hydrophobicity, and chemistry were obtained. The cell responses of cerebellar granular neurons to the changed nanophysical properties of the new versus aged tissue scaffolds were also investigated using atomic force microscopy (AFM). Cerebellar granular neurons (CGNs) were selected because this is a comparatively homogeneous CNS cell population. In a healthy situation, CGNs respond by well-fasciculated (branched) neurite (dendrites and axon) outgrowth. They can also display a guidance response to nano-patterning in their environment, including nanofibers [1]. In the present research, the CGN responses to the new versus aged tissue scaffolds were shown to be different. A new result was that a strong CGN drive toward obtaining a 3-dimensional environment, achievable with the aged nanofibrillar scaffolds, was observed.

EXPERIMENTAL PROCEDURES

Preparation of scaffold samples

The polyamide nanofibrillar scaffolds electrospun on Aclar substrates were purchased from Donaldson Co., Inc. (Minneapolis, MN). The polyamide nanofibers were electrospun on Aclar substrates for use as cell culture surfaces. Astrocyte cell cultures were prepared as previously reported [1,2]. The old and new nanofibrillar scaffolds were kept in dark storage for about 3 years and 1 year, respectively, except for experimental usage.

Preparation of cell samples

Purity-enhanced cultures of primary CGNs were prepared from postnatal day P8 Sprague Dawley rats and grown to confluence in 75 cm^2 tissue culture flasks. The culture medium was composed of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) + 10% fetal bovine serum (Invitrogen), 1% penicillin and streptomycin, (Sigma-Aldrich, St. Louis, MO) to which 25 mM KCl was added. CGNs were then subcultured in 0.5 mL medium at a density of 30,000 cells/well directly on Aclar coverslips coated with nanofibers in 24 well plates. Cells were fixed with paraformaldehyde at 24 h.

Analytical techniques

AFM height images of the neuron cell cultures were captured with a Nanoscope IIIA (Bruker AXS Inc, Madison WI, formerly Veeco Metrology) as previously reported [5].

TEM sample preparation was done as previously described [5]. TEM and SAED investigations of old and new nanofibers 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).

Contact angle measurements of culture surfaces were acquired using VCA Optima contact angle analysis equipment (AST Products Inc., Billerica, MA). One sample each from the culture surfaces was analyzed in five randomly selected areas.

Raman spectroscopy investigation was performed with using Kaiser Optical Systems HoloProbe Micro-Raman Spectrograph (Kaiser Optical Systems Inc., Ann Arbor, MI) coupled to an Olympus BX-60 optical microscope (Olympus, Center Valley, PA). For surface enhanced

Raman spectroscopy (SERS), glass coverslips were coated with approximately 20 nm thick gold in an Emscope Sputter Coater model SC 500 (Ashford, Kent, England) purged with argon gas. Old and new nanofibers were deposited by direct scraping with a clean razor blade to avoid chemical contamination.

EXPERIMENTAL RESULTS

TEM and SAED investigation of new and aged nanofibers

TEM investigation of individual nanofibers did not show obvious structural differences between the new and aged nanofibers, e.g., the hollow, bamboo, and solid core structures described in Ref. [6]. A light contrast interior with darker contrast walls suggestive of a hollow core structure was observed for both, as shown in the representative examples of Figure 1a and 1b. A similar conclusion of structural homogeneity was drawn from AFM investigation of new and aged nanofibrillar matrix surfaces (not shown). However SAED results indicated first, that many nanofibers both new and aged were substantially crystalline and next, that there were differences in the crystallinity between the new and the aged nanofibers. The aged nanofibers showed more pronounced diffraction spots as shown in Figure 1c and 1d. Dark contrast inclusions (arrows, Fig. 1a) were also noted for the aged nanofibers investigated in this study.

Contact angle measurements of new and old nanofibers

The results of a series of five contact angle measurements of the new and aged nanofibrillar surfaces are given in Table 1. The new and aged nanofibers had 52.3 ± 2.2 ° and 63.3 ± 2.5 ° (mean ± standard deviation) contact angles respectively. These results indicated that the culture surfaces became more hydrophobic as time passed.
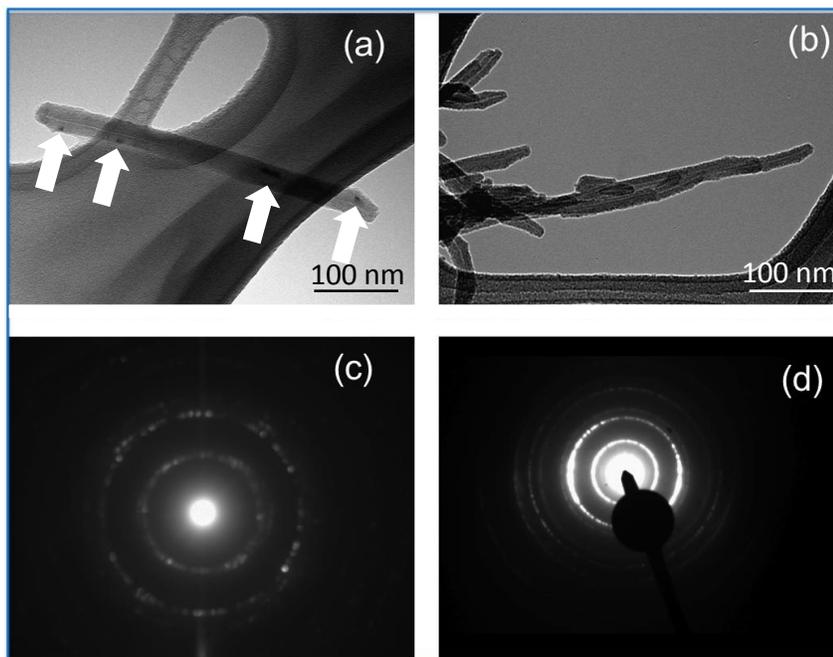

