

Feasibility of Electrospinning the Globular Proteins Hemoglobin and Myoglobin

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Disclosure: Several authors have United States and International patents pending concerning technology presented in this manuscript, and this technology has been licensed to NanoMatrix, Inc., of which several authors have a financial interest.

ABSTRACT

Various concentrations of the globular protein hemoglobin were successfully electrospun to create micro-fibrous mats of varying physical and mechanical characteristics. The electrospinning parameters are reported. One concentration of myoglobin was electrospun into a mat for comparison to the hemoglobin mats. Scanning electron microscopy revealed ribbon-like morphologies for the hemoglobin and myoglobin structures. Mean fiber width and thickness for each mat electrospun from a different hemoglobin concentration increased from 2.68 ± 0.83 to 3.55 ± 1.49 μm and from 0.49 ± 0.08 to 0.99 ± 0.41 μm , respectively, for increasing hemoglobin solution concentrations (from 150 to 225 mg/mL). For calculations of surface area to volume ratio for the four different electrospun hemoglobin concentrations, there was a negative correlation ($r = -0.84$) with concentration; the surface area to volume ratio ranged between 0.50 ± 0.16 and 1.53 ± 0.24 m^2/cm^3 . Also, there appears to be a positive correlation between electrospun hemoglobin concentration and porosity, which increased with increasing concentration from 69.5 to 83.3 %. Following cross-linking with glutaraldehyde, the mechanical

properties of two constructs were evaluated via uniaxial tensile testing to demonstrate handling capability. Results indicated that increased cross-linking time produced stiffer structures, as peak stress and modulus increased while strain at break decreased when the mats were cross-linked for 30 minutes with glutaraldehyde versus the 20 minute cross-linking time.

INTRODUCTION

Hemoglobin and myoglobin are proteins responsible for binding and releasing oxygen in the blood and muscles, respectively. The hemoglobin protein consists of four polypeptide chains, and the myoglobin protein is a single polypeptide. Each polypeptide chain (of both macromolecules) contains one heme group that holds an iron ion; thus, hemoglobin contains four iron ions and myoglobin contains only one iron ion. Each iron ion binds reversibly with one oxygen molecule. Maintenance of this heme iron in the reduced state (Fe^{2+}) is imperative for oxygenation to occur; oxidation of the iron into Fe^{3+} can result in the formation of methemoglobin or metmyoglobin and, subsequently, inhibit the oxygen-binding capabilities of the proteins [1]. Hemoglobin exhibits two major conformations: the R state in which oxygen has a higher affinity for binding to hemoglobin (oxyhemoglobin), and the T state which is more stable in the absence of oxygen (deoxyhemoglobin). When oxygen binds to hemoglobin in the R state, this state is stabilized. When oxygen binds to hemoglobin in the T state, a change in conformation to the R state occurs, and the $\alpha\beta$ dimers of hemoglobin slide past each other and rotate. Having both more than one ligand-binding site and a difference in affinities between oxygen and hemoglobin in the different conformational states results in cooperative binding [2, 3]. Current research into blood substitutes, and specifically red blood cell substitutes, focuses on increasing the oxygen carrying capacity of therapeutics used in the delivery of oxygen to tissues; hemoglobin has been extensively investigated for this use [4-6].

The electrospinning processing technique has been described in detail elsewhere [7]. Briefly, a liquid jet is created by imparting charge to a polymer solution (or melt), which is then drawn through a small nozzle or tube (such as a blunt-end needle). As electrostatic forces within the solution overcome the surface tension of the solution, a liquid jet is initiated at the nozzle tip. The charged solution is attracted to a grounded target, such as a rotating mandrel, placed some distance away from the nozzle. As the solution travels toward the target the solvent evaporates, leaving a continuous polymer fiber that accumulates on the mandrel. The product of this process is a nonwoven fibrous mat. Fibers with micron or nanometer sized diameters can be produced depending on the selected materials and solution concentrations. Fibers of these dimensions have an extremely high surface area to volume ratio [8], a desirable feature in optimizing cellular interaction with tissue engineering scaffolds and drug delivery devices, to name a few applications. In an effort to more closely mimic the structural and functional profile of the extracellular matrix, natural polymers, including collagen, elastin and fibrinogen, have been successfully electrospun by our laboratory [9-12].

Here we describe the successful electrospinning of the two globular proteins hemoglobin and myoglobin. Our efforts are focused on combining the flexibility of electrospinning with the physiologic activity of hemoglobin and myoglobin to prepare a biological construct with the potential to be used as an oxygen delivering scaffold for optimal wound healing in future work.

In this study, we have assessed the feasibility of electrospinning these proteins and evaluated their electrospinning parameters, three-dimensional structure, and preliminary tensile characteristics.

