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Enhancing the Stiffness of Electrospun Nanofiber Scaffolds with Controlled Surface Coating and Mineralization

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Abstract

A new method was developed to coat hydroxyapatite (HAp) onto electrospun poly(lactic-co-glycolic acid) (PLGA) nanofibers for tendon-to-bone insertion site repair applications. Prior to mineralization, chitosan and heparin were covalently immobilized onto the surface of the fibers to accelerate the nucleation of bone-like HAp crystals. Uniform coatings of HAp were obtained by immersing the nanofiber scaffolds into a modified 10 times concentrated simulated body fluid (m10SBF) for different periods of time. The new method resulted in thicker and denser coatings of mineral on the fibers compared to previously reported methods. Scanning electron microscopy measurements confirmed the formation of nanoscale HAp particles on the fibers. Mechanical property assessment demonstrated higher stiffness with respect to previous coating methods. A combination of the nanoscale fibrous structure and bone-like mineral coating could mimic the structure, composition, and function of mineralized tissues.

Keywords

electrospinning; nanofiber; surface modification; biomineral; biomaterials

Introduction

The reattachment of tendon to bone after an injury is a challenging problem in the clinical setting. For example, the failure rate of rotator cuff repair surgery can be as high as 94%.¹ This high failure rate is in large part due to the difficulty in re-attaching tendon to bone. The two materials differ dramatically in composition, structure, and mechanical properties--tendon is a compliant tissue with no mineral content while bone is a stiff tissue with an abundant mineral content.² To address this clinical problem, it is critical to develop a tissue engineering scaffold that mimics the native tendon-to-bone insertion site, which has a characteristic gradient in mineral composition.³ This gradation can produce a spatial variation in the stiffness of the scaffold, and can potentially be used to enhance tendon-to-bone healing through a tissue engineering approach.²

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The desired scaffold can be prepared by biomineralization of a nonwoven mat of electrospun nanofibers. Electrospinning is a versatile technique capable of generating large quantities of nanofibers from a wide variety of biocompatible and biodegradable polymers (both natural and synthetic), as well as composites containing inorganic materials.⁴ Because of their small feature size, scaffolds derived from electrospun nanofibers typically exhibit high porosities and large surface areas and can thus mimic the hierarchical structure of extracellular matrix (ECM) critical to cell attachment and nutrient transport.⁵⁻⁷ Additionally, the nanofibers can be readily assembled into a range of hierarchically structured arrays by manipulating their alignment, stacking, and/or folding.^{8,9} All these unique attributes make electrospun nanofibers well-suited as scaffolds for tissue engineering. Since a functional tendon-to-bone insertion site requires a mineral gradient spanning the length of the scaffold, biomineralization--deposition of calcium phosphate from a simulated body fluid--needs to be applied to generate the desired mineral coating on a nonwoven mat of nanofibers.¹⁰

Our group previously developed a simple method for fabricating scaffolds of electrospun nanofiber with gradients in mineral content.¹¹ In the previous study, 10 times concentrated simulated body fluid (10SBF) was gradually added into a glass vial containing the electrospun nanofiber scaffold with an orientation close to the vertical direction. The mineral gradient was formed along the length of the scaffold based on the immersion time along the vertical direction. Although this method was effective in creating a gradient in scaffolds stiffness, the mechanical properties of the fully mineralized end did not approach those of bone tissue. A possible reason for this is the highly porous structure of the mineral coating caused by the relatively large grain sizes of the mineral phase and their random orientations. The mineral coating typically exhibited a flower-like morphology leading to large void spaces between adjacent grains. Reducing this porosity may enhance the mechanical properties of the scaffolds. Previous reports have shown simultaneous increases in both hardness and toughness of calcium phosphate films as the grain sizes were reduced from micrometer to nanometer scales.¹² To this end, a modified 10 times concentrated simulated body fluid (m10SBF) was developed in the current study to facilitate the reduction of grain size for mineral coating of electrospun nanofibers. The major difference between 10SBF and m10SBF was the concentration of bicarbonate ion (HCO_3^-) in the solution. In m10SBF, an increased concentration of HCO_3^- was used because it was reported that ionic species favor the attachment of calcium phosphate mineral to a substrate by reducing the crystal size of calcium phosphate.¹³ Our hypothesis for the current study was that increasing the concentration of HCO_3^- ions in the m10SBF solution should enhance the mechanical properties of the mineralized nanofibers. It has also been reported that negatively charged surfaces are favorable for the heterogeneous nucleation of calcium phosphate.¹⁴⁻¹⁷ The accepted interpretation is that the accumulation of Ca^{2+} ions due to electrostatic attraction increases supersaturation near a negative surface, and as such, initial nucleation can be preferentially induced. Therefore, immobilization of negatively charged species onto the nanofiber surface may enhance the heterogeneous nucleation of calcium phosphate on that surface and subsequently increase the thickness of the mineral coating. Increased mechanical properties could therefore be achieved by reducing the grain size and increasing the thickness of the mineral coating.

