Biomedical Materials

PAPER

Incorporation and release of dual growth factors for nerve tissue engineering using nanofibrous bicomponent scaffolds

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Keywords: dual-source dual-power electrospinning, bicomponent scaffold, *in vitro* release, *in vitro* degradation, growth factors

OPEN ACCESS

RECEIVED
28 November 2017

REVISED
1 March 2018

ACCEPTED FOR PUBLICATION
14 March 2018

PUBLISHED
4 May 2018

Abstract

Electrospun fibrous scaffolds have been extensively used as cell-supporting matrices or delivery vehicles for various biomolecules in tissue engineering. Biodegradable scaffolds with tunable degradation behaviors are favorable for various resorbable tissue replacements. In nerve tissue engineering, delivery of growth factors (GFs) such as nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) from scaffolds can be used to promote peripheral nerve repair. In this study, using the established dual-source dual-power electrospinning technique, bicomponent scaffolds incorporated with NGF and GDNF were designed and demonstrated as a strategy to develop scaffolds providing dual GF delivery. NGF and GDNF were encapsulated in poly(D, L-lactic acid) (PDLLA) and poly(lactic-*co*-glycolic acid) (PLGA) nanofibers, respectively, via emulsion electrospinning. Bicomponent scaffolds with various mass ratios of GDNF/PLGA fibers to NGF/PDLLA fibers were fabricated. Their morphology, structure, properties, and the *in vitro* degradation were examined. Both types of core–shell structured fibers were evenly distributed in bicomponent scaffolds. Robust scaffolds with varying component ratios were fabricated with average fiber diameter ranging from 307 $\pm$ 100 nm to 688 $\pm$ 129 nm. The ultimate tensile stress and elastic modulus could be tuned ranging from 0.23 $\pm$ 0.07 MPa to 1.41 $\pm$ 0.23 MPa, 11.1 $\pm$ 3.0 MPa to 75.9 $\pm$ 3.3 MPa, respectively. Adjustable degradation was achieved and the weight loss of scaffolds ranged from 9.2% to 44.0% after 42 day degradation test. GDNF and NGF were incorporated with satisfactory encapsulation efficiency and their bioactivity were well preserved. Sustained release of both types of GFs was also achieved.

1. Introduction

Biodegradable polymers including poly(D, L-lactic acid) (PDLLA) and poly(lactic-*co*-glycolic acid) (PLGA) have been employed for various biomedical applications such as tissue engineering scaffolds and delivery systems with controlled release. Biodegradable biomaterials are favorable with appropriate degradation time, mechanical properties and supply of biomolecular stimuli to match the healing or regeneration process. Considering the unique requirements for specific medical application, a combination of biodegradable materials may be adopted as a strategy to perform collaboratively and accomplish complex tasks.

Electrospinning and as-spun fibrous scaffolds have attracted great attention owing to its ease of operation, feasibility with various natural and synthetic polymers, structural resemblance with the extracellular matrix (ECM) and excellent cell response [1–3]. Biomolecules or drugs can also be incorporated in electrospun scaffolds to improve their biological functionality [4, 5]. However, there is concern about incorporation efficiency of biomolecules through traditional electrospinning due to potential exposure of biomolecules to organic solvent and resultant destabilization and denaturation. Emulsion electrospinning has been employed as an efficient method to preserve the bioactivity of incorporated biomolecules, which

could also help alleviate the burst release and provide a sustained release of biomolecules [6, 7].

In neural tissue engineering, repairing of peripheral nerve injuries is a major challenge in reconstructive surgery. Peripheral nerves can regenerate to some extent after surgical nerve repair. However, appropriate device for bridging and guiding axons is necessary when there is a severe nerve defect [8]. Due to the limited availability of donor nerves and function loss of donor site, autografting is greatly hampered [9]. In recent years, tissue engineering scaffold-based nerve guide conduits have been extensively studied as a promising alternative. These artificial scaffolds and scaffold-based conduits are able to guide axonal elongation and neurite outgrowth [10–12], and can also serve as a reservoir of biological cues, such as laminin and growth factors [13, 14]. Local delivery of neurotrophic factors including nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) at surgical sites have been proposed to enhance peripheral nerve regeneration. These neurotrophic factors play important roles in neuronal survival, neural differentiation, and axonal regeneration independently or synergistically at different effective concentrations [15–20]. Sustained stimulation of neurotrophic factors is also required to induce neural differentiation [21], which is the physiological reason for choosing these growth factors. Continuous supplementation and co-administration of GDNF and NGF promoted axonal elongation and branching synergistically [20]. Considering the complexity of the whole system with specific requirements for mechanical, chemical and physiological properties, the production of an 'ideal' implantable and biocompatible scaffolds or conduits for peripheral nerve tissue repair is challenging. The driving hypothesis of this study is that whether nanofibrous scaffolds with dual growth factor release capability and a bi-phasic degradation behavior could be fabricated through the dual-source dual-power electrospinning (DSDP-ES), which might be promising for designing tissue engineering scaffolds with dual release capability. Nanofibrous scaffolds having NGF-loaded PDLLA fibrous component and GDNF-loaded PLGA fibrous component might be advantageous in simultaneously realizing neurite outgrowth and neural differentiation.