Figure 1. No structural differences were evident in TEM images of (a) aged and (b) new nanofibers but SAED showed stronger diffraction for (c) aged than for (d) new nanofibers.

Table 1. Contact angles of new and aged nanofibrillar surfaces indicated that these culture surfaces became more hydrophobic as a function of time. Std Dev is standard deviation.

#	NEW (< 1 yr)	AGED (> 3 yr)
1	50.09 °	61.3 °
2	51.5 °	62.3 °
3	56.3 °	66.0 °
4	53.4 °	65.8 °
5	52.1 °	60.9 °
Mean ± Std Dev	52.3 ± 2.2 °	63.3 ± 2.5 °

Raman spectroscopy investigation

SERS Raman investigation of seven regions for each sample showed a typical polyamide spectrum with an Amide I: C=O stretch at about 1300 cm^{-1} and an Amide III: C=O/C=N stretch at about 1630 cm^{-1} for both new and aged samples. A cis N-H peak at about 1400 cm^{-1} that has previously been reported for polyamide nanofibers was also observed for both samples. However, full width at half maximum (FWHM) increases on the polyamide peaks and the development of a broad disordered carbon peak [7] for the aged sample clearly indicated that changes in chemical bonding had occurred over time. It was noted that the Amide I C=O stretch appeared to be stronger in spectra from the aged nanofibrillar matrices. Representative spectra from both new and aged nanofibrillar matrices are shown in Figure 2.

AFM investigation of cerebellar granular neuron responses

High pass filtered AFM height images of cerebellar granular neurons are shown in Figure 3. The most frequently observed result for CGNs cultured on the aged > 3 yr scaffolds was that the
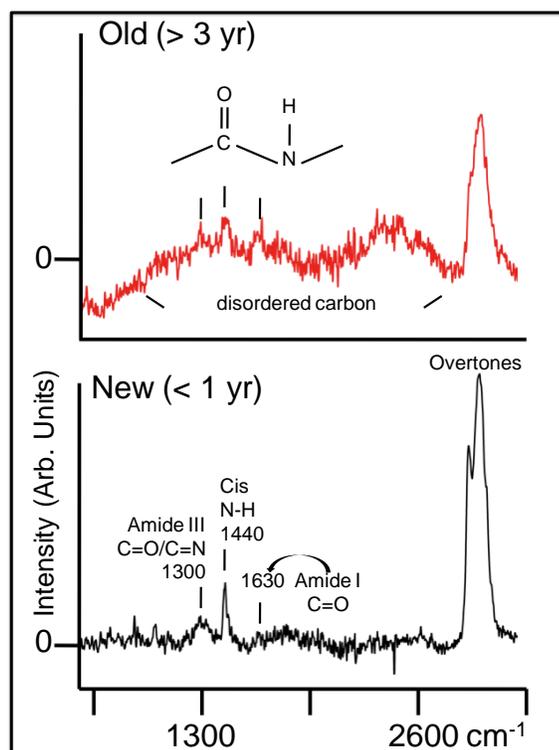

Figure 2. Raman spectroscopy showed FWHM increases on the polyamide peaks and development of a broad disordered carbon peak for the > 3 yr samples (red).

