Materials Letters 79 (2012) 14–17

Contents lists available at SciVerse ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/matlet

Quantitative characterization of the electrospun gelatin–chitosan nanofibers by coupling scanning electron microscopy and atomic force microscopy

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ARTICLE INFO

Article history:
Received 24 October 2011
Accepted 13 March 2012
Available online 21 March 2012

Keywords:
Electrospinning
Gelatin–chitosan nanofibers
Atomic force microscopy
HarmoniX
Elastic properties
DMT modulus

ABSTRACT

Nanofibers were electrospun from gelatin and chitosan at different mass ratios (gelatin/chitosan: 0/100, 25/75, 50/50, 75/25, 100/0). Atomic force microscopy (AFM) combined with scanning electron microscopy (SEM) was utilized in this study to evaluate the morphological and mechanical properties of the gelatin–chitosan nanofibers. The SEM images showed that the electrospun gelatin–chitosan nanofibers possessed more uniform morphologies than the pure gelatin or chitosan nanofibers. Moreover, AFM-HarmoniX mode was used to quantitatively assess the Derjaguin–Müller–Toporov (DMT) modulus of gelatin–chitosan nanofibers. After modified by two correction factors, the DMT modulus of gelatin–chitosan nanofibers showed higher values than the pure gelatin or chitosan nanofibers, which indicated the existence of intermolecular interaction within the electrospun gelatin–chitosan nanofibers.

1. Introduction

Natural extracellular matrix (ECM) of tissues is a complex composite of nano-scale fibrous proteins and glycosaminoglycans in a living organism. Its structural organization and biological function are now used to be mimicked for the scaffold design in tissue engineering [1]. Gelatin (an excellent fibrous protein) and chitosan (an ideal glycosaminoglycan material) are considered as the scaffold materials to make artificial ECM because of their advantages in terms of biocompatibility, as well as biochemical functionality by showing similarity to structures in animal tissues. Various gelatin–chitosan based ECM scaffolds have been constructed in macroscopic scale by blending, freeze-drying and cross-linking [2]. However, these macroscopic-scale gelatin–chitosan scaffolds are not sophisticated enough to mimic the natural ECM.

Electrospinning has recently received substantial attention as a way to produce nanofibers and this method can be used to construct nano-scale artificial gelatin–chitosan based ECM [1]. In order to determine the mechanical properties of the fibers on the nano-scale, nanoindentation, force volume AFM and nano tensile testing system have been applied [3]. However, the demanding and time-consuming testing processes have limited their broad applications.

Very recently, the novel HarmoniX mode of AFM has provided an excellent solution. This mode of AFM can quantitatively obtain the DMT modulus (one kind of elastic modulus) and the real-time information of materials with high spatial resolution and gentle forces [4]. HarmoniX imaging can efficiently save time by introducing relatively higher scan speed than the conventional AFM [5]. Moreover, AFM-HarmoniX is particularly useful in the elastic modulus measurements of heterogeneous samples, because it is able to observe a large dynamic range of samples [4]. In contrast, the conventional AFM could not achieve this, since only a limited number of spots from samples are selected and evaluated. Until now, the HarmoniX mode of AFM has not been used to quantitatively measure the mechanical properties of the electrospun nanofibers.

In this study, the gelatin–chitosan nanofibers were prepared by electrospinning and quantitatively assessed by AFM-HarmoniX. Since the horizontal dimension measurements of AFM were overestimated [6], we also used SEM to get the horizontal information of the nanofibers.

2. Materials and methods

2.1. Materials

Gelatin (isoelectric point: 6; Mw: 60 kDa) and chitosan (75–85% deacetylated; Mv: 50 kDa-190 kDa) were purchased from Sigma-Aldrich Co. Ltd. Trifluoroacetic acid (TFA) from Aladdin Reagent Co. Ltd. (China) and Dichloromethane (DCM) from Sinopharm Chemical Reagent Co. Ltd. (China) were of analytical grade and were used without further treatment.