In this work, fibrous bicomponent scaffolds were designed and fabricated through DSDP-ES technique [22], with NGF and GDNF incorporated into PDLLA and PLGA nanofibers, respectively, via emulsion electrospinning. The mass ratios of GDNF/PLGA fibers to NGF/PDLLA fibers in bicomponent scaffolds were varied through a multiple-syringe strategy. The morphology, structure and properties of produced scaffolds were investigated. The encapsulation efficiency (EE) of NGF and GDNF in produced scaffolds and their release profiles were determined. Rat adrenal pheochromocytoma (PC12) cell line was used to assess the bioactivity of growth factors (GFs) released from fibrous scaffolds. The *in vitro* degradation of fibrous scaffolds and degradation mechanisms were also investigated.

2. Materials and methods

2.1. Materials

PLGA (LA:GA = 50:50) and PDLLA with molecular weight of 100 kDa (as indicated by their inherent viscosity 0.6–0.8 dl g^{-1}) were purchased from Lakeshore Biomaterials, USA. Chloroform was supplied by Uni Chem Co., Korea. Dimethyl sulfoxide (DMSO) was supplied by Fisher Scientific Inc., US. The human β-NGF with Enzyme Linked Immunosorbent Assay (ELISA) Kit, human GDNF with ELISA Kit were purchased from Peprotech Inc. and R&D Systems, Inc., respectively. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S) and trypsin/EDTA were purchased from Invitrogen, Inc., USA. Mouse anti-neurofilament (M + H) primary antibody, goat anti-mouse IgG secondary antibody (Alexa Fluor® 594), phalloidin (Alexa Fluor® 488) and DAPI (4',6-diamidino-2-phenylindole) were purchased from Life Technologies. Triton X-100, Span 80, phosphate buffered saline (PBS) tablets, heparin, bovine serum albumin (BSA) and paraformaldehyde were Sigma-Aldrich products. Other chemicals were used as received.

2.2. Fabrication of scaffolds

The fibrous scaffolds incorporated with GFs were fabricated by emulsion electrospinning. For water-in-oil (water/oil) emulsion formulation, PLGA/chloroform or PDLLA/chloroform solution with a certain concentration was used as the oil phase whereas NGF or GDNF was dissolved in 0.5 wt% BSA solution as the water phase. The volume ratio of the oil phase to the water phase was fixed at 10:1. 5 wt% of Span-80 (with respect to the weight of polymer used) was added in the polymer solution for the formation and stabilization of emulsions. The oil phase and water phase were mixed for 10 min through magnetic stirring at 300 rpm to form homogeneous water/oil emulsions. Polymer concentration was optimized both for PLGA and PDLLA and served as control variable to manipulate fiber diameter. The electrospinning parameters including applied voltage, inner diameter of needle tip, needle-to-collector distance and feeding rate of emulsions were optimized as 16 kV, 0.8 mm, 8 cm and 2 ml h^{-1}, respectively. To produce bicomponent nanofibrous scaffolds, DSDP-ES was employed (figure 1). GDNF/PLGA fibers and NGF/PDLLA fibers were collected simultaneously by a rotating drum collector, forming non-woven bicomponent scaffolds. By using a multiple-syringe strategy, the mass ratio between GDNF/PLGA fibers and NGF/PDLLA fibers in bicomponent scaffolds was adjusted. The formulation of emulsions for producing different mono- and bicomponent scaffolds was summarized in table 1. The produced

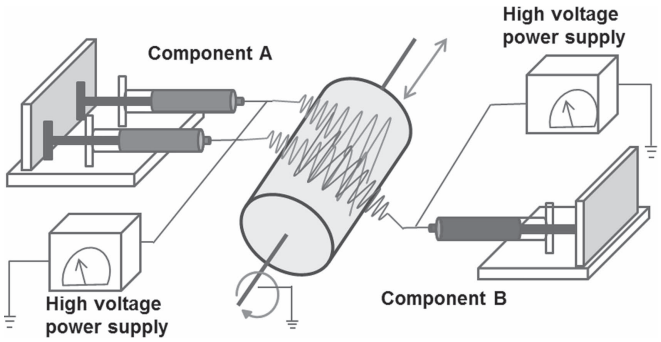

Figure 1. Schematic diagram of the DSDP-ES setup for fabricating nonwoven bicomponent scaffolds.

Table 1. Composition of emulsions for producing nonwoven mono- and bicomponent electrospun scaffolds.

	GDNF/PLGA emulsions		NGF/PDLLA emulsions	
Scaffold sample	Water phase 1 ml H_2O	Oil phase 10 ml $CHCl_3$	Water phase 1 ml H_2O	Oil phase 10 ml $CHCl_3$
G1 (0:1)				
G1 (1:2)				
G1 (1:1)	5 μg GDNF	1.5 g PLGA	5 μgNGF	2 g PDLLA
G1 (2:1)	5 mg BSA	75 μl Span-80	5 mg BSA	100 μl Span-80
G1 (1:0)				
G2 (0:1)				
G2 (1:2)				
G2 (1:1)	4 μg GDNF	1.2 g PLGA	3.75 μg NGF	1.5 g PDLLA
G2 (2:1)	5 mg BSA	60 μl Span-80	5 mg BSA	75 μl Span-80
G2 (1:0)				

electrospun scaffolds were freeze-dried for 24 h before any further experiments.

