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Quantitative Investigations of Nanoscale Elasticity of Nanofibrillar Matrices

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ABSTRACT

Recent research indicates that nanophysical properties as well as biochemical cues can influence cellular re-colonization of a tissue scaffold. It has also been shown nanoscale elasticity can strongly influence cellular responses. In the present work, quantitative investigations of the elasticity of a nanofibrillar matrix scaffold that has demonstrated promise for spinal cord injury repair are compared with complementary transmission electron microscopy investigations, performed to assess nanofiber internal structures. Interpretive model improvements are identified and discussed.

INTRODUCTION

Tissue scaffold engineering is an active research field that merges the disciplines of life sciences and physical sciences to develop functional synthetic tissues that can maintain, restore, or improve damaged organs [1]. One of the major challenges is to develop synthetic scaffolds that mimic all significant aspects of a native extracellular matrix. It has been recently recognized that significant aspects include nanophysical properties such as local elasticity [2,3] and 3D nanofibrillar architecture [4] in addition to the biochemical cues provided by directive growth factors [5].

Nanoscale elasticity may be especially important for actin-based cells such as astrocytes or fibroblasts that can exert strong traction force to actively probe their local environments [2]. Nanoscale elasticity can be investigated using atomic force microscope-based force curves, typically acquired as a raster scan of regularly spaced nano-indentations (force volume imaging). The nano-indentation measurements must then be interpreted using an elasticity model, such as a Hertz or more sophisticated model [6]. All models assume that the indenting tip is normal to the sample surface. For a nanofibrillar matrix, this means taking force curves exclusively at the median points along individual nanofiber and not on the sides or in between. It is not possible to achieve this condition for every data point in a conventional force volume imaging raster scan.

Scanning Probe Recognition Microscopy (SPRM) is a dynamic new mode of atomic force microscopy (AFM) developed within our group that enables auto-tracking along individual nanofibers within a nanofibrillar matrix through incorporation of recognition-based tip control [7,8,9]. In our previously published work [9], local force curves were collected exclusively at nanofiber median points with the tip normal to the nanofiber cylinder axis. Measurements from several nanofibers were compiled into a statistical representation of the nanofibrillar matrix as a whole. The measurements were related to a Young's modulus using a Hertz model. The nanofibrillar matrices under investigation are from a prototype neural cell prosthesis composed of non-woven electrospun polyamide nanofibers that has shown promise for spinal cord injury repair, in in-vivo studies (rat model) following penetrating injury [10,11].

However, several calibration issues remain with the elasticity values thus obtained. One is that the Hertz model assumes a uniform and homogenous substrate. We have previously reported transmission electron microscopy (TEM) results for a different set of electrospun nanofibers that demonstrated that different types of internal structures are possible as a function of different electrospinning conditions [7]. Both hollow and filled core nanofibers were observed. Spatial variations were also observed: the hollow core nanofibers had bamboo-like constrictions at roughly 200 nm intervals and the filled core nanofibers had phase contrast inclusions. Any of these internal structures would affect an elasticity measurement performed locally at the nanoscale. Therefore, an assessment of the nanofiber internal structure for a given set of synthesis conditions is required to select a correct interpretive model.

In the present work, first results from a systematic TEM study of nanofiber internal structures for the present neural cell prosthetic nanofibrillar matrix samples are presented. Both plan-view TEM, with the electron beam perpendicular to nanofiber axis, and cross-section TEM, with the electron beam normal to the cross section, were performed to assess nanofiber internal structures. Selected area electron diffraction (SAED) was also performed to assess nanofiber crystallinity.

EXPERIMENTAL DETAILS

The nanofibrillar matrices were randomly oriented polyamide nanofibers. The continuous nanofibers were collected as a nonwoven fabric. The samples were electrospun by Donaldson Co., Inc. (Minneapolis, MN) from a blend of two polymers, $(C_{28}\ O_4\ N_4\ H_{47}\ )_n$ and $(C_{27}\ O_{4.4}\ N_4\ H_{50})_n$ onto plastic (Aclar) coverslips [10].

The conventional and SPRM atomic force microscopy investigations were performed using a specially adapted Veeco Instruments (Woodbury, NY) Nanoscope IIIa operated in contact mode in ambient air. Other instrumental parameters include the use of an E scanner with a maximum $13x13\mu m^2$ x-y scan range and Veeco DNP silicon nitride probes with a $35^o \pm 2^o$ cone angle and a nominal 20 nm tip radius of curvature.