2.2. Electrospinning

Gelatin and chitosan were dissolved in TFA/DCM (v/v, 70/30) separately to make 30% gelatin solution and 7% chitosan solution. A series of gelatin–chitosan solutions with different mass ratios (gelatin/chitosan:

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doi:10.1016/j.matlet.2012.03.044

0/100, 25/75, 50/50, 75/25, 100/0) were prepared by mixing. The mixed solutions were stirred for 24 h before loading into a 2 ml syringe with a 0.4 mm inner diameter needle. The grounded collector with aluminum foil was placed 15 cm below the needle. The applied voltage and flow rate for all the solutions were set at 25 kV and 0.3 ml/h.

2.3. Collection of nanofibers on mica

The collecting devices were built according to [7]. The collection part was modified in order to collect fibers on the substrate mica (Fig. S1). 1.5 cm × 1.5 cm mica was glued on a piece of weighing paper. Then the paper was attached to the collecting cardboard with mica-side toward the electrospun needle. After electrospinning, mica was detached from the paper and detected with AFM.

2.4. Scanning electron microscopy analysis

Hitachi S-4800 cold field emission SEM with accelerating voltages of 5 kV was used to observe the morphologies of the gold coated nanofibers. Nanofiber diameters were determined by image processing software (ImageJ, NIST). 150 fibers per sample were sized to generate reliable statistics.

2.5. Atomic force microscopy analysis

The electrospun nanofibers on mica were examined using a Multimode AFM with a NanoScope V controller (Veeco, Santa Barbara, CA, USA). Torsional cantilevers with a nominal spring constant of 2.8108 N/m were used. The vertical and torsional resonance frequencies were 63 kHz and 1.1 MHz, respectively. The tip radius (R_t) was 10 nm. Cantilevers were calibrated using a standard PS/LDPE sample. Imaging was performed at 0.5 scan rates. Data analysis was performed with the NanoScope 7.30 software. Three fibers per sample were tested. 10 heights and 120 DMT modulus per fiber were measured to generate reliable statistics.

3. Results and discussion

3.1. Morphologies of the electrospun gelatin–chitosan nanofibers

As previously mentioned, SEM was used here as a complementary technique for AFM. Namely, SEM measured the horizontal diameters, while AFM measured the vertical heights. Although these two techniques were different in many ways, direct comparisons of SEM and AFM results had already been adopted by researchers for a long period of time [8]. In order to minimize the changes of tested samples, we did not introduce any pretreatment other than the gold coating for the SEM experiment. It was well known that the error introduced by gold coating was not significant for analysis of nanostructures composed of > 30 nm diameter [8]. For the AFM experiment, we did not introduce any pretreatment at all.

The SEM images showed the gelatin–chitosan nanofibers had uniform cylindrical shape and their mean diameters distributed between 250 nm and 470 nm (Fig. 1b to d, Table 1). Comparatively, the cylindrical-shaped chitosan nanofibers had smaller diameters (between 50 and 250 nm) (Fig. 1a, Table 1). While the gelatin nanofibers had a broader distribution of diameters (between 300 and 900 nm)