2.3. Characterization of fibers and electrospun scaffolds

Morphological and structural characterization of electrospun fibers and fabricated scaffolds was conducted. Freeze-dried samples of electrospun fibers and scaffolds were sputtered with a thin gold coating for 30 s by a sputter coater (BEL-TACSCD005) and their morphology was examined using SEM (Hitachi S-4800 FEG SEM, Japan). Electrospun fibers were also collected during electrospinning on a copper grid covered with carbon film and their structures were observed using TEM (Philips EM208s TEM, the Netherlands). Wettability of electrospun fibrous scaffolds was investigated by measuring their water contact angles (WCAs) at room temperature with a WCA measuring machine (SL200B, Shanghai Solon Tech Inc. Ltd, China). Measurements were performed 3 s after a DI water drop was placed on the sample surface. Tensile tests were performed using a bench top Instron mechanical testing machine (Model 5848, USA) with a load cell of 10 N and a cross-head speed of 2 mm min^{-1}. Each fibrous scaffold with similar thickness was cut into rectangular shape (5 mm $\times$ 30 mm) for testing. The ultimate tensile strength (UTS), elastic modulus (EM) and elongation at break (EB) of scaffold samples were determined according to the recorded stress–strain curves. At least three replicates were tested for each type of fibrous scaffold and results were expressed as mean $\pm$ SD.

2.4. Encapsulation efficiency of GFs

The EE of NGF or GDNF in electrospun fibers (and hence scaffolds) was calculated using the equation:

$$EE\% = E/E_0 \times 100\%, \quad (1)$$

where E is the actual amount of growth factor encapsulated and E_0 is the amount used for encapsulation. The actual amount E was measured using an extraction method. Briefly, a scaffold sample (around 20 mg) was dissolved in 2 ml DMSO. 20 ml 0.05 M NaOH solution with 0.5% Tween-20 was added to form a mutually soluble mixture. Subsequently, 100 µl of the emulsion were pipetted and the concentration of NGF or GDNF was measured using corresponding ELISA Kit for the growth factors.

2.5. *In vitro* release test of NGF and GDNF

For *in vitro* release investigations, experiments followed the procedures as used by other research groups [23]. Briefly, scaffold samples (about 50 mg each) were immersed in 12-well plates filled with 3 ml release

medium and incubated in shaking water bath at 37 °C. At pre-set times, 0.4 ml of supernatant was retrieved from each well and replaced by 0.4 ml of fresh release medium. The supernatant sample was frozen at −20 °C for further measurement or directly used for analysis. The concentration of growth factor in the supernatant was determined using ELISA kit. The release medium was prepared by adding 0.5% BSA, 0.05% Tween-20, 0.02% NaN_3 and 0.1% heparin in PBS solution.

2.6. Bioactivity assay of NGF and GDNF

The bioactivity of incorporated NGF and GDNF was evaluated on a culture-to-culture basis. Briefly, mono-component scaffold GDNF/PLGA and NGF/PDLLA scaffold samples (100 mg each) were sterilized by ^{60}Co γ-irradiation at a dose of 15 kGy before usage then immersed in 10 ml DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin and incubated in shaking water bath at 37 °C for 24 h. The concentration of released NGF or GDNF was determined before the medium with released growth factors was used and 10 ng ml^{-1} GDNF and 5 ng ml^{-1} NGF was used for further bioactivity comparison study. As-received GFs were reconstituted in culture medium at same concentration as comparison. About 8×10^3 PC12 cells (rat pheochromocytoma cell line, Biowit Technologies, Shenzhen, China) were seeded on glass slide in each well supplemented with 1 ml culture medium at 37 °C and 5% CO_2. After 1 day, the culture medium was replaced by release medium or reconstituted medium described above and refreshed every 2 days. At day 1, 4, and 7, the cells were washed twice with PBS and fixed on the glass slides with 4% paraformaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were permeabilized with 0.1% Triton X-100 solution and incubated in (1% w/v) BSA block solution for 30 min, followed by the incubation in mouse anti-neurofilament (M + H) primary antibody (1:100 dilution) containing block solution for 1 h. After washing 2 times with PBS, the cells were incubated with goat anti-mouse IgG 594 secondary antibody (2 drops ml^{-1}) for 30 min FITC phalloidin (1:40 dilution) was simultaneously added for the staining of F-actin and DAPI (1:1000 dilution) was added for the staining of nuclei. Confocal imaging of cells was performed under a confocal microscope (LSM 710, Carl Zeiss).

2.7. *In vitro* degradation tests

In vitro degradation tests of scaffolds were conducted following established procedures. Each sample (about 20 mg) was accurately weighed, sterilized, fully immersed in 3 ml phosphate buffer solution (pH = 7.4) and placed in a separate container incubated in shaking water bath at 37 °C. The degradation medium was replaced with fresh PBS every 3 days. 0.02% NaN_3 was added in PBS to prevent bacterial growth. At preset time, samples were taken out from degradation medium, rinsed with distilled water for 3 times and lyophilized for weight measurements. At least 3 repetitive samples were taken at each time point. Morphology of degraded scaffold samples was also examined under SEM.