In present work, we coated electrospun PLGA nanofibers with HAp (a major component of bone mineral) under a number of different conditions. Specially, chitosan and heparin were sequentially deposited onto the nanofibers to produce negatively charged surfaces to trigger the preferential nucleation of HAp. The effect of HCO_3^- ion concentration on both the grain size and thickness of coated HAp was also investigated. Mechanical properties were then tested to confirm the hypothesis that both the thickness and porosity of a mineral coating had a significant impact on the strength, toughness, and modulus (i.e., stiffness) of the scaffold.

Experimental Section

Materials

Poly(lactic-*co*-glycolic acid) (PLGA, $M_w \approx 50,000$ -75,000, lactide:glycolide = 85:15), dichloromethane (DCM), dimethylformaldehyde (DMF), acetic acid, chitosan (low M_w), heparin, 1-ethyl-dimethylaminopropyl carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid (MES), and all the chemicals for preparation of the 10 times concentrated simulated body fluid (10SBF) were obtained from Sigma-Aldrich (St. Louis, MO). All of them were used as received.

Fabrication of PLGA nanofiber scaffolds by electrospinning

The electrospinning setup used in the present work was described in detail in our previous publications.⁵ The solution for electrospinning was prepared by dissolving PLGA in a solvent mixture of DCM and DMF with a volume ratio of 80:20 at a concentration of 25%. The solution was loaded into a 5 mL plastic syringe with a 22-gauge needle attached, and dispensed using a syringe pump. The injection rate was 0.5 mL/h. The working distance between the tip of the needle and the collector was ~15 cm, and a voltage of 15 kV was applied. Alignment of the PLGA nanofibers was achieved by electrospinning onto a rotating mandrel collector.

Pretreatment of the PLGA nanofiber scaffolds with chitosan and heparin

In order to enhance the efficiency of immobilizing chitosan and heparin onto the nanofibers, the PLGA surface (typically hydrophilic) was modified by plasma treatment for 8 minutes. After treatment, the PLGA nanofiber scaffold was soaked in an aqueous solution containing 0.2% chitosan and EDC/NHS/MES (0.40 g EDC and 0.097 g NHS in 50 mL de-ionized water with 50 mM MES buffer) for 4 h. Afterwards, the sample was transferred into an aqueous solution of 1% heparin and EDC/NHS/MES for an additional 4 h. The sample was then washed with distilled water and dried in air at room temperature.

Mineralization of the nanofiber scaffolds using m10SBF

A supersaturated solution of 10SBF was prepared from NaCl, CaCl₂, NaHCO₃ and Na₂HPO₄. The ion concentrations in the 10SBF were 1000 mM for Na⁺, 25 mM for Ca²⁺, and 10 mM for HPO₄⁻. In order to investigate the role of HCO₃⁻ in mineralization with the 10SBF, the solutions were modified by using three different concentrations for HCO₃⁻ (m10SBF). In order to remove the impurities, the solutions were filtered through a 0.22 μ m pore size filter system. The chitosan- and heparin-coated PLGA scaffolds were then immersed in m10SBF in a capped plastic tube and kept at 37 °C for 0.5-9 h. The m10SBF solution was changed every 3 h when necessary. After removal from m10SBF, the sample was gently washed with water and then dried in air at room temperature.