TEM investigations were performed using both a JEOL 100CX TEM operated at 100 kV and a JEOL FS2200 TEM operated at 200 kV (Japan Electron Optics Laboratories, Tokyo, Japan). The JEOL FS2200 TEM was used to obtain the selected area electron diffraction (SAED) of nanofibers with the camera length set to 60 cm. TEM sample preparation was as follows. For the plan-view TEM, the nanofibers were gently detached from the nanofibrillar surface with tweezers and suspended in 100% ethanol. The dispersion was then ultrasonicated for 30 sec (Branson Ultrasonic Corporation, Danbury, CT). A droplet was placed on a lacey film coated TEM grid by pipette and dried in ambient air. For the cross-section TEM, nanofibrillar matrix samples electrospun onto 200 μm plastic coverslips were embedded in Poly/Bed 812 epoxy resin

(Polysciences) and polymerized for 24h at 60 °C. Thin sections (70 nm thickness) of embedded nanofibers, were obtained using a MTX ultramicrotome (RMC, Boeckeler Instruments, Tucson, AZ) and using glass knives. Glass knives were prepared with a Glass Knife Maker (RMC, Boeckeler Instruments, Tucson, AZ). Thin sections were placed on 200 mesh copper grids and positively stained with 2% uranyl acetate in 50% ethanol and lead citrate (Reynold's formulation). Positively stained samples resulted in high contrast TEM images.

DISCUSSION

TEM investigation of nanofiber internal structures

An AFM image of the nanofibrillar matrix is shown in figure 1. While the 3D matrix architecture is apparent, it was not possible to determine whether or not the nanofibers were homogenous in composition. Therefore, TEM was used to investigate possible nanofiber internal structures.

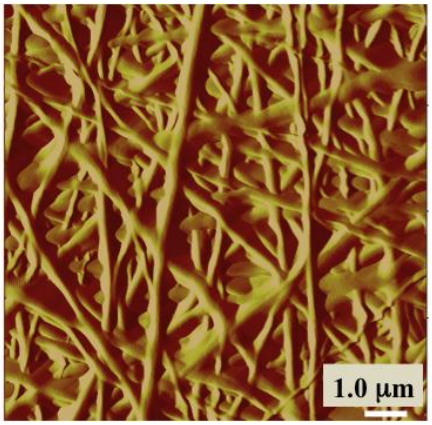

Figure 1. AFM deflection image of nanofibrillar matrix, top view.

TEM images indicated that the nanofiber diameters ranged from 30-200 nm, which is consistent with previous measurements. Bright field images indicated that almost all nanofibers had filled cores (the hollow structure on the left of figure 2 (a) is carbon lacey film). Almost all nanofibers also showed dark regions, which could range in size from the small 5-10 nm isolated regions shown in the figure 2 nanofibers to the large > 100 nm regions shown in the figure 3 nanofiber. Plan-view and cross-section view TEM images of the first type of nanofibers are shown in figure 2 (a) and (b). The small isolated dark regions appeared to be consistent in size and uniformly distributed throughout the nanofiber volume. This type of nanofiber was observed most frequently.

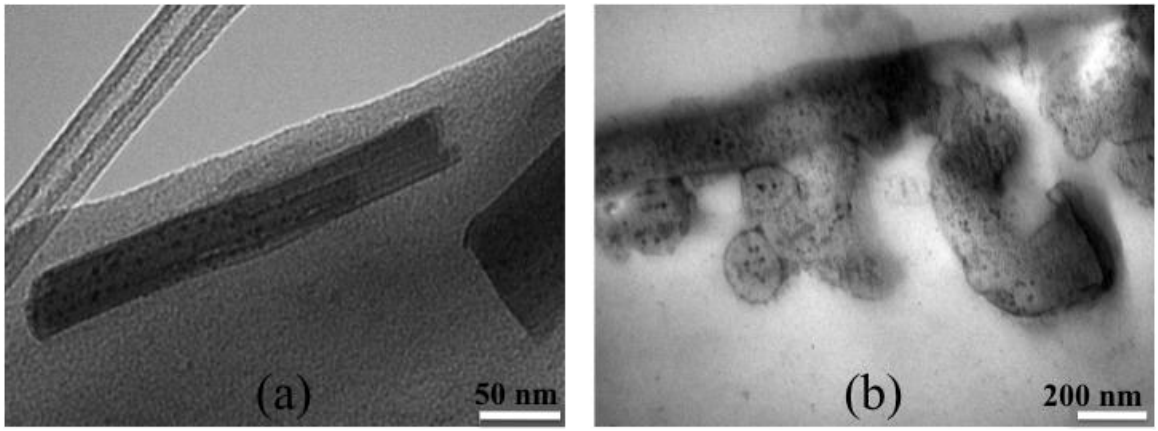

Figure 2. (a) Perpendicular view TEM image of dispersed nanofibers. (b) TEM image of a nanofiber cross-section embedded in Poly/Bed 812 and positively stained. Filled core nanofibers with small isolated dark contrast regions throughout are observed in both images.