2.8. Image analysis

SEM images were loaded into Image J (National Institute of Health, USA) and 100 fibers in each scaffold were randomly selected to determine average fiber diameters. Confocal microscopic images were loaded into Image J and morphometric data were obtained. The presence of neurites was examined and cell differentiation was quantified by measuring the neurite length. Only protrusions originating from the cell body and longer than 28 μm (about two times of average cell body diameter) were counted as neurites [24].

2.9. Statistical analysis

The unpaired student t-test (for comparison of two groups) was used for statistical analyses. Statistical difference was condisdered at $p < 0.05$.

3. Results

3.1. Surface morphology and wettability

PLGA and PDLLA-based mono- and bicomponent nanofibrous scaffolds with a thickness of approximate 500 μm were obtained through emulsion electrospinning and DSDP-ES technique. The surface morphology of fibrous scaffolds as well as structures of GDNF/PLGA and NGF/PDLLA fibers are shown in figure 2. The average fiber diameter and wettability of each scaffold sample are shown in figures 3 and 4.

SEM images of mono- and bicomponent nanofibers showed a randomly interconnected structure and smooth surface morphology without any bead (figure 2(a)). A uniform distribution of GDNF/PLGA and NGF/PDLLA fibers in bicomponent scaffold was obtained. Through emulsion electrospinning, discontinuous core–shell structure was formed in both GDNF/PLGA fibers and NGF/PDLLA fibers, which is shown in figure 2(b). The water phase core was enclosed by the polymer shell in both types of fibers but appeared differently. The phase boundary of core–shell structure in PDLLA fibers was unambiguous, while it appeared in a dispersive pattern in PLGA fibers.

Fiber diameter of fibrous scaffolds was varied by changing polymer concentration during emulsion electrospinning, which was 12% (w/v), 15% (w/v) for PLGA and 15% (w/v), 20% (w/v) for PDLLA correspondingly. Based on the fiber diameter, the fibrous scaffolds were divided into thick fiber group (G1 group, 15% (w/v) for PLGA and 20% (w/v) for PDLLA) and thin fiber group (G2 group, 12% (w/v) for PLGA and 15% (w/v) for PDLLA). The average