Structural characterization

The morphologies of the PLGA nanofibers (before and after surface modification with chitosan or heparin) and the PLGA nanofibers after mineralization in m10SBF were examined by scanning electron microscopy (SEM, FEI Nova 200 Nanolab). The samples were sputter-coated with gold for 60 s prior to imaging, and an accelerating voltage of 5-15 kV was used.

Mechanical property characterization

Samples mineralized for different periods of time (0, 2 and 6 h) were tested in tension to determine their mechanical properties.¹¹ The scaffold was cut into strips of 3×14 mm² in size for uniaxial tensile mechanical testing (N=2-4 per group). The scaffold thickness was

measured using a laser micrometer (LK-081, Keyence) while the width was measured from calibrated digital images. The cross-sectional area was calculated as width multiplied by thickness. A texture-rich pattern of alizarin red dye was sprayed onto the test strip for optical deformation tracking. Mechanical testing was then performed in uniaxial tension under displacement control at a strain rate of ~0.5% per second using a materials testing system (ElectroPuls, Instron). Testing was recorded by video at 1360×1024 resolution at 2.5 fps using a CCD camera (DP70, Olympus). Engineering stress was calculated as load divided by initial cross-sectional area, and finite strain was determined from image data using custom Matlab (Mathworks, Natick MA) routines. Modulus was calculated as the slope of the linear region of the stress-strain curve. Toughness was calculated as the area under the stress-strain curve, reflecting the energy absorption of the material. Strength was calculated as the maximum stress during the tensile test.

Results and Discussion

Both the thickness and porosity of the mineral coating can affect the mechanical properties of the nanofiber scaffold. First, four different methods of surface treatment were investigated to promote the formation of a thick coating of mineral. The effect of HCO_3^- on the morphology and grain size of the mineral coating was then examined. Finally, mechanical tests were performed to determine the effect of these modifications on the mechanical properties of the scaffolds. The experimental parameters for the preparation of all samples are listed in Table 1.

Figure 1 shows the influence of surface treatment prior to mineral coating. The SEM images were taken from samples 2, 3, 4, and 5, respectively. The major differences among these samples are that they were pre-treated in different ways: sample 2 was not pretreated, sample 3 was treated with plasma only, sample 4 was treated with plasma and then chitosan, and sample 5 was treated with plasma, chitosan, and then heparin. These samples were then mineralized in the same coating solution for 3 h. While samples 2, 3, and 4 had no or very little mineral deposited on their surfaces, sample 5 had a thick and dense mineral coating. A thick mineral layer was also evident from the apparent increase in fiber diameter. For pristine PLGA nanofibers (i.e., sample 1), the average diameter was ~600 nm. Samples 2, 3, and 4 had approximately the same average diameter. The diameter of sample 5 was increased to ~1 μm . This increase in diameter indicates that the thickness of the mineral layer was ~200 nm. These results indicate that only a negatively charged surface could induce the nucleation of calcium phosphate on the surface of PLGA nanofibers and facilitate the mineral crystallization and growth. This phenomenon is likely due to the specific interactions between the functional groups on the surface of a macromolecule and the ions on the surface of a crystal nucleus.¹⁸ In Figure 1C, the nanofibers were first treated with plasma and then coated with chitosan. Chitosan is likely to be positively charged in the coating solution.¹⁹ Previous studies have demonstrated that a positively charged surface tended to inhibit the nucleation of HAp.¹⁵ That is why the nanofibers in Figure 1C were barely coated with HAp. The nanofibers in Figure 1D were first treated with plasma, and then coated with chitosan and heparin. Heparin is likely to be negatively charged in the solution.¹⁹ A negatively charged surface promotes the heterogeneous nucleation of HAp, leading to a more uniform, thick, and dense coating. Heterogeneous nucleation only occurs on the surface of an object once the interaction favors a decrease in the free energy. Anionic groups such as $-\text{COO}^-$ brought to the surface of an organic polymer by heparin may, for example, attract Ca^{2+} , resulting in local supersaturation and nucleation of crystallites in the vicinity of the nanofiber and minimization of homogeneous nucleation in the bulk solution. In comparison, the surface of nanofibers in sample 4 was positively charged due to the existence of chitosan, which presents amine groups to the fiber surface thereby inhibiting the precipitation of calcium phosphate on the surface of the scaffold (Fig. 1C).

