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Phosphate salts facilitate the electrospinning of hyaluronic acid fiber mats

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Abstract Electrospinning is a cost effective and facile method to manufacture fiber mats appropriate for biomedical applications. Due to its high molecular weight and charged backbone, hyaluronic acid (HA) fiber mats with consistent fiber morphology have been difficult to electrospin from neutral pH solutions. Here, we present that the electrospinning of HA fibers in aqueous dimethylformamide solutions is facilitated by the addition of three phosphate salts. The salts—glycerol phosphate (GP), sodium phosphate (SP), and tripolyphosphate (TPP)—facilitated electrospinning of the solutions as characterized by conductivity measurements and fiber morphology. From tensile experiments, HA mats electrospun with SP demonstrated improved Young's modulus (12 MPa) over HA mats spun with either GP or TPP (5 and 3 MPa, respectively). This work demonstrates that a new neutral solvent system can be employed to spin HA fibers, which offers the potential for using the fibers for biomedical applications, such as a bone biomimetic.

Introduction

Hyaluronic acid (HA) is a major glycosaminoglycan found in the extracellular matrix (ECM) of mammalian connective tissue. It is a linear natural polysaccharide composed of the repeating disaccharide β -1-4-D-glucuronic acid and

β -1-3-N-acetyl-D-glucosamine units. Since HA is a primary ECM component, it has been investigated as scaffolding material for soft tissue engineering applications [1–4]. HA is biocompatible and a key component in bone and cartilage tissue engineering applications, and thus, a wide range of additional biomedical applications have also been explored [5, 6].

Currently, electrospinning is the most effective method for producing nonwoven fibrous mats. The process is simple, inexpensive, and the mats produced typically have enhanced properties over fibers produced using traditional methods, including increased surface area-to-volume ratios and smaller pore sizes [7–9]. During electrospinning, applying a voltage to a polymer solution results in charge accumulation and the formation of unstable droplets. These droplets become fiber jets that procure nanofibers, which are then deposited onto a conductive material [10]. While thorough investigations conducted on well-characterized uncharged polymers [11–13] have resulted in sound guidelines concerning their spinnability, application to natural polyelectrolyte solutions, such as HA, is not straightforward [14]. However, the production of pure HA fibrous mats via electrospinning is desirable and has been sought after for use in biomedical applications.

Additionally, for electrospinning to ensue, a polymer solution must reach or exceed a critical chain entanglement. The anionic nature of HA contributes to electrostatic repulsion among chains, thus restricting chain entanglements. Therefore, viscous HA solutions arise at polymer concentrations less than 5 wt%. To overcome this challenge, Um et al. [14] have investigated a modified “electroblowing” system to produce HA nanofibrous mats. Additionally, HA has been electrospun from organic solvent systems consisting of dimethylformamide DMF:H₂O [15, 16], sodium hydroxide NaOH:DMF [17], ammonium

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hydroxide $\text{NH}_4\text{OH}:\text{DMF}$ [18], and formic acid $\text{FA}:\text{DMF}:\text{H}_2\text{O}$ [19].

Maleki et al. [20] have shown that when HA is dissolved in solutions at the extreme ends of the pH spectrum (i.e., $\text{NaOH}:\text{DMF}$ and $\text{FA}:\text{DMF}$) the polymer degrades and solution viscosity decreases within 30 min. Therefore, to reduce these degradation effects, electrospinning of HA from neutral solutions is a required step toward unveiling their full potential in future biomedical applications. In an effort to produce robust fibrous mats from neutral pH solutions while preserving the possible benefits of additional phosphate ions for creating a bone biomimetic, this work integrated several phosphate salts into the HA fiber spinning solution. The ability of GP, SP, and TPP to assist in the production of fibrous mats was investigated.

We hypothesize that the addition of phosphate salts to the HA solutions will affect the electrospinning solution in several ways. Depending on solution pH, the salts will dissociate in the aqueous environment causing a dramatic increase in the number of charge carriers in solution and affect chain entanglement. These charge carriers may aid in pendant drop formation, which is a prerequisite to the creation of electrified jets during electrospinning [21]. Addition of salts may alter the jet formation by affecting the surface tension and distribution of charge groups within the solution [22]. Additionally, researchers have shown that the addition of sodium chloride to HA solutions reduces the solution viscosity by up to 70 % [16]. In this study, electrospinning HA solutions with varying ratios of the three phosphate salts, SP, GP, and TPP, were investigated to correlate solution properties with fiber mat production.

Experimental

Materials

All compounds were used as received. Hyaluronic acid (HA, cosmetic grade, $M_w = 2000000$ Da) was purchased from Dali Chemical Co. (China). Hydrochloric acid (HCl), sodium hydroxide (NaOH), glycerol phosphate (GP), sodium phosphate (SP), tripolyphosphate (TPP), sodium chloride, sodium sulfate, and dimethylformamide (DMF, 99.8 % ACS) were purchased from Sigma-Aldrich (St. Louis, MO).