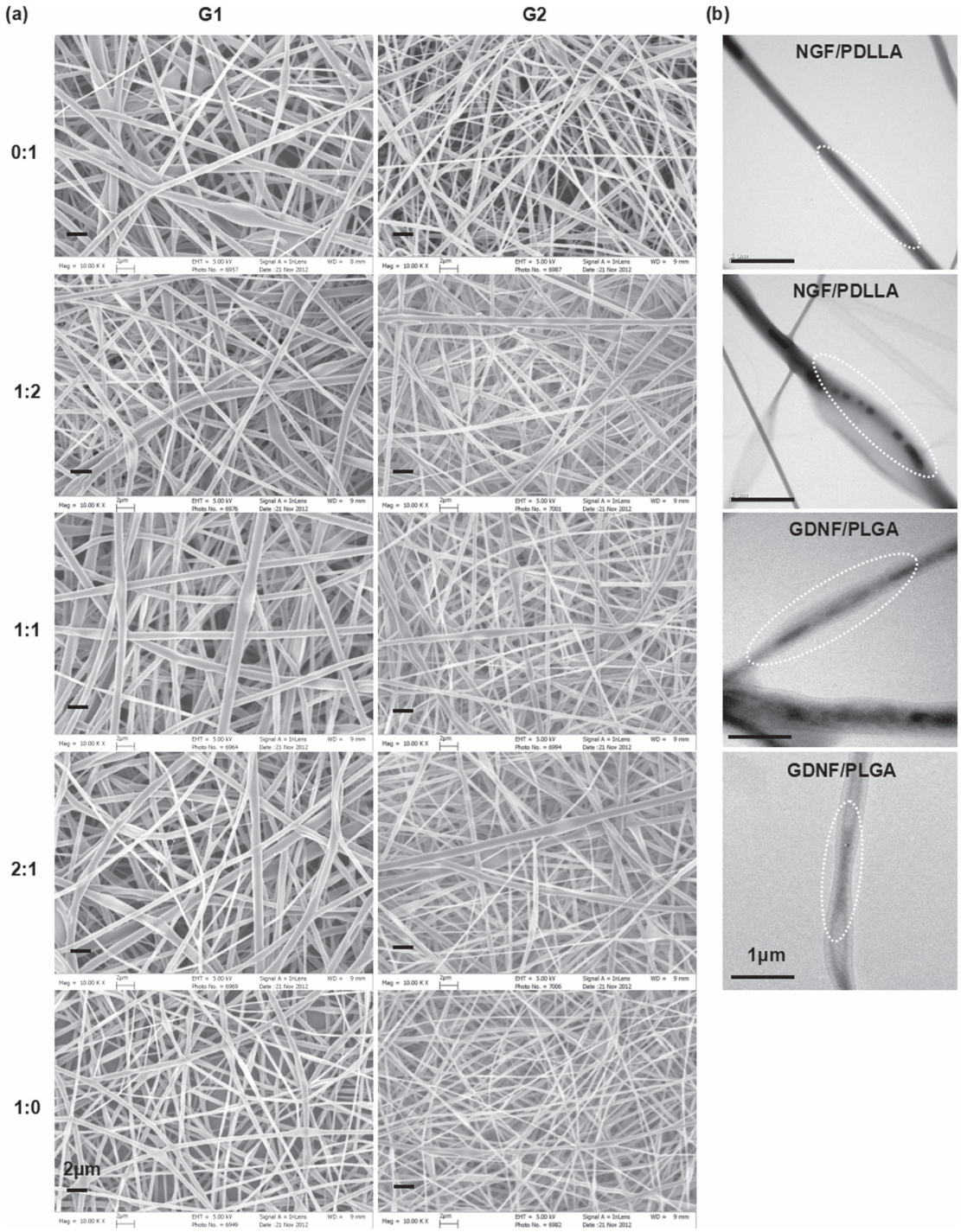

Figure 2. Morphology and structures of electrospun fibers and scaffolds: (a) SEM images of mono- and bicomponent fibrous scaffolds, (b) TEM images of core–shell structured NGF/PDLLA and GDNF/PLGA fibers and discontinuous core–shell structures were noted with dashed ellipse.

fiber diameter decreased from 688 ± 129 nm to 447 ± 107 nm, 502 ± 102 nm to 307 ± 100 nm in G1 and G2 group, respectively, the ratio of GDNF/PLGA fibers to NGF/PDLLA fibers increased from 0:1 to 1:0 (figure 3).

The static WCA of mono- and bicomponent fibrous scaffolds was measured and the shape of a water droplet on each scaffold was captured by a camera to determine the surface hydrophilicity/hydrophobicity (figure 4). WCA of NGF/PDLLA monocomponent scaffold, which was 137.0 ± 3.3° and 134.0 ± 1.0° in G1 and G2 group, was much higher than that of GDNF/PLGA scaffold which was 81.0 ± 2.0° and 65.5 ± 0.5°, respectively. The result showed that the NGF/PDLLA scaffold was more hydrophobic than GDNF/PLGA scaffold. Increasing the GDNF/PLGA fiber to NGF/PDLLA fiber ratio in bicomponent scaffolds did not significantly decrease their surface hydrophobicity. The WCA value of G1 group was decreased as compared to G2 group,

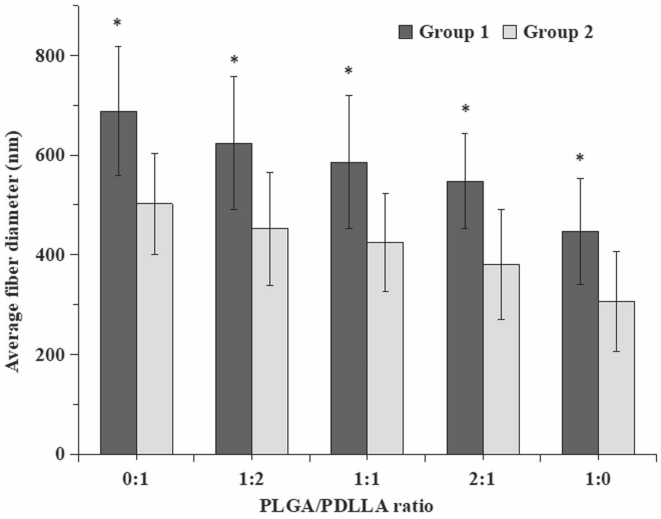

Figure 3. Average fiber diameter in fibrous scaffolds with different fiber component ratios. Fiber diameter in Group 1 statistically different than that in Group 2 for respective PLGA/PDLLA ratios. $^{*}= p < 0.05$.

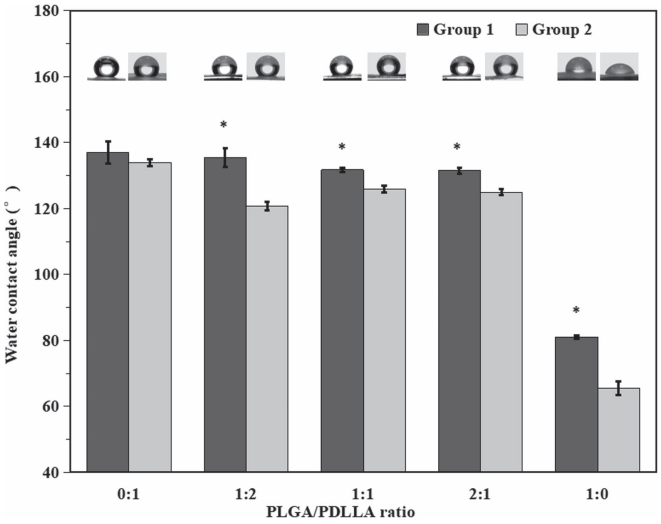

Figure 4. Water contact angle of mono- and bicomponent scaffolds. Water contact angle in Group 1 statistically different than that in Group 2 for respective PLGA/PDLLA ratios. $^{*}= p < 0.05$.

suggesting that thinner fiber diameter could result in a slight decrease of hydrophobicity.

3.2. Mechanical properties

Tensile tests of produced scaffolds were performed and their UTS, EM and EB were determined using obtained stress–strain curves (figure 5).