neurons burrowed beneath the scaffold as shown in the composite image in Figure 3a, and in the close-up in Figure 3b. They also developed dendrites and axons as shown in Figure 1a. In contrast, CGNs cultured on the newly electrospun < 1 yr scaffolds showed clustering behavior and lacked development of neurite outgrowth, as shown in Figure 3c.
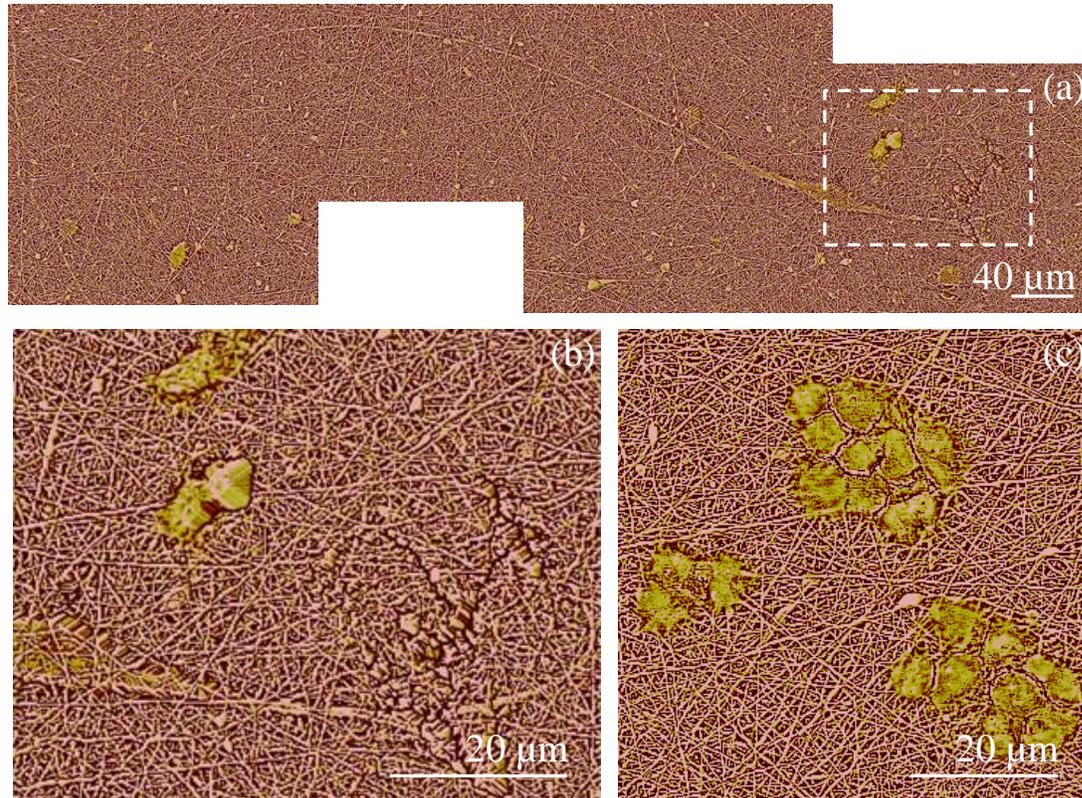

Figure 3. The composite AFM image (a) shows the burrowing response to use of an aged (> 3 years) nanofibrillar scaffold for a recent cerebellar granular neuron culture. The close-up of the dashed box in (a) is shown in (b) clearly shows that the neurons with associated neurite outgrowth are underneath the nanofibers. (c) Neurons cultured on new nanofibers clustered on the top and lacked neurite outgrowth.

DISCUSSION

This study demonstrated a cell migration/growth difference due to aging of tissue scaffolds. Careful investigation of the nanofibrillar layers on the Aclar surfaces after the burrowing response was observed showed no obvious breaks. The neurons were therefore strongly driven to reach a 3-dimensional environment, either by penetrating through the nanofiber meshes or by infiltrating between the nanofiber and the Aclar layers at their edges and then migrating considerable distances (0.5-1.0 cm) to reach some of the regions investigated by AFM. Contact angle measurements showed that there was a measurable difference in the surface polarity of new and aged scaffolds. Raman spectroscopy results confirmed the degradation of the basic polyamide chemistry over time. The SAED results also supported the idea that aging changed the

crystallinity; however, the SAED data showed that aging resulted in stronger diffraction, which indicates that the degree of order was increasing. This apparent contradiction can possibly be resolved if the degree of order was increasing locally, as crystalline inclusions within a polyamide matrix that, overall, was breaking down. The dark contrast inclusions identified by arrows in Fig. 1a were noted in all of the aged samples. Similar inclusions were not observed in any of the new samples.

The experimental results therefore suggest that a combination of polymer degradation with local crystallization was present in the aged nanofibers. An expected consequence of nanofiber degradation would be loss of elasticity. Facilitation of neuron growth cone development into axons on hydrogel matrices with compliances comparable to those of brain tissue (100-400 Pa, considered soft) has been reported [8]. It was further reported [8] that neuron single cultures grew *only* on soft hydrogels, which suggests a strong response to the mechanical environment. In the present study, healthy neuron morphologies with neurite outgrowth were observed in association with the aged scaffolds while pathological non-process bearing morphologies were observed on the new. A new observation is reported, that the properties of the aged nanofibrillar scaffolds evoked a response for neuron burrowing into a more 3-dimensional environment in addition to facilitation of neurite outgrowth.

CONCLUSIONS

The > 3 yr old nanofibrillar scaffolds showed definite physical and chemical aging effects compared with the < 1 yr old nanofibrillar scaffolds. These differences or degradation enabled neuron burrowing into a more 3-dimensional environment. Superior neurite outgrowth suggests that the aged nanofibers that enabled neuron access to the 3-dimensional environment were more biomimetic of growth supporting tissue. As individual properties are quantified and correlated with neural cell responses, new scaffold manufacture can emulate the nanophysical characteristics of environments that best support neural cell regeneration.

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