EXPERIMENTAL

Hemoglobin from bovine blood (64.5 kDa) and myoglobin from equine skeletal muscle (17.6 kDa), both purchased from Sigma-Aldrich Co., were solubilized in 2,2,2-Trifluoroethanol (TFE, Fisher Scientific) at various concentrations. The authors have found that several natural polymers electrospin equally well from TFE as compared to the more commonly used 1,1,1,3,3,3 hexafluoro-2-propanol (HFP) (unpublished data). The hemoglobin solutions were prepared at concentrations ranging between (and including) 100 and 250 mg/ml, while the myoglobin solution was prepared at a single concentration of 250 mg/mL for comparison to the hemoglobin. These solutions were placed in a Becton Dickinson 3 ml syringe affixed with an 18-gauge blunt-end needle. A KD Scientific syringe pump (Model 100) was used to dispense all solutions at a rate determined during testing. The positive output lead of a high-voltage power supply (Spellman CZE1000R, Spellman High Voltage Electronics Corp.), set to 22 kV, was attached to the needle. A rotating, grounded mandrel was placed 12.7 cm from the needle tip, and the rotational speed was maintained at 500 RPM to ensure random fiber organization. All solutions were electrospun onto a 303 stainless steel rectangular mandrel (2.5 cm x 7.6 cm x 0.4 cm). *Figure 1* illustrates the electrospinning process used in our laboratory.

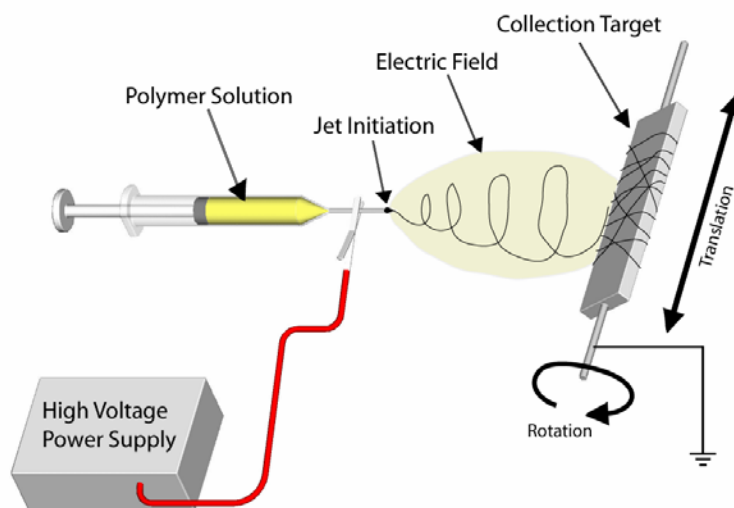


Figure 1. Schematic of the electrospinning process.

Fiber characteristics of the resulting mats, including fiber width and thickness, porosity, surface area to volume ratio and overall morphology, were analyzed with scanning electron microscopy (SEM) using a JEOL JSM-820 JE microscope (Japanese Electron Optical Ltd., Japan). The

micrographs were digitized (via scanning using an HP Scanjet 5550c) and examined with imaging software (ImageJ 1.36b, NIH freeware). Means and standard deviations of fiber dimensions were determined from 15 measurements per micrograph and calibration was made with the scale bar on each micrograph. Fiber width and thickness were measured only on fibers that twisted within the micrograph to ensure accurate measurements of the respective dimensions. The following equations were used to calculate porosity and surface area to volume ratio. Values for the known material specific volume (constant at 0.74 cm³/g [13]), the measured mass and volume of the mats, and the fiber width and thickness measurements are used in these calculations; cross-sectional area was determined as the product of width and thickness.

$$\text{Porosity} = [1 - (\text{calculated density of mat} * \text{known material specific volume})] * 100\%$$

Surface Area to Volume Ratio

$$= \frac{\frac{\text{measured mass of mat} * \text{known material specific volume}}{\text{cross - sectional area}} * \text{cross - sectional perimeter}}{\text{calculated volume of mat}}$$

Electrospun samples of the 175 mg/mL concentration of hemoglobin and the 250 mg/mL concentration of myoglobin were cross-linked using a vapor fixation technique with 50% w/w glutaraldehyde solution. The mats were laid flat on a screen fixed above the bottom of a glass dish; 300 μL of 50% w/w glutaraldehyde solution was placed in the bottom of the dish below the mats; the dish was covered with a glass lid and placed on a heating plate set to 100°C. The hemoglobin mats were exposed to the heated vapors for two different time periods: 20 minutes and 30 minutes. The 20-minute time period allowed only for hot vapor fixation, while the 30-minute time period allowed the hot vapors to condense within the fixation vessel, thereby providing a combination of hot vapor and hot liquid fixation. Since glutaraldehyde is cytotoxic [14], the fluid must be removed from the structure following the cross-linking procedure. It is desirable to avoid liquid contact with the mats, as removal of the gas phase from the electrospun mats is much simpler (by degassing) than removal of the liquid phase. The electrospun myoglobin mat afforded less useable area available for subsequent tensile testing; thus, the myoglobin mat was cross-linked at only one time period (20 minutes) due to the limited material.