The results showed that UTS of scaffolds increased from 0.30 ± 0.08 MPa to 1.41 ± 0.23 MPa in G1 group, 0.23 ± 0.07 MPa to 1.34 ± 0.21 MPa in G2 group as GDNF/PLGA fiber to NGF/PDLLA fiber ratio increased from 0:1 to 1:0 (figure 5(a)). Similar trends were observed in EM and EB value. EM value increased from 21.5 ± 3.0 MPa to 75.9 ± 3.3 MPa in G1 group, 11.1 ± 3.0 MPa to 53.5 ± 4.0 MPa in G2 group as GDNF/PLGA fiber to NGF/PDLLA fiber ratio increased from 0:1 to 1:0 (figure 5(b)). EB value increased from 34 ± 8% to 112 ± 16% in G1 group, 37 ± 7% to 95 ± 14% in G2 group as GDNF/PLGA fiber to NGF/PDLLA fiber ratio increased from 0:1 to 1:0 (figure 5(c)). These results indicated that GDNF/PLGA fibrous scaffolds had much higher UTS, EM and EB values comparing with NGF/PDLLA ones. Increasing fiber diameter and GDNF/PLGA fiber to NGF/PDLLA fiber ratio in bicomponent scaffolds could both significantly improve the mechanical properties of fibrous scaffolds.

3.3. Encapsulation efficiency of GFs and release profile of GFs

EE (in percentage) of GFs incorporated in fibers was measured using ELISA kits through an extraction method and calculated using equation described before and shown in figure 6. The EE of NGF was

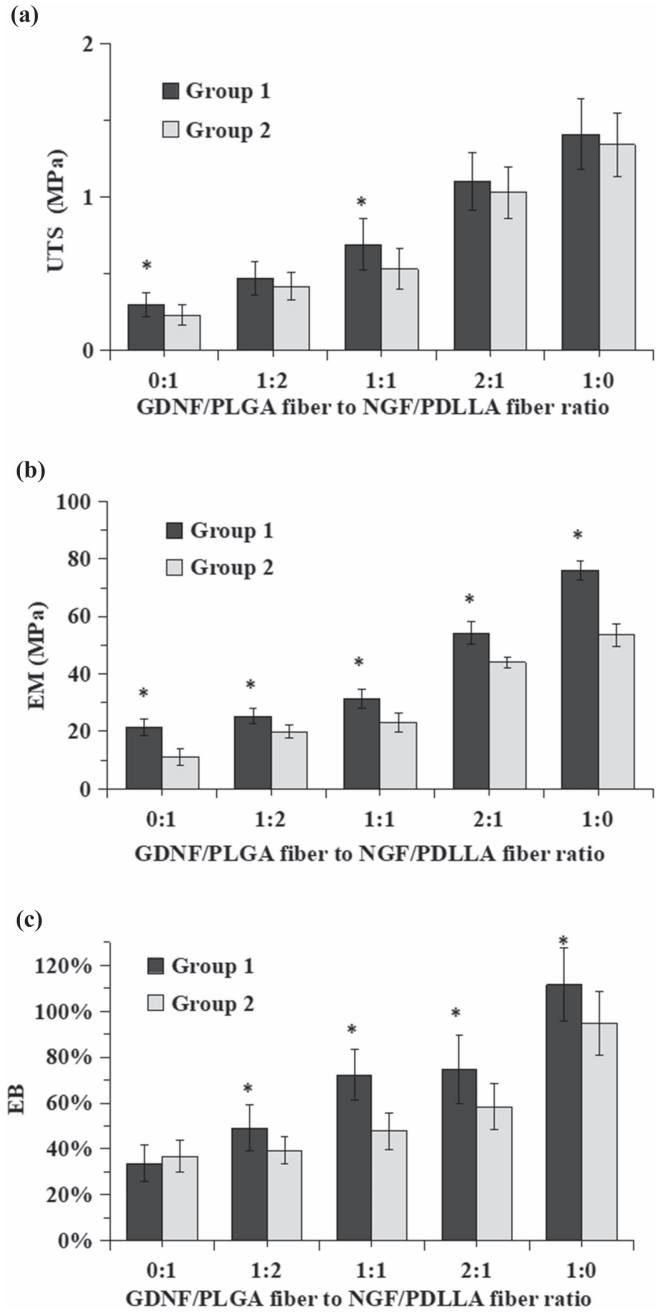

Figure 5. Tensile properties of electrospun scaffolds: (a) ultimate tensile strength, (b) elastic modulus and (c) elongation at break. UTS, EM and EB in Group 1 statistically different than that in Group 2 for respective PLGA/PDLLA ratios. $^{*} = p < 0.05$.