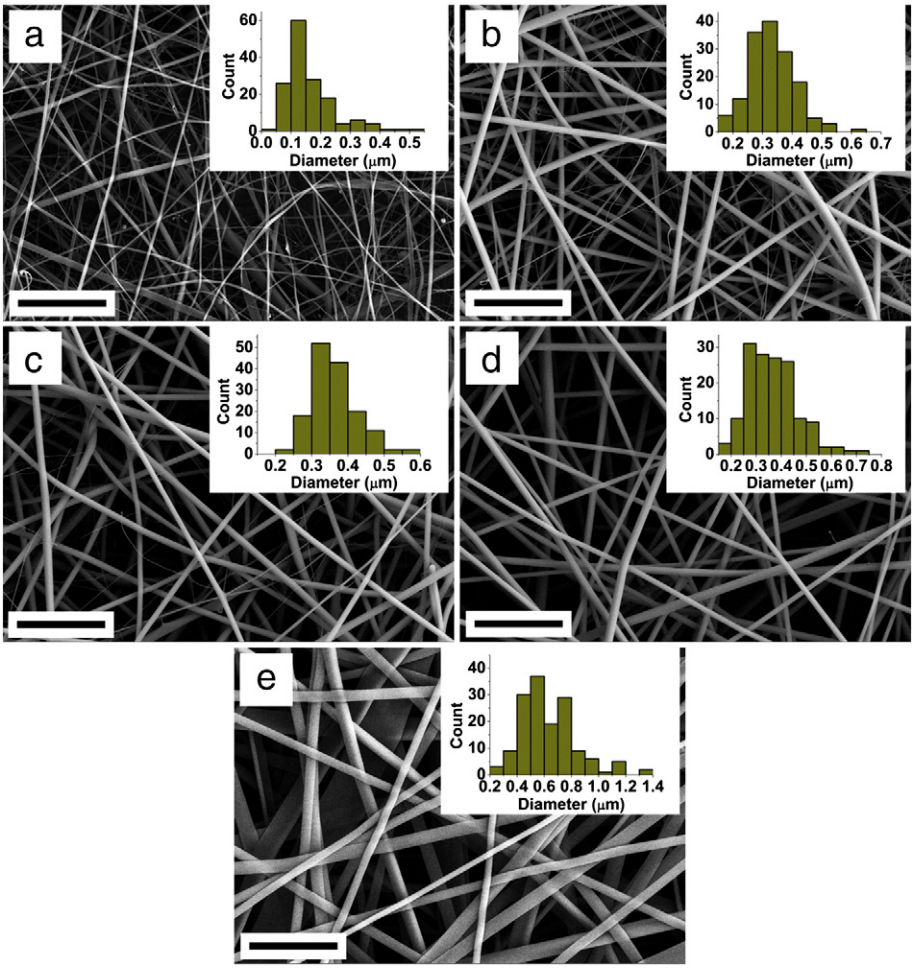

Fig. 1. SEM micrographs of electrospun gelatin–chitosan nanofibers in different mass ratios (gelatin/chitosan). (a) 0/100; (b) 25/75; (c) 50/50; (d) 75/25; (e) 100/0. The insets showed the fiber size distributions. Scale bar: 5 μm.

Table 1
Properties of electrospun gelatin–chitosan single nanofibers.

Gelatin/chitosan mass ratio	0/100	25/75	50/50	75/25	100/0
Diameter (nm)	162 ± 84	334 ± 77	364 ± 65	369 ± 97	634 ± 203
Height of single nanofibers (nm)	88 ± 10	188 ± 42	279 ± 72	296 ± 189	139 ± 105
Uncorrected DMT modulus (MPa)	952 ± 73	1334 ± 89	1226 ± 196	1331 ± 176	1109 ± 216
Corrected DMT modulus (MPa)	1000 ± 77	1374 ± 92	1250 ± 200	1357 ± 180	1109 ± 216

and some of them presented flat ribbon like shape (Fig. 1e, Table 1), presumably due to the slow solidification or solvent evaporation during the electrospinning process. In general, the gelatin–chitosan nanofibers possessed more uniform morphologies than the pure gelatin and chitosan nanofibers.

At single nanofiber scale, more morphological details of each sample were obtained by the corresponding height and phase images of AFM. The AFM height imaging revealed the heights of the gelatin–chitosan nanofibers (Fig. S2b to S2d, Table 1) were larger than the chitosan nanofibers (Fig. S2a, Table 1), similar with their mean diameters obtained from SEM images. The diameter/height ratios were 1.83, 1.77, 1.31, 1.24 and 4.56 (gelatin/chitosan: 0/100, 25/75, 50/50, 75/25, 100/0). Comparatively, the heights of pure gelatin nanofibers were much smaller than their corresponding mean diameters (Fig. S2e, Table 1), verifying the flat ribbon like shape from the SEM images (Fig. 1e). Moreover, the corresponding AFM phase imaging showed the differences between central and marginal areas of a single nanofiber (Fig. S2f to S2j). According to [9], the differences were caused by changing contact area.