Uniaxial tensile testing of the dry, cross-linked mats was performed on a MTS Bionix 200 mechanical testing system (MTS Systems Corp., Eden Prairie, MN), incorporating a 50N load cell with an extension rate of 10.0 mm/minute to failure. Dog-bone shaped samples (2.67 mm wide at their narrowest width and a gage length of 7.49 mm) were punched from the mats for the mechanical testing. Six samples were taken from the 20 minute cross-linked mat and three samples were taken from the 30 minute cross-linked mat.

Statistical analyses of the measurements was based on a Kruskal-Wallis one way analysis of variance on ranks and a Tukey-Kramer pairwise multiple comparison procedure. An *a priori* significance level of $\alpha = 0.05$ was used and the calculations were performed with the JMP[®]IN 4.0.3 statistical software package (SAS Institute Inc.). Pairwise correlations were also analyzed using the JMP[®]IN 4.0.3 statistical software package, and the Pearson's correlation coefficient (r) is reported.

RESULTS

Electrospun hemoglobin and myoglobin solutions resulted in fibrous, nonwoven meshes (*Figure 2*) that maintained mechanical integrity for gentle handling. As shown in *Figure 2*, the electrospun myoglobin structure contained many more defects, resulting in a much more delicate structure than the hemoglobin mat. *Table 1* gives the electrospinning parameters necessary to produce fibers from hemoglobin. Myoglobin was electrospun using the same parameters as hemoglobin with the exception of the range of concentrations; because myoglobin has a lower molecular weight, at 17.6 kDa compared to 64.5 kDa for hemoglobin, a greater minimum concentration must be used when electrospinning myoglobin. There must be sufficient polymer interactions, i.e. the viscosity of the solution must be great enough, for fiber creation via electrospinning to occur [15]. Viscosity is dependent on concentration and size, or molecular weight. Thus, the concentrations of myoglobin that are capable of being electrospun will be greater than those of hemoglobin.

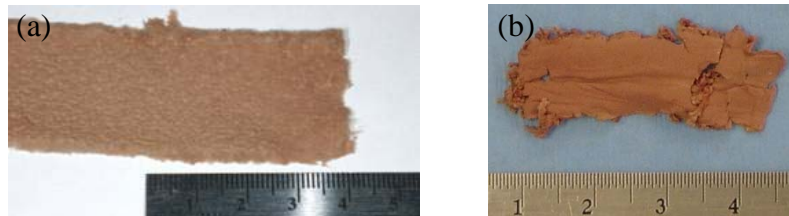


Figure 2. Photographs of electrospun (a) hemoglobin and (b) myoglobin mats after removal from the collection target.

Table 1. Electrospinning parameters for hemoglobin.

Electrospinning Parameter	Value
Range of concentrations	150 – 225 mg/mL
Rate, syringe pump	1.8 mL/hr
Voltage	22 kV
Distance between needle and mandrel	12.7 cm
Solvent	2,2,2-Trifluoroethanol (TFE)

The electrospun mats were composed of fibers with a ribbon-like morphology, as can be seen in *Figures 3 and 4*. The scanning electron micrographs shown in *Figures 3 and 4* also depict the random (unaligned) orientation of fibers within the electrospun structures. The results of the fiber width and thickness measurements are given in *Table 2* and illustrated in *Figure 5*. Note that there are positive correlations between electrospun hemoglobin concentration and both fiber width and fiber thickness, though this relationship is weaker for concentration and width ($r = 0.26$) than for concentration and thickness ($r = 0.57$).