Hyaluronic acid (HA)–phosphate mat fabrication

HA solutions of 1.5 % (w/v) in DMF were added to aqueous phosphate solutions so that the final volume ratio of $\text{H}_2\text{O}:\text{DMF}$ was 1:1. Different HA–phosphate ratios (by weight) were investigated to observe the effect on fiber morphology. These included 1:1 and 4:1 for the GP and

TPP solutions, and 1:1 and 1:2 for the SP solutions. The mixture was then placed on an Arma-Rotator A-1 (Bethesda, MD) mixer until a completely dissolved, transparent solution was obtained (24 h). The pH of each HA–phosphate solution was measured using pH indicator strips (Macherey-Nagel, Bethlehem, PA). The pH of the mixture containing HA–GP and HA–SP was 8.5; similarly, the pH was 7.5 for HA–TPP.

The electrospinning apparatus utilized was previously described [23]. Briefly, a 5-mL Luer-lock syringe was loaded with the polymer solution (Becton–Dickinson, Franklin Lakes, NJ) and a Precision Glide 21-gage needle (Becton–Dickinson, Franklin Lakes, NJ) was attached. The positive electrode of a high voltage supply (Gamma High Voltage Research, Ormond Beach, FL) was directly connected to the needle by an alligator clip. The syringe was then placed on an advancement pump (Harvard Apparatus, Plymouth Meeting, PA) located a fixed distance from the negative electrode. Voltage was then applied, thus creating a positive and negative anode as the solution was advanced at a constant rate. To electrospin HA–GP and HA–TPP, an advancement speed of 0.01 $\mu\text{L}/\text{min}$, a separation distance of 6.0 cm, and an applied voltage of 15 kV were used. HA–SP was electrospun using an advancement speed of 0.008 $\mu\text{L}/\text{min}$, a separation distance of 6.7 cm, and an applied voltage of 15 kV. All mats were electrospun onto a copper plate wrapped with aluminum foil. The temperature and relative humidity were monitored by a digital thermohygrometer (Fisher Scientific, Pittsburgh, PA).

Solution characterization

Solution conductivity

The aqueous DMF solutions were too viscous for accurate conductivity measurements, and thus exact electrospun ratios could not be used. Instead, consistent 0.5 % (w/v) aqueous HA solutions were prepared neat with the SP, GP, and TPP salts, as well as sodium chloride and sodium sulfate for comparison, using ultrapure water from a Milipore QPAK system. Solution conductivity measurements were taken using a CON 510 conductivity meter (Oakton, Vernon Hills, IL), which was calibrated using a 1413 μS standard solution (potassium chloride, Fluka) prior to each use. Measurements were taken in triplicate.

Fiber characterization

Scanning electron microscopy

A Denton vacuum desk II sputtering machine was utilized to coat the samples for 10 s with platinum–palladium. Micrographs of electrospun fiber mats were acquired using

a Zeiss Supra 50 VP field emission scanning electron microscope (FE-SEM). Average fiber diameter was determined by first calibrating the picture scale and then measuring the diameter of 50 fibers from at least three different micrographs using ImageJ 1.410 software (National Institutes of Health, Bethesda, MD).

Chemical analysis

Thick fiber mats were electrospun and peeled from the aluminum foil. The chemical composition of the various fiber mats was analyzed with Smith's IlluminatIR attenuated total reflectance Fourier transform infrared spectroscopy (ATR-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} .

Tensile testing

A uniaxial tensile test was conducted on the electrospun HA-phosphate mats using a Kawabata KES-G1 microtensile tester. Methods described by Donius et al. [24] were utilized. HA-phosphate solutions were electrospun for 8 h to produce a robust mat. Three samples of each HA-phosphate combination were tested. Each phosphate mat was desiccated for 24 h in order to ensure that all residual solvent evaporated, before being cut into three rectangular strips (5.5 cm $\times$ 1 cm). Mat thickness was determined with an optical microscope and each sample was also weighed. The ends of each HA-phosphate sample were adhered with glue (Avery Permanent Glue Stick, Brea, CA) to a cardboard frame and tested at a rate of 0.1 cm/s.