3.2. Mechanical mapping of the electrospun gelatin–chitosan nanofibers

The AFM HarmoniX stiffness imaging was used to investigate the DMT modulus of each single nanofiber (Fig. 2a to j). In order to avoid instability of the edges (Fig. S2f to S2j), the DMT modulus was acquired from the central part of each single nanofiber.

It should be noted that the DMT modulus from the stiffness images were based on two assumptions. The first assumption was that the radius (R) used to calculate the relative elastic modulus (E, namely DMT modulus in this experiment, as shown in Eq. (1)) in Hertz theory [10] equaled the radius of tip (R_t). The second assumption was that the sample thickness was infinite [11]. For the nanofiber samples, two independent corrections should be calculated to measure the elastic modulus more accurately [9].

$$E = \frac{3F\left(1-\sigma^2\right)}{4R^{1/2}\delta^{3/2}} \quad (1)$$

where F is the applied force, σ is the Poisson ratio of the sample, R is the radius, and δ is the indentation.

As for the first assumption, the real R was smaller than Rt for the nanofiber samples [3]. For an infinitely long cylinder nanofiber in contact with a spherical indenter, the R is given by:

$$R = \sqrt{\frac{R_t{}^2 R_f}{R_t + R_f}} \quad (2)$$

and

$$R_f = H/2 \quad (3)$$

where R_f is the fiber radius, and H is the height of fiber.

After this correction, the calculated correction factors were 0.90, 0.95, 0.97 and 0.97 for R of gelatin–chitosan nanofibers (gelatin/chitosan: 0/100, 25/75, 50/50, 75/25). Whereas the pure gelatin sample partly possessed flatter ribbon-like shape, it was not included for this correction. According to Eq. (1), the corresponding correction factors were 1.05, 1.03, 1.02 and 1.02 for E of gelatin–chitosan nanofibers (gelatin/chitosan: 0/100, 25/75, 50/50, 75/25).

As for the second assumption, the finite-thickness fibers with heights less than 100 nm could cause substantial increments to the elastic modulus [12]. For the chitosan fibers with height below 100 nm, Akhremitchev and Walker [11] established the following equation to calculate normalized force ($\tilde{F}$), which combined with E