85.3 ± 3.0%, 86.8 ± 2.2%, 86.2 ± 2.8%, and 88.2 ± 2.9% in G1 group and 87.0 ± 3.1%, 86.3 ± 2.4%, 87.6 ± 2.6%, and 85.5 ± 3.2% in G2 group, corresponding to GDNF/PLGA fiber to NGF/PDLLA fiber ratio at 0:1, 1:2, 1:1, and 2:1, respectively. The EE of GDNF was 83.0 ± 2.8%, 79.6 ± 2.9%, 82.6 ± 3.0%, and 81.0 ± 3.0% in G1 group and 81.3 ± 2.7%, 80.7 ± 2.4%, 79.1 ± 2.3%, and 78.6 ± 3.1% in G2 group, corresponding to GDNF/PLGA fiber to NGF/PDLLA fiber ratio at 1:2, 1:1, 2:1, and 1:0, respectively. All the scaffolds achieved high EE of growth factors which was above 79%, demonstrating that PLGA and PDLLA nanofibers fabricated by emulsion electrospinning could be good delivery vehicles for hydrophilic proteins. Regardless of the component ratio and fiber diameter, the EE of NGF in bicomponent fibrous scaffolds was at the same level with that in their monocomponent counterparts. This observation was also made for the EE of GDNF, although the average EE of GDNF was 80.7 ± 1.6%, slightly lower than that of NGF which was 86.6 ± 1.0%.

The release profiles of NGF and GDNF from bicomponent scaffolds (G1 (1:1), G2 (1:1)) including cumulative release amount and cumulative release percentage of GFs were shown in figure 7. All scaffolds exhibited similar release profile including an initial burst release within 24 h, followed by a much slower

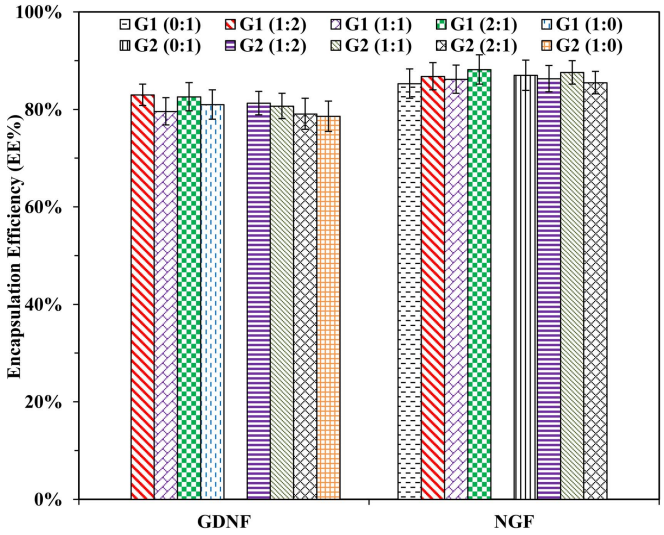

Figure 6. Encapsulation efficiency of NGF and GDNF in mono- and bicomponent fibrous scaffolds.

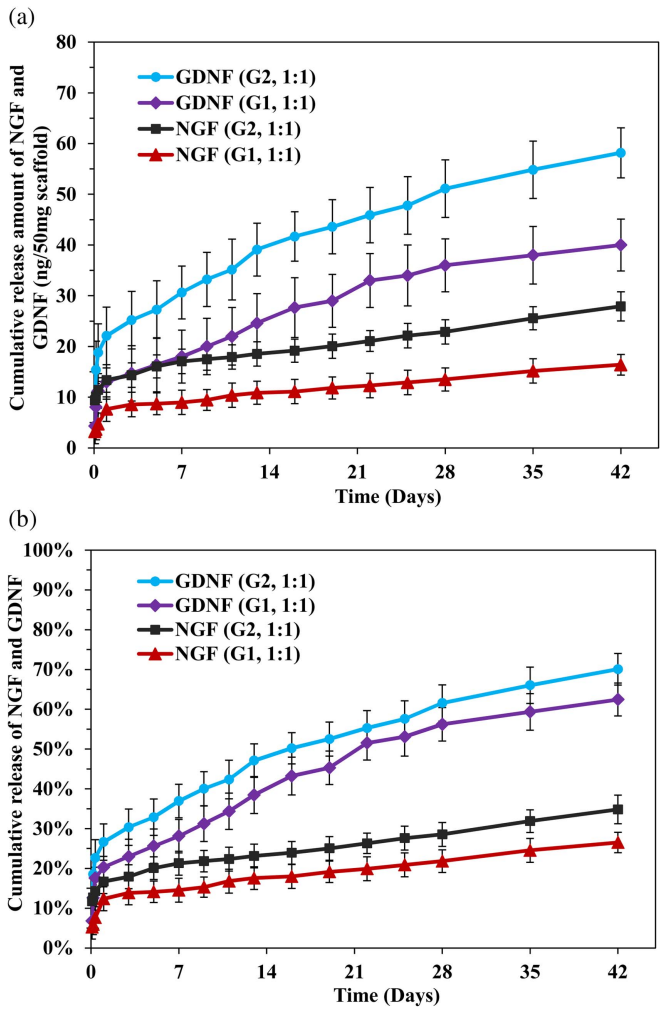

Figure 7. *In vitro* release of growth factors from bicomponent scaffolds with 1:1 component ratio of GDNF/PLGA to NGF/PDLLA fibers in 42 day release tests: (a) cumulative release amount of NGF and GDNF, (b) cumulative release percentage of GFs.

and sustained release. After 42 day release, the cumulative release amount of NGF increased to 16.4 ± 2.0 ng and 27.9 ± 2.9 ng in G1 and G2 group, respectively, comparing to 40.0 ± 5.1 ng and 58.2 ± 4.9 ng of that of GDNF. The cumulative release of NGF within 1 day reached 12.4% and 16.7%

in G1 and G2, respectively, comparing to 20.3% and 26.6% of that of GDNF. After 42 days, the cumulative release increased gradually to 26.5% and 34.8% in G1 and G2, respectively. As for GDNF, the cumulative release ascended steadily, reaching 62.5% and 70.1% in G1 and G2, respectively, after 42 days. The results of *in vitro* release tests revealed that concurrent and sustained release of NGF and GDNF could be successfully achieved using bicomponent scaffolds made by DSDP-ES. The release rate of GDNF was much faster than that of NGF. Thicker fiber diameter could help alleviate the initial burst release and slow down the release rate of both growth factors.