A plan-view TEM image of another type of nanofiber sometimes observed is shown in figure 3 (a). Large > 100 nm dark regions fill most of the nanofiber volume. The volume is non-uniform and the surface was rough. A close-up of the region marked by the arrow in figure 3 (a) is shown in figure 3 (b). An irregular surface with possible layers of polymeric material was observed. The surface is electron transparent, which means that it is < 30 nm thick. Dark, meaning non-electron transparent, regions are observed in the nanofiber interior. SAED investigation of a dark region (circle in figure 3 (a)) revealed a diffraction pattern consistent with a polycrystalline material. The polycrystalline pattern is made up of rings with discrete spots due the relatively large size (> 10 nm) of individual grains within the diffraction area. Care was taken to locate the diffraction area on a part of the nanofiber that was suspended over a lacey film hole.

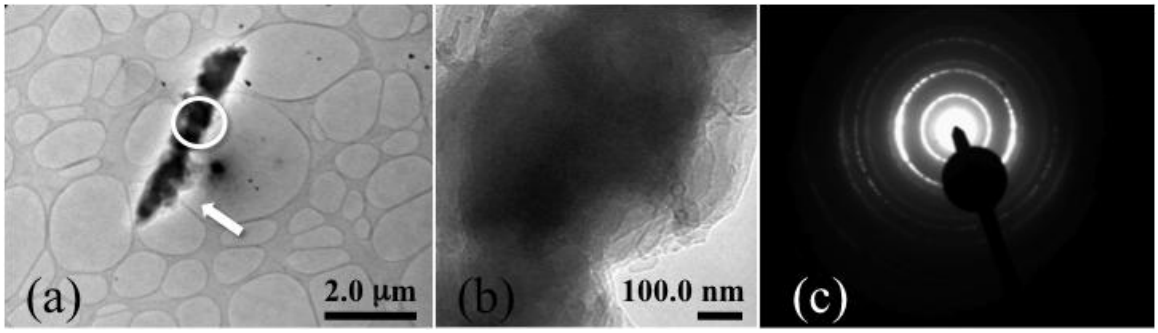

Figure 3. (a) Plan-view TEM image of nanofiber with large dark regions. (b) Close-up of region identified by the arrow shows an irregular electron transparent surface and dark non-electron transparent regions in the interior (c) The SAED diffraction pattern of a dark region indicates a polycrystalline material.

Comparison with Elasticity Measurements

We have previously reported SPRM results for the same nanofibrillar matrix samples, which indicated a range of Young's modulus values between 500 and 1000 Pascal (Pa) with a mean value of about 725 Pa [7]. Our TEM present work indicates that the present set of electrospinning conditions produces a majority of nanofibers with filled cores as opposed to hollow cores. However, our present work also indicates that the filled core nanofibers typically contained dark, non-electron transparent inclusions, which, while uniform in individual nanofibers, could range in size from 5 nm to > 100 nm. Furthermore, our present and also previous [7] SAED work indicates that the filled core nanofibers may contain a crystalline component. We are presently investigating a possible correlation between a range of Young's modulus values and a range of internal structures that may be crystalline in nature. We are also investigating whether there is a correlation between nanofiber diameter and the sizes of the dark contrast regions.

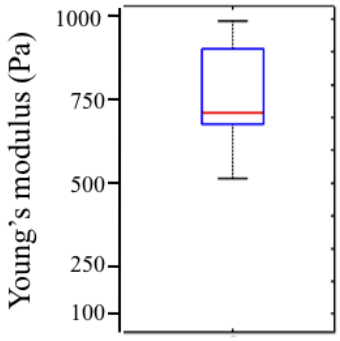

Figure 4. (a) SPRM elasticity measurements showed a range of values. Figure is adapted from Ref. [7]

The Young's modulus values shown above were obtained by minimizing the error between experimental and theoretical force values. The theoretical force values were obtained from a

Hertz model, with the assumption of a single component homogenous material. For the present nanofibrillar matrix nanofibers, the TEM results indicate that the original polymers are self-assembling into two distinct phases during the electrospinning process. Therefore, the theoretical Young's modulus should be replaced with the effective elastic modulus of a two-component material.

CONCLUSIONS

An internal structure assessment of synthetic non-woven electrospun polyamide nanofibers was performed with both plan-view and cross-section TEM investigation. This is essential for the correct interpretation of the elasticity evaluations. The nanofibers were filled core, with well-separated dark and light contrast regions observed in bright-field TEM images. This indicates that any interpretive model for elasticity measurements on these nanofibers should include an effective elastic modulus for a two-component material. The nature of the dark and light contrast regions is currently under investigation. Materials may be non-electron transparent due to high atomic number, thickness, or crystalline structure formation. The nanofiber synthesis conditions did not include any step that would introduce a high atomic number element into the composition. Small 5-10 nm dark regions were observed within even ~30 nm diameter nanofibers. A 30 nm thick sample composed of an amorphous blend of low atomic number polymers would be expected to be electron transparent. Crystallography of the nanofibers with SAED indicated that the nanofibers could have a polycrystalline component. Therefore, the images and the diffraction patterns suggest that the dark electron dense regions in the nanofibers may be crystalline structures. Additional TEM and high-resolution TEM experiments are continuing to further investigate the nanofiber internal structures.

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