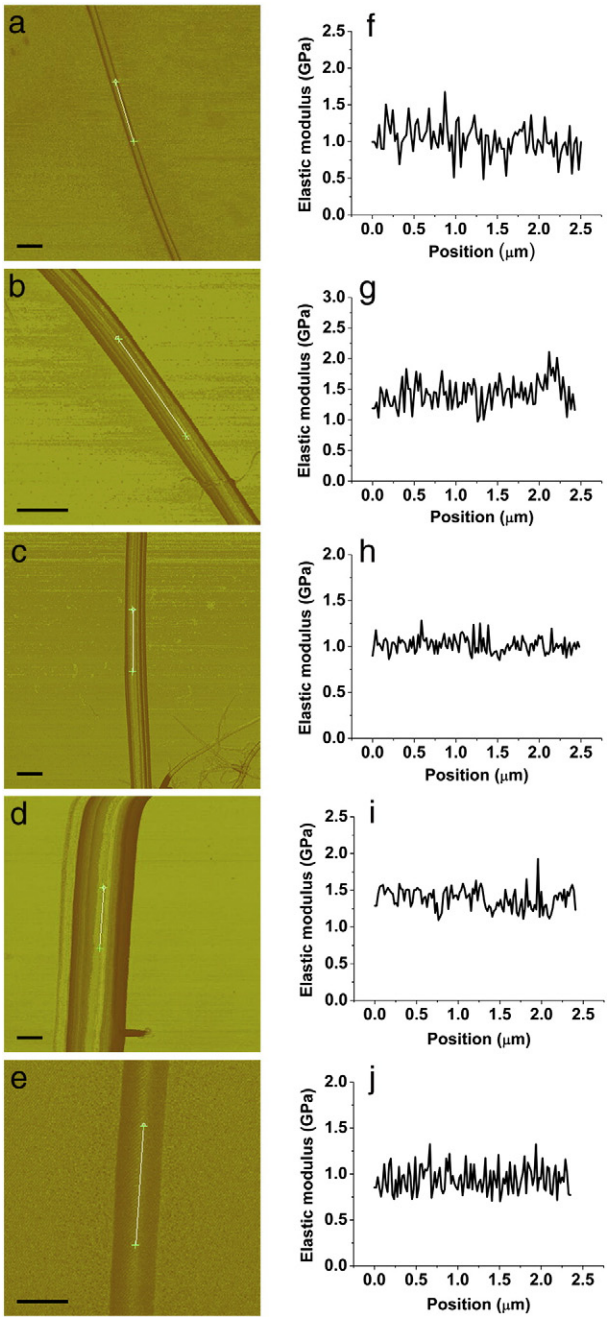

Fig. 2. Stiffness images (left) and the DMT modulus (right) of a single electrospun gelatin–chitosan nanofiber in different mass ratios (gelatin/chitosan). (a, f) 0/100; (b, g) 25/75; (c, h) 50/50; (d, i) 75/25; (e, j) 100/0. Around 2.5 μm along the axle wire of each single fiber was selected for averaging and was used to determine the DMT modulus. Scale bar: 1 μm. Z-scales: stiffness: 10.0 GPa.

could calculate the correction factor. Normalized force ($\tilde{F}$) is given by:

$$\tilde{F} = \frac{FR\left(1-\sigma^2\right)}{H^3} \quad (4)$$

where F is the peak force applied (acquired by AFM-HarmoniX, data not shown), a Poisson ratio value $\sigma = 0.3$ is taken as a reasonable value [13].

According to Eq. (4), the calculated normalized force ($\tilde{F}$) of chitosan nanofiber was 6.7×10^{-4} nN/nm^2. For the finite-thickness sample with the E around 1 GPa and $\tilde{F}<10^{-3}$ nN/nm^2, the correction factor was 1 [11]. In other words, the finite sample thickness had no significant influence on the DMT modulus of chitosan nanofibers. A reasonable explanation was that the harmonic imaging model caused smaller deformation depth than other methods, and the smaller deformation depth decreased the substrate's effect on the measured modulus for ultrafine samples [5].

Through such two corrections, the corrected DMT modulus of various nanofibers showed that the elastic modulus of the gelatin–chitosan nanofibers were larger than pure gelatin or chitosan nanofibers (Table 1). In addition, the effects of gelatin/chitosan mass ratio on the corrected DMT modulus were evaluated. More specifically, parametric methods including one-way analysis of variance (ANOVA) and Bonferroni post hoc analysis for multiple group comparisons were used to determine the statistical significance of the results (with $p<0.05$ considered significant). The analysis results indicated that the mixed samples showed significant improvement in mean corrected DMT modulus compared with pure samples ($p<0.05$). This phenomenon indicated the presence of intermolecular interaction within the electrospun gelatin–chitosan nanofibers, which was in good agreement with the nano tensile test results [14].

4. Conclusions

The SEM and AFM-HarmoniX analyses exhibited that the gelatin–chitosan nanofibers had more uniform morphologies and enhanced elastic properties than pure gelatin or chitosan nanofibers. The DMT modulus results indicated the existence of intermolecular interaction within the electrospun gelatin–chitosan nanofibers. In summary, AFM-HarmoniX was proved to be a convenient and efficient tool to examine electrospun nanofibers.

Acknowledgements

Thanks for the electroscope support of Public Center for Characterization and Test, Suzhou Institute of Nano-tech and Nano-bionics, Chinese Academy of Sciences. The authors thank Dr. Huibin Zou for valuable advices and linguistic revisions. This work was supported by the National Natural Science Foundation of China (NSFC, Grant No. 21106170), Shandong Province Natural Science Foundation (ZR2011CQ049), the Technologies Research and Development Program of Qingdao (No.07-2-1-nsh) and the Specialized Fund for Excellent Young Scientists from Youth Innovation Promotion Association, CAS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.matlet.2012.03.044.

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