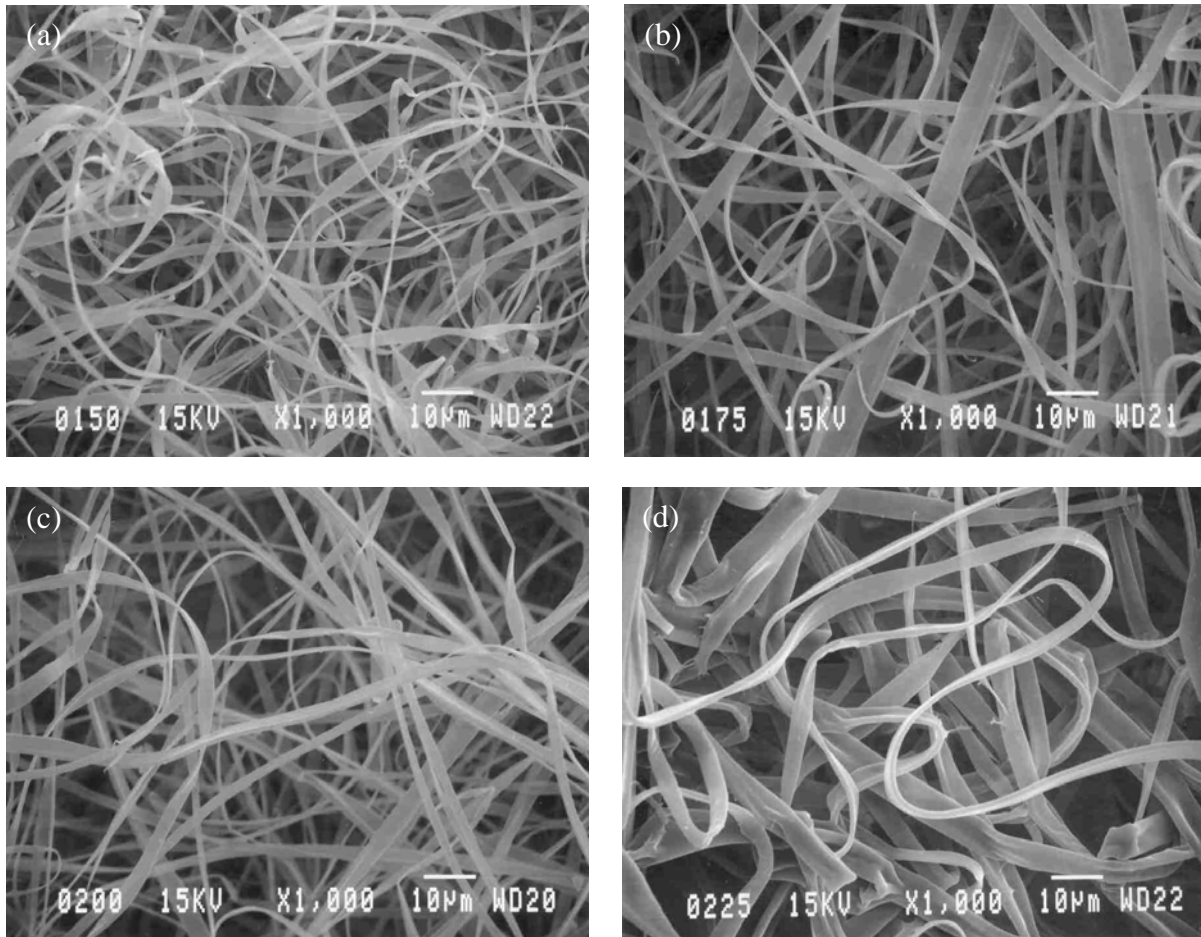


Figure 3. Scanning electron micrographs of electrospun hemoglobin in TFE at the following concentrations: (a) 150 mg/mL, (b) 175 mg/mL, (c) 200 mg/mL, and (d) 225 mg/mL (1,000x magnification, scale bar is 10 μ m).

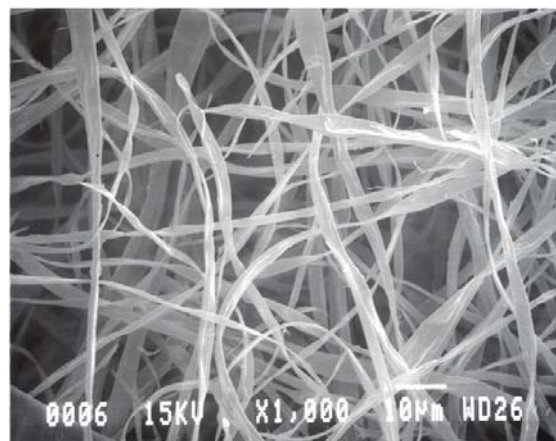


Figure 4. Scanning electron micrograph of electrospun myoglobin in TFE at 250 mg/ml (1,000x magnification, scale bar is 10 μ m).

Table 2. Electrospun hemoglobin (Hb) fiber and mat characteristics.

Hb Concentration (mg/ml)	Fiber width (μm)	Fiber thickness (μm)	Porosity (%)	Surface area/volume (m^2/cm^3)
150	2.68 ± 0.83	0.49 ± 0.08	69.5	1.53 ± 0.24
175	2.71 ± 1.08	0.66 ± 0.26	78.4	0.91 ± 0.28
200	2.82 ± 0.82	0.72 ± 0.17	80.4	0.72 ± 0.15
225	3.55 ± 1.49	0.99 ± 0.41	83.3	0.50 ± 0.16