The Kawabata raw load data including time, displacement of fiber mats, and corresponding voltage were recorded in Labview (National Instruments) and exported into Microsoft Excel. Elastic modulus (E) and Ultimate tensile strength (UTS) were determined by first transforming the raw load data obtained from the Kawabata machine to corresponding stress measurements. Stress ($\sigma = F/A$) was calculated using the voltage (load) output (F) from Labview divided by the cross sectional area (A) of each fiber mat tested. Cross sectional area was calculated using the dimensions of the fiber mat, including the measured thickness and the weight [24]. The thickness of the mats varied based on phosphate HA-GP ($249 \pm 167 \mu\text{m}$); HA-TPP ($68 \pm 21 \mu\text{m}$); and HA-SP ($18 \pm 2 \mu\text{m}$). The weight of the mats varied based on phosphate HA-GP (7.7 mg); HA-TPP (9.7 mg); and HA-SP (7.9 mg). The initial strain rate (ϵ_0) was 10 mm/s with each fiber mat measuring 5.5 cm in length. The linear region of the slope of the stress-strain curve provided the elastic modulus

($E = \sigma/\epsilon$) and the maximum stress was determined to be the UTS.

Statistical analysis

All HA-phosphate mats were fabricated in triplicate, with each fiber diameter or mechanical property value reported as an average with $n = 3$. A Student's unpaired t test was utilized in order to determine level of statistical significance, with $p < 0.05$.

Results and discussion

HA solution conductivity and fiber morphology

The three phosphate structures used in the fabrication of HA-phosphate mats are shown in Fig. 1. HA fiber mats containing GP, SP, and TPP were successfully electrospun as seen in Fig. 2. HA-SP fibers spun using a 1:1 and 1:2 ratio both exhibited continuous fiber morphology. However, increased homogeneity in fiber morphology was achieved using the higher (1:2 HA-SP) salt concentration (Fig. 2a, b). This was in contrast to the morphology observed when spinning high GP or TPP salt concentrations. An equal ratio (1:1) of HA-GP or HA-TPP produced flattened, ribbon-like fibers with uneven morphology, and a film-like distribution (Fig. 2c, e). The flattening of the fibers can be due to a salt effect on the evaporation of the H_2O :DMF solvent from the fibers as they are spun. Continuous, randomly oriented fibers are present in fibers spun from the lower, 4:1, ratio of GP and TPP to HA (Fig. 2d, f). Fiber diameter distributions with median values for the HA-phosphate solutions that spun into consistent fibrous mats are provided in Fig. 3. The average fiber diameter of the HA-GP and HA-TPP fibers was 110 ± 34 and 143 ± 34 nm, respectively, significantly thicker than the average HA-SP fiber diameter of 88 ± 17 nm. Mitupatham et al. [25] reported that polyamide fibers increased in diameter when solution conductivity increased. Conductivity measurements on all phosphate containing solutions revealed that GP and TPP containing solutions were higher compared to that of SP [25]. This may explain the larger fiber diameter obtained for the more conductive GP and TPP solutions (Fig. 4).

Conductivities are shown in Fig. 4a for HA-phosphate solutions containing GP, SP, and TPP. It was expected that higher solution conductivity would be observed in all solutions compared to an aqueous HA solution. While this is the case for GP containing solutions, the remaining solutions did not show marked increases. The HA-GP salt solution has a higher conductivity compared to the pure

Fig. 1 Structures of the three phosphate salts used in this study **a** sodium phosphate (SP), **b** glycerol phosphate (GP), and **c** tripolyphosphate (TPP)

Fig. 2 SEM micrographs with 500-nm marker displaying fiber mats electrospun at **a** 1:1 and **b** 1:2 HA-SP ratio; **c** 1:1 and **d** 4:1 HA-GP ratio; and **e** 1:1 and **f** 4:1 HA-TPP ratio

HA solution (control, no salt). Both the TPP and SP solutions demonstrate a similar trend, but do not display higher conductivity when compared to aqueous HA. The HA-SP solutions yielded the most consistent fibers, even though the conductivity was lower.

HA solutions were also prepared using additional sodium salts of different anion sizes and ionic strengths to compare to the phosphate results, Fig. 4b. The radius of the chloride ion is 1.81 Å, while the apparent ionic radii for sulfate and phosphate ions are 2.3 and 2.38 Å, respectively

Fig. 3 Fiber diameter distribution for HA mats electrospun with phosphate salts: 1:1 SP, 1:2 SP, 4:1 GP, and 4:1 TPP. Data determined using ImageJ software on SEM micrographs

[26]. In Fig. 4b, HA concentration was held constant at 0.5 w/v % while the salt concentration was varied. Increasing concentrations of the larger salts, sodium sulfate and sodium phosphate show a minimal effect on solution conductivity. In contrast, solutions containing higher concentrations of sodium chloride, the smallest ion, display a solution conductivity that is an order of magnitude greater. The results not only suggest a potential ionic strength effect on conductivity, but also an anion size dependence. These results suggest that a smaller ionic radius and a lower ionic strength may allow for the observed increases in conductivity with sodium chloride. Under normal aqueous conditions in the semi-dilute region, there is a strong hydrogen bonding effect between water molecules and the HA molecule. This effectively creates a water cloud surrounding the sodium HA chains, including the carboxyl groups, which are saturated by sodium ions. The sodium chloride seems to be having a larger effect on the conductivity of the polyelectrolyte similar to the effects

Fig. 4 Conductivity of HA solutions containing **a** no salt (control) and various concentrations (w/v %) of GP, SP, and TPP. For comparison, HA solution containing **b** sodium sulfate, sodium chloride, and sodium phosphate are displayed

observed with a synthetic polyelectrolyte, polyacrylic acid [27].