The influence of HCO_3^- concentration on mineralization is shown in Figure 2. The SEM images were taken from samples 6, 7, and 8. The only difference among these samples is the concentration of HCO_3^- in the mineralization solution: 21 mM (sample 6); 42 mM (sample 7); and 63 mM (sample 8). The pH of 10SBF (21 mM) was ~ 6.1 while the pH of m10SBF (42 mM) was ~ 6.7 . The increase in pH resulting from the additional HCO_3^- could lead to an accelerated rate of HAp precipitation. In order to remove this precipitate, we filtered the m10SBF before coating the nanofibers. The concentration of HCO_3^- influenced the grain size, and thus surface roughness of the mineral coating. After incubation in 10SBF containing 21 mM HCO_3^- , plate-like crystals were formed on the fiber surface (Fig. 2A). When the concentration of HCO_3^- was doubled, there was a significant change in grain size for the mineral coating (Fig. 2B). The morphology of the mineral coating changed from a plate-like structure to a dense layer with a great reduction in both grain size and surface roughness. After mineralization with an even higher concentration of HCO_3^- in the 10SBF (sample 8), all of the fibers were fully encased in thick mineral sheaths with smooth surfaces (Fig. 2C). The reduction in grain size may have been due to molecular interactions between the precipitated HAp and HCO_3^- ions.¹⁸ Specifically, the HCO_3^- ions are believed to act as an inhibitor of HAp crystal growth in a simulated body fluid. In the absence of growth, more nucleation events are favored for HAp. The grain size of HAp likely decreased with increasing the concentration of HCO_3^- through this mechanism.¹³ In summary, a denser and smoother coating of HAp was produced when the concentration of HCO_3^- was increased.

To determine the thickness of the mineral coating, sample 7 was immersed in DCM to selectively dissolve the PLGA fibers in the core and obtain tubular fibers made only of the mineral phase (Fig. 2D). The thickness of these tubes (i.e., the thickness of the mineral coating) was ~ 420 nm. We then quantified the level of mineral content using an electronic balance and energy dispersive X-ray spectroscopy (EDX). The mineral content (Ca/(Ca+C) ratio) increased from 0.5% to 57.7% as the mineralization time was increased from 0.5 h to 9 h; at the same time, the mass percentage of the mineral increased from 0.08% to 3.13% during the course of mineralization for 9 h. As expected, the coating thickness increased from 230 nm to 450 nm with increasing immersion time (Fig. 3B).

Raman spectra were acquired of the mineralized PLGA nanofibers to better understand the composition and structure of the mineral phase (Fig. 4A). We observed dramatic changes to the Raman spectra after the mineral coating had been formed. Changes were especially apparent at 960 cm^{-1} (this peak represents symmetric stretching of the phosphate group). It has been reported that peaks in the region of $950 - 960\text{ cm}^{-1}$, are characteristic of crystalline HAp.²⁰ The Raman spectra also showed peaks corresponding to the molecular vibrations of the PLGA component, including the C-H (1450 cm^{-1}) and C-COO (875 cm^{-1}) stretch modes of lactic acid.

X-ray diffraction (XRD) was used to investigate the crystal structure of the mineral phase after mineralization in m10SBF for 3 hours. As shown in Figure 4B, the XRD pattern only shows peaks for HAp. The peak was not as sharp as those for HAp single crystals due to some disorder in the lattice structure as well as the randomness of orientation for different grains. Figure 4C shows a typical transmission electron microscopy (TEM) image of the HAp tube. It is clear that the HAp grains wrapped together very tightly around the PLGA nanofiber. Figure 4D is a high-resolution TEM image, which showed a well-resolved lattice fringe for a single mineral grain. Overall, the HAp tubes were comprised of numerous randomly-oriented pieces of such grains that wrap together to form a tube, as can be seen in the inset of Figure 2D.