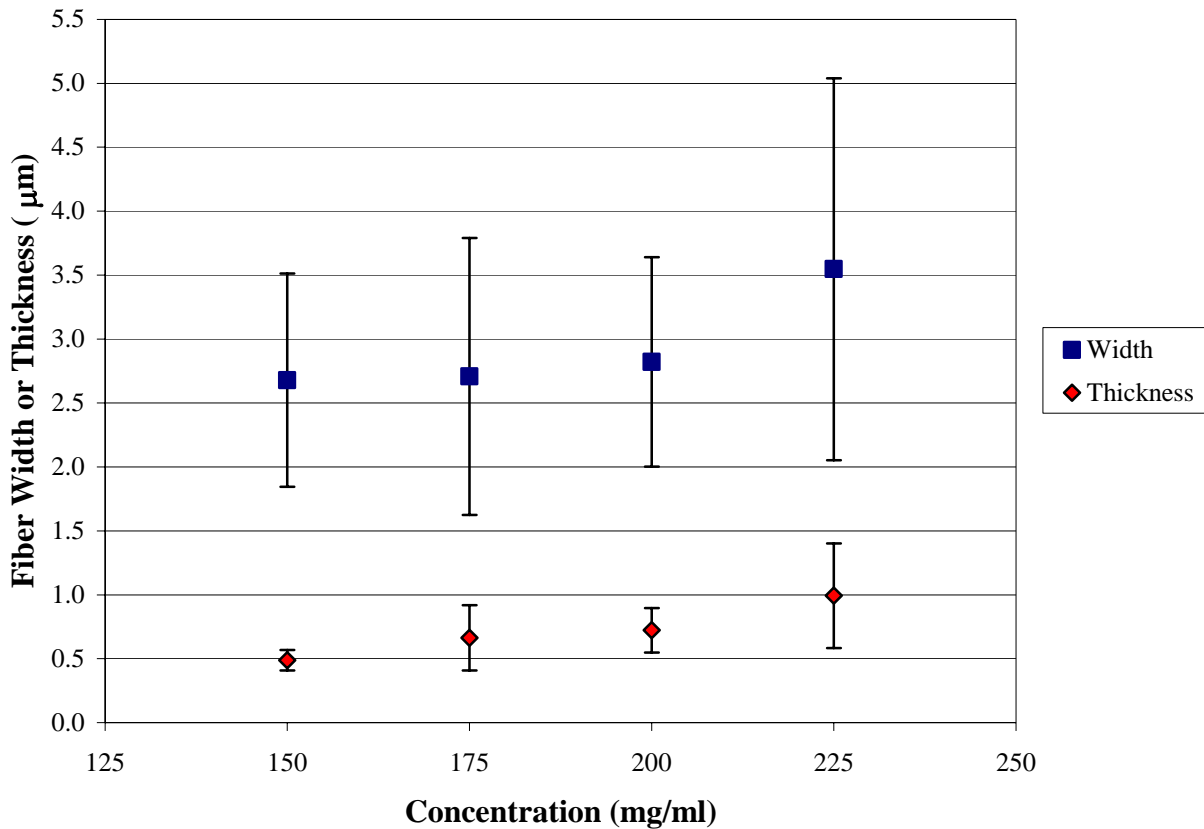


Figure 5. Fiber width and thickness measurement analysis to illustrate the relationship between concentration of electrospun hemoglobin in TFE and fiber dimension; the correlation between concentration and width is $r = 0.26$, and the correlation between concentration and thickness is $r = 0.57$.

Two other important characteristics that influence the interaction of cells with tissue engineering scaffolds are porosity and the ratio of surface area to volume. Structures that are highly porous will more readily permit cell migration and infiltration throughout the thickness of the scaffold.

Porosity results are given in *Table 2* and indicate the void space in the scaffolds as a percent of total scaffold volume. In *Figure 6*, there appears to be a positive correlation between electrospun hemoglobin concentration and porosity, which would indicate that as concentration increases, the percent void space of the scaffold also increases. This is most likely due to a decrease in the number of smaller fibers (as concentration increases), which tend to fill the gaps between the larger fibers. Surface area to volume ratio is another characteristic that affects the interaction of a structure with the host environment. For example, the biconcave disc shape of a red blood cell gives increased surface area over a normal, cylindrical disc shape, thereby allowing more surface area for oxygen exchange with tissues. The results of the surface area to volume analysis for the electrospun hemoglobin mats are given in *Table 2* and are graphically represented in *Figure 7*. There is a strong negative correlation ($r = -0.84$) between electrospun hemoglobin concentration and surface area to volume ratio, indicating that structures electrospun at lower concentrations, which have more fibers due to being dimensionally smaller, have increasingly more surface area available for cellular interactions.

