that this solution disrupts the rigid structure of HA and permits the critical chain entanglement to be reached. Thus, the electrospinning of continuous, cylindrical, and randomly oriented HA fibrous mats devoid of remnant solvent was demonstrated. Working with solvents at either end of the pH spectra can pose compatibility issues for the bulk biopolymer and biocompatibility of the mats. Thus, our fabricating nanofibrous HA mats with an unaltered electrospinning system and a less basic solvent system is encouraging for their potential use in biomedical applications.

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