ATR-FTIR of HA-phosphate fiber mats

ATR-FTIR spectra acquired for HA fiber mats produced with TPP, GP, and SP salts all display the characteristic carbohydrate peaks for HA (3300 and $1200\text{--}930\text{ cm}^{-1}$), Fig. 5. To determine if the phosphates were bound to the HA, the characteristic region for phosphorous-based peaks [26], between 900 and 400 cm^{-1} , were compared in spectra acquired on the HA-phosphate mats to that of the bulk phosphate salts. No evidence was present that the phosphates were bound in the final HA fibers.

HA crosslinking via phosphates could also potentially occur based upon our previous observation of ionic crosslinking and gelation from phosphates added to polysaccharide chitosan solutions [28]. The bonding mechanisms associated with potential crosslinking would be evident by the formation of a P–O–alkyl bond, designated

Fig. 5 ATR-FTIR spectra acquired on electrospun HA-TPP, HA-GP, and HA-SP fiber mats

Fig. 6 The **a** Young's moduli, E , and **b** ultimate tensile strength, UTS of HA-phosphate mats are plotted versus their average fiber diameter

by a broad peak at $1050\text{--}970\text{ cm}^{-1}$. However, this peak was not evident in any of the spectra, coupled with the dissolution of the mats in aqueous solutions indicated that no crosslinking occurred.

Mechanical behavior of HA-phosphate fiber mats

HA mats containing SP, GP, and TPP were evaluated using uniaxial tensile testing in order to determine their Young's modulus (E) and ultimate tensile strength (UTS). Figure 6a displays that the HA-SP fibers had the highest E compared to both the GP and the TPP fibers. The HA-SP fiber mats had an average modulus of 12 MPa, whereas the HA-GP and HA-TPP fiber mats had moduli of 5 and 3 MPa, respectively (Fig. 6b). The UTS value for the HA-SP fiber mats was an average of 1.5 MPa, while HA-GP and HA-TPP displayed values of 0.5 and 0.4 MPa, respectively. While it may appear that the smallest average fiber diameter yielded higher moduli and tensile strength, Fig. 6a, b these values were not determined to be statistically significant.

The fiber diameter [29, 30], number of fiber-fiber contacts [31, 32], contact area between fibers [31, 33], and fiber-to-fiber bond strength [19, 20] have been noted as the most influential parameters on the mechanical properties of randomly oriented nanofibrous mats. In previous work, we have determined that Young's modulus of fiber mats can be greatly affected by the quantity of fiber junctions formed and their interconnectivity [24]. The HA-phosphate mats displayed varying morphologies depending upon the type and quantity of phosphate incorporated into the spinning solution. Their fiber morphology ranged from cylindrical uniform structures in the case of HA-SP and HA-TPP, to flattened, ribbon-like formations as demonstrated by HA-GP. The flattened, film-like morphology of the HA-GP fibers may have led to a decrease in fiber junctions and mat interconnectivity, thus decreasing the Young's modulus and UTS. Because HA-SP fibers were cylindrical and yielded a thick mat, there may have been an increase in fiber junctions, contributing to their higher Young's modulus and UTS. An additional predictor of mechanical properties is the porosity of the electrospun fibrous mat.

Although porosity measurements of the HA-phosphate mats were not performed, Thomas et al. [34] reported that increased porosity of polymer mats has a detrimental effect on the mechanical properties, leading to weaker fiber mats. Thus, measuring the porosity of the phosphate mats will be a goal for future work [34].

Conclusion

Fiber mats electrospun from natural polymers and neutral solutions could potentially serve as a platform for numerous biomedical applications. For the first time, we have demonstrated that the spinning of HA fibers from aqueous dimethylformamide (DMF) solutions is facilitated by the addition of GP, SP, and TPP salts. HA-SP and HA-TPP displayed more consistent fiber morphology than those spun with HA-GP. Tensile experiments determined that HA-SP demonstrated the highest mechanical properties over HA mats spun with either GP or TPP. This work demonstrates that phosphate salts can be employed to spin robust HA fibers for use in future applications.

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