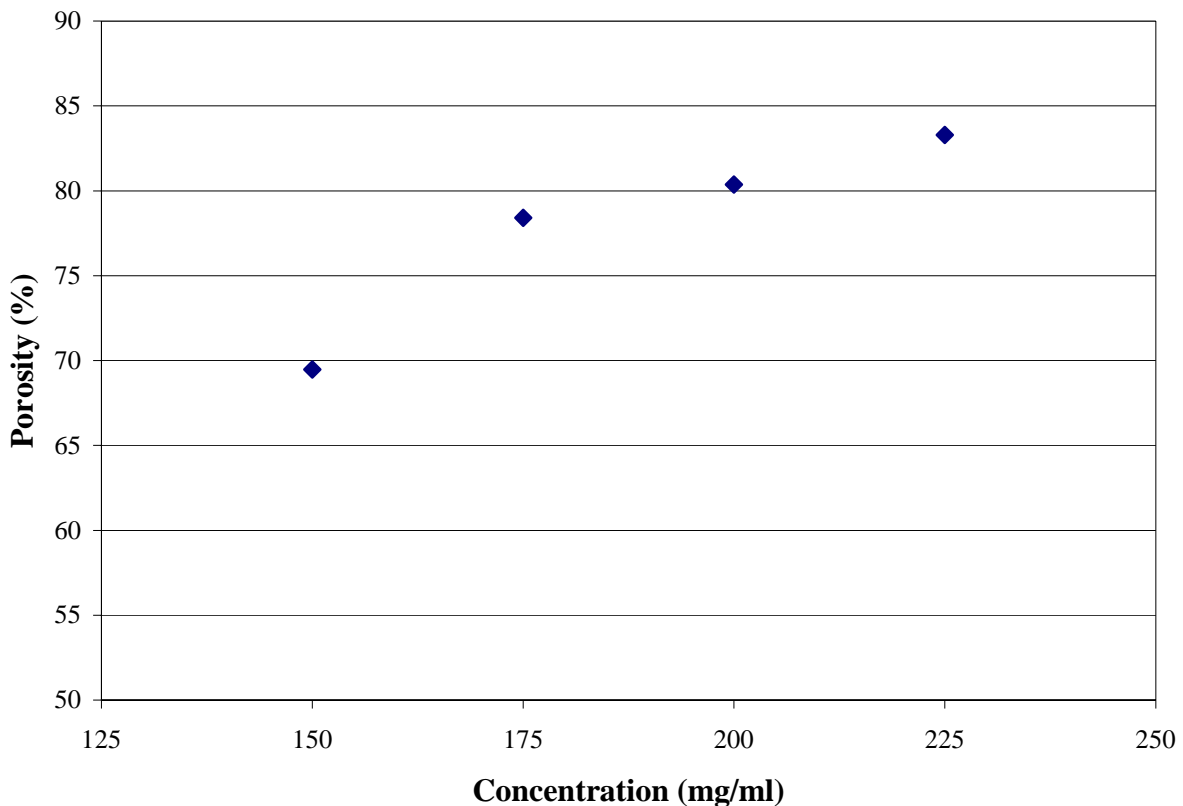


Figure 6. Porosity analysis of the electrospun hemoglobin mats to illustrate the relationship between concentration of electrospun hemoglobin in TFE and percent void space in the resulting structure (n=1).

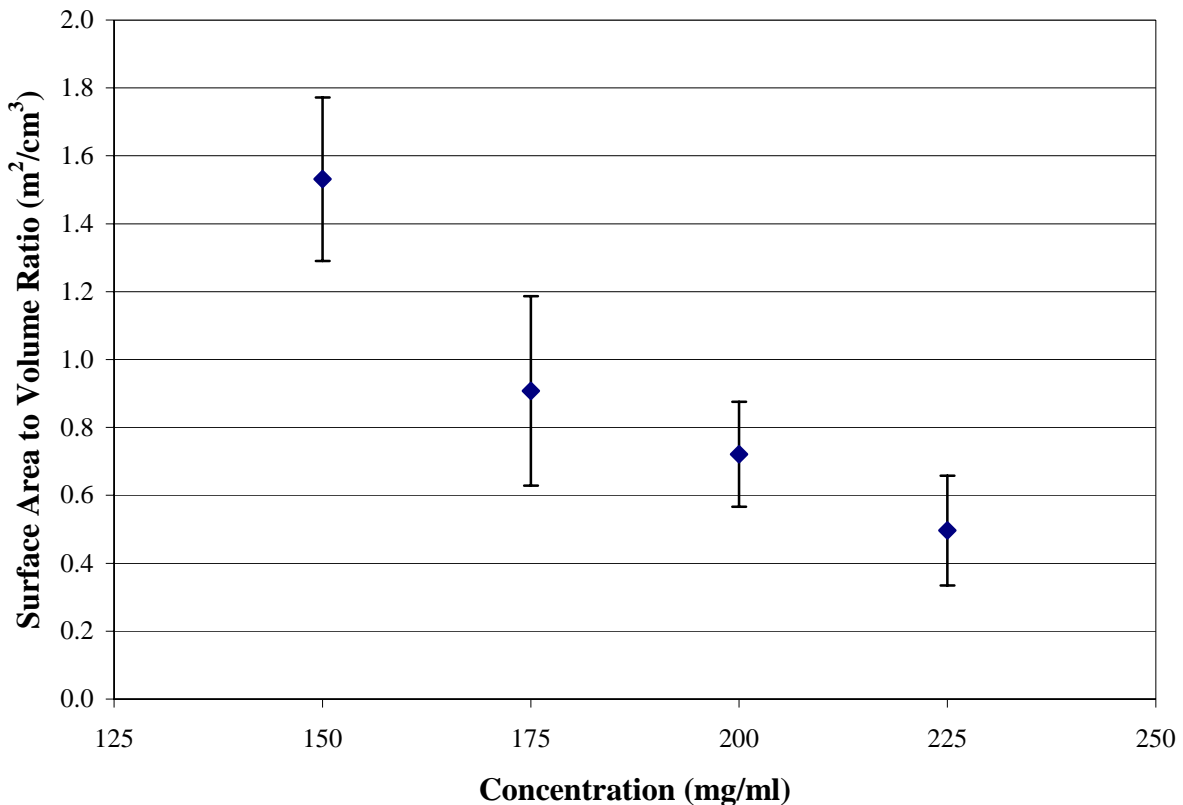


Figure 7. Surface area to volume ratio analysis to illustrate the relationship between concentration of electrospun hemoglobin in TFE and the surface area to volume ratio; $r = -0.84$.