In terms of mechanical properties, the modulus greatly increased with increasing mineral content (Fig. 5A). Conversely, the toughness of the scaffolds decreased with increasing

mineral content (Fig. 5B), indicating that the scaffolds had become more brittle (and more bone-like) with longer mineralization time. Despite the increase in stiffness, increasing mineral did not cause the yield stress to change, indicating that the coated mineral did not increase the stress at which permanent deformation began (Fig. 5C). These findings demonstrate that the mineral coatings produced using the new m10SBF solution could enhance the mechanical properties of nanofiber-based scaffolds. For the scaffolds fabricated using the conventional 10SBF, a modulus of only 100 MPa was achieved on the portion of the scaffold with the highest mineral content.¹¹ In comparison, with the use of a modified solution described in the present work, moduli as high as 500 MPa could be routinely achieved due to the drastic reduction in grain size for the mineral coatings. In summary, increased mineralization time led to increases in modulus, suggesting a stiffening effect of the mineral.

Conclusions

A class of biomimetic scaffolds was developed by coating the surface of electrospun PLGA fibrous mats with HAp in high density and controllable thickness. Negatively charged heparin was immobilized first, and HAp nanocrystallites were then deposited by mineralization with m10SBF. We demonstrated that these treatments could facilitate the formation of thick, uniform HAp coatings with greatly reduced grain sizes. The mineral coating had a significant impact on the mechanical properties of the scaffolds, including a significant increase in modulus. The scaffold developed in this study can be further modified with a gradation in mineral content and then employed for tendon-to-bone insertion site repair applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

SEM images of PLGA electrospun nanofibers (A) without (sample 2) and (B-D) with different types of surface treatments, followed by immersion in m10SBF (with a concentration of 42 mM for HCO_3^-) for 3 h: (B) treated with plasma (sample 3), (C) treated with plasma and then chitosan (sample 4), and (D) treated with plasma, chitosan, and then heparin (sample 5).

Figure 2.

SEM images of PLGA electrospun nanofibers that had been immersed in m10SBF solutions with different concentrations for HCO_3^- : (A) 21 mM (sample 6), (B) 42 mM (sample 7), and (C) 63 mM (sample 8). These samples were all pre-treated with plasma, chitosan and heparin before mineral coating. (D) SEM image of tubular structures made of nanocrystalline HAp that were obtained by dissolving the PLGA nanofibers (sample 7) in the core.

Figure 3.

(A) Quantification of the mineral content by EDX and direct weighing measurements, and
 (B) dependence of the mineral coating thickness on mineralization time.

Figure 4.

(A) Raman spectrum, (B) X-ray diffraction pattern, (C) TEM image, and (D) high-resolution TEM image taken from sample 7, which was pre-treated with plasma, chitosan, and heparin, followed by immersion in mSBF for 9 h with change to the fresh solution at t=3 and 6 h.

Figure 5. Mechanical testing results for samples mineralized for 0, 2, and 6 h, respectively. (A) Modulus increased after 6 hours of mineralization, (B) toughness (i.e., energy absorption) was decreased with increasing mineralization, and (C) yield stress (i.e., strength) was unchanged due to mineralization. (* $p < 0.05$)

Table 1

Summary of experimental parameters for sample preparation

Sample No.	Surface Treatment			HCO ₃ ⁻ Concentration (mM)			Coating Round*
	plasma	chitosan	heparin	21	42	63	
1	-	-	-	-	-	-	0
2	-	-	-	-	+	-	1
3	+	-	-	-	+	-	1
4	+	+	-	-	+	-	1
5	+	+	+	-	+	-	1
6	+	+	+	+	-	-	3
7	+	+	+	-	+	-	3
8	+	+	+	-	-	+	3
9	+	+	+	-	+	-	2/3
10	+	+	+	-	+	-	2

*. Each round of coating lasted 3 hours. For example, two rounds of coating means that the mIOSBF was replaced with the fresh solution at t=3 h.