Preliminary mechanical testing was performed on samples from the 175 mg/mL hemoglobin mat (20 and 30 minutes cross-linking times) and the 250 mg/mL myoglobin mat (cross-linked for 20 minutes), to get an initial idea of the structural characteristics. Though both the hemoglobin and myoglobin mats can be handled immediately following removal from the mandrel without compromising their integrity, the mats must be cross-linked before mechanical testing can be performed. *Table 3* gives the mechanical data recorded after testing the mats; peak stress, strain at break, and modulus are reported. As can be expected, increased cross-linking time did result in stiffer structures, as peak stress and modulus increased while strain at break decreased when the mats were cross-linked for 30 minutes with glutaraldehyde vapors versus the 20 minute cross-linking time. To further illustrate these results, *Figure 8* gives three representative stress-strain curves for the electrospun mats. As can be seen, the 30 minute cross-linking time period (of hot vapor and hot liquid fixation) resulted in effectively curing the hemoglobin mat such that the biopolymer has been hardened.

Table 3. Mechanical properties of cross-linked, electrospun hemoglobin (175 mg/mL concentration) and myoglobin (250 mg/mL concentration).

Property	Hemoglobin		Myoglobin
	20 min fixation	30 min fixation	20 min fixation
Peak Stress (MPa)	0.13 ± 0.05	2.2 ± 0.3	0.43 ± 0.05
Strain at Break (%)	13.5 ± 1.4	8.7 ± 2.5	17.2 ± 5.2
Modulus (MPa)	5.4 ± 3.0	60.1 ± 7.4	6.2 ± 1.9

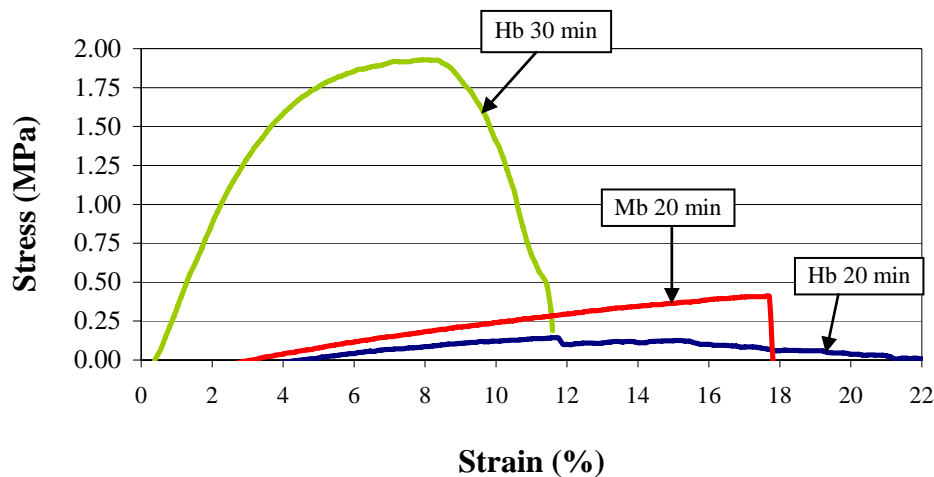


Figure 8. Representative stress-strain diagrams for the uniaxial tensile testing of the hemoglobin mats (cross-linked for 20 and 30 minutes, labeled “Hb 20 min” and “Hb 30 min”, respectively) and the myoglobin mat (labeled “Mb 20 min”).

DISCUSSION

In this feasibility study, our aim was two-fold: to demonstrate the feasibility of electrospinning globular proteins and to characterize the morphological and mechanical properties of the mats created in this manner. Comparing our mechanical data to data previously reported on the natural biopolymers collagen and fibrinogen [10, 16, 17], all fixed with glutaraldehyde, it is noteworthy that electrospun hemoglobin mats cross-linked for 20 minutes exhibit larger tangential modulus values than fibrinogen and are in the range of both collagen types I and II, but 30 minute cross-linked hemoglobin exceeds both collagen types as well as fibrinogen. Peak stress means for electrospun hemoglobin are comparable to those of electrospun collagen type I and fibrinogen. On the other hand, the hemoglobin mats have percent strain at break values that are smaller than those for electrospun fibrinogen and collagen, though the hemoglobin values are close to the lower end of the range reported for collagen type II [17]. Through this comparison, it is reasonable to conclude that the electrospun hemoglobin structures can maintain the

mechanical integrity necessary for use in wound dressings. Ideally, the use of these mats for such applications would be desirable due to the oxygen-carrying capacity of hemoglobin.

Oxygen is essential to proper wound healing of tissues, including skin, muscle and bone. Current techniques to optimize oxygen delivery to tissues include hyperbaric oxygen administered within a pressurized chamber (systemic) or via local devices placed directly over the wound (topical) [18]. While both of these techniques have demonstrated improved wound healing, cellular toxicities due to extended treatments of oxygen have been recorded [18]. The major challenge in oxygen delivery-induced wound healing is finding a conduit that optimally delivers oxygen to tissues under physiological conditions. A biological oxygen-delivering protein would be an ideal conduit, as its delivery characteristics may follow the oxygen dissociation curve at various physiological partial pressures. Both hemoglobin and myoglobin accomplish this aim.

Though acute hypoxia may chemotactically stimulate the inflammatory response and cell arrival at a site of injury, sustained wound hypoxia may be a limiting factor in the normal healing process. Chronic hypoxia slows cell proliferation, collagen synthesis and the bactericidal action of leukocytes [19]. Wound dressings supplemented with hemoglobin or myoglobin that are saturated with oxygen could potentially release that oxygen (over a finite period of time) at the time of application of the dressing to the wound, thereby satisfying the increased local oxygen demand (created by the increased metabolic rate) and possibly enhancing resistance to infection. The concentration of hemoglobin/myoglobin in the scaffolds can be modified to account for the varying demands of oxygen by individual tissues and rates of cellular metabolism. This combination of natural materials has the potential to improve wound healing via faster cell proliferation and collagen production.

Electrospinning is a highly flexible technique through which the characteristics of each scaffold can be modified to accommodate the needs of individualized wound sites. These include scaffold shape, size, thickness, rate of bioresorption, oxygen binding capacity and rate of oxygen release. Of particular interest for oxygen delivery is the high surface area to volume ratio conveyed by electrospun fibers. This feature will allow maximal interaction with the surrounding host environment for oxygen delivery to hypoxic tissues; this may prove to have a similar efficiency as a red blood cell with its biconcave disc shape. A normal red blood cell has a volume of 81–98 femtoliters [20] and contains approximately 280 million hemoglobin molecules [21]; hence, there are about $2.9\text{--}3.5 \times 10^{18}$ molecules of hemoglobin/cm³ of red blood cells. An electrospun hemoglobin mat at the concentrations used in this feasibility study may contain between 1.4×10^{18} and 2.1×10^{18} molecules of hemoglobin/cm³ (based on the lower and upper concentrations of hemoglobin electrospun, i.e. 150 mg/mL and 225 mg/mL, respectively). Thus, the electrospun hemoglobin mat has the potential to have the oxygen-carrying capacity of a comparable volume of red blood cells.

The appeal of combining fibrinogen [12] with hemoglobin or myoglobin lies in the prospect of being able to supply oxygen to a wound site where chronic hypoxia could adversely affect the healing process. Thus, a more comprehensive study is underway to investigate the use of an electrospun hemoglobin or myoglobin wound dressing to improve cellular matrix production. Cell studies will involve the seeding of fibroblasts onto the scaffolds to investigate the effect of

hemoglobin and myoglobin fiber dimensions and oxygen-carrying capacity on both cell proliferation within the constructs and on collagen synthesis by the seeded fibroblasts over numerous time periods. Mechanisms by which the iron in methemoglobin or metmyoglobin in the scaffolds can be reduced to the ferrous state to enable the oxygenation of both proteins will be explored and a key focus will be to cross-link the scaffold structures without restricting the ability of the heme proteins to transition between the conformations that allow binding and release of oxygen. It is important that the native three-dimensional structures of hemoglobin and myoglobin are maintained throughout the electrospinning process as this determines functionality. Thus, structural biology techniques (including x-ray diffraction and spectroscopy) must be utilized to help in understanding the three-dimensional structure of the electrospun hemoglobin tetramer or myoglobin dimer, and thereby ensure that the scaffold functionality is optimal for oxygen binding. Other considerations that will be given to the scaffold design include the following: collagen [10] and/or fibrinogen may be combined with hemoglobin or myoglobin and the effects of these materials as electrospun scaffolds versus hemoglobin/myoglobin alone can be compared or a wound dressing with layers could be created such that a mixture of fibrinogen and hemoglobin/myoglobin resides on top of a layer of collagen and fibrinogen.

CONCLUSION

The electrospinning of hemoglobin and myoglobin into fibrous structures is a noteworthy achievement since these are globular proteins. In this preliminary study, we have demonstrated the capability of adjusting physical properties of the scaffolds by simply changing the concentration of the polymeric components. Additionally, cross-linking of the materials to different degrees enables modification of the mechanical properties of the scaffold. These tools for regulating the characteristics of the scaffolds are critical for tailoring the structures to meet the needs of specific tissue engineering applications. The feasibility study presented here merely scratches the surface of the tremendous potential a bandage composed of electrospun hemoglobin/myoglobin has to offer.

ACKNOWLEDGEMENTS

This study was supported by the Whitaker Foundation (TF-02-0013) and the Virginia Commonwealth University School of Medicine. Microscopy was performed at the VCU - Dept. of Neurobiology & Anatomy Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core grant (5P30NS047463).

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