3.4. Bioactivity assay of incorporated GFs

In order to assess the bioactivity of GFs incorporated in fibrous scaffolds, morphology of PC12 cells cultured with different neurotrophic factor medium was examined by CLSM at different culture time and is shown in figure 8. GDNF-release group and NGF-release group represented that cells were cultured with medium containing GDNF and NGF released from their corresponding monocomponent scaffolds. In GDNF-control and NGF-control groups, virgin GDNF and NGF were used, respectively.

As shown in figure 8, cells adhered and spread well, showing a spindle-like shape on glass slides in all groups at day 1. With the stimulation of GDNF, cells exhibited with neurite sprouting and improved neurite elongation in random directions as neurite protrusions were evident by observing morphology of F-actin filaments (stained with phalloidin in green) in GDNF-release group at day 4. Weak signals of intracellular neurofilaments (stained in red) were also noticed. At day 7, more polarized cells bearing neurite outgrowth and further elongated neurite protrusions were found. Neurite branching was not observed. Cells in GDNF-control group behaved similarly as neurite sprouting and elongation was found at day 4 and enhanced neurite outgrowth was also observed at day 7. Similar results were found in NGF-release group and NGF-control group. The addition of released NGF and virgin NGF both promoted neurite outgrowth and cell differentiation, although the NGF concentration used was half of GDNF concentration.

To quantify the bioactivity of GFs and their effects on cells, neurite outgrowth and neural differentiation percentages were determined and are shown in figure 9. Neurite length was defined as the distance between cell body and distant end of neurite protrusion. Only cells bearing neurite protrusions longer than 28 μm were counted as differentiated cells. Normalized neurite length is defined as average neurite length of both differentiated and undifferentiated cells in which the neurite length of undifferentiated cells is assumed as 28 μm. At day 4, cell differentiation percentages increased to 58.8% and 70.3% in GDNF-release group and GDNF-control group, respectively, as compared to 68.0% and 72.1% in NGF-release group and NGF-control group, respectively. At day 7, cell differentiation percentages further increased to 86.7% and 96.0% in GDNF-release group and GDNF-control group, respectively, as compared to 84.6% and 94.1% in NGF-release group and NGF-control group, respectively. In control group without the addition of growth factors, cell differentiation percentages barely changed and maintained at very low level. Neurite length of cells in different groups is shown in figure 9(b) and the dotted line insert represents neurite length at the level of 28 μm. In GDNF-release group, the average neurite length was 36.3 μm and 67.2 μm at day 4 and 7, respectively. In comparison, the average neurite length was 40.3 μm and 76.0 μm at day 4 and 7 in GDNF-control group. The average neurite length was 38.2 μm and 59.4 μm in NGF-release group, corresponding to 41.2 μm and 68.2 μm in NGF-control group at day 4 and 7, respectively. There was no change in the average neurite length in control group without addition of growth factors. These results revealed that released GDNF and NGF both remained bioactive to a large extent and promoted neural differentiation, although the bioactivity of released growth factors was marginally lower as compared to the bioactivity of virgin growth factors.

3.5. *In vitro* degradation behavior of growth factor-incorporated scaffolds

The *in vitro* degradation tests of scaffolds with various compositions were conducted. The weight losses of scaffolds were monitored in the tests and results are shown in figure 10. The remaining weight of each sample decreased as immersion time increased. After 6 weeks, the weight loss of each sample was 9.4%, 16.4%, 18.2%, 21.2% and 44.0% in G1 group (GDNF/PLGA fiber to NGF/PDLLA fiber component ratio increased from 0:1 to 1:0) and 9.2%, 16.3%, 22.0%, 24.0% and 39.5% in G2 group correspondingly. The GDNF/PLGA monocomponent scaffolds degraded much faster than the other ones and the NGF/PDLLA monocomponent scaffolds had slowest degradation. The degradation rate of bicomponent scaffolds increased with the increase of GDNF/PLGA fiber to NGF/PDLLA fiber component ratio. The degradation profile was also affected by diameter of fibers in scaffolds. Generally, the degradation rate of scaffolds with larger fiber diameter (G1 group) was lower than that of scaffolds with smaller fiber diameter (G2 group). The weight loss of monocomponent PLGA scaffold in G1 group was lower than that in G2 group in the first 3 weeks; however, it exceeded that in G2 group in the subsequent period of degradation tests.

The morphology and structure of fibers and scaffolds at 2, 4 and 6 week degradation times were examined and are shown in figure 11. In the first 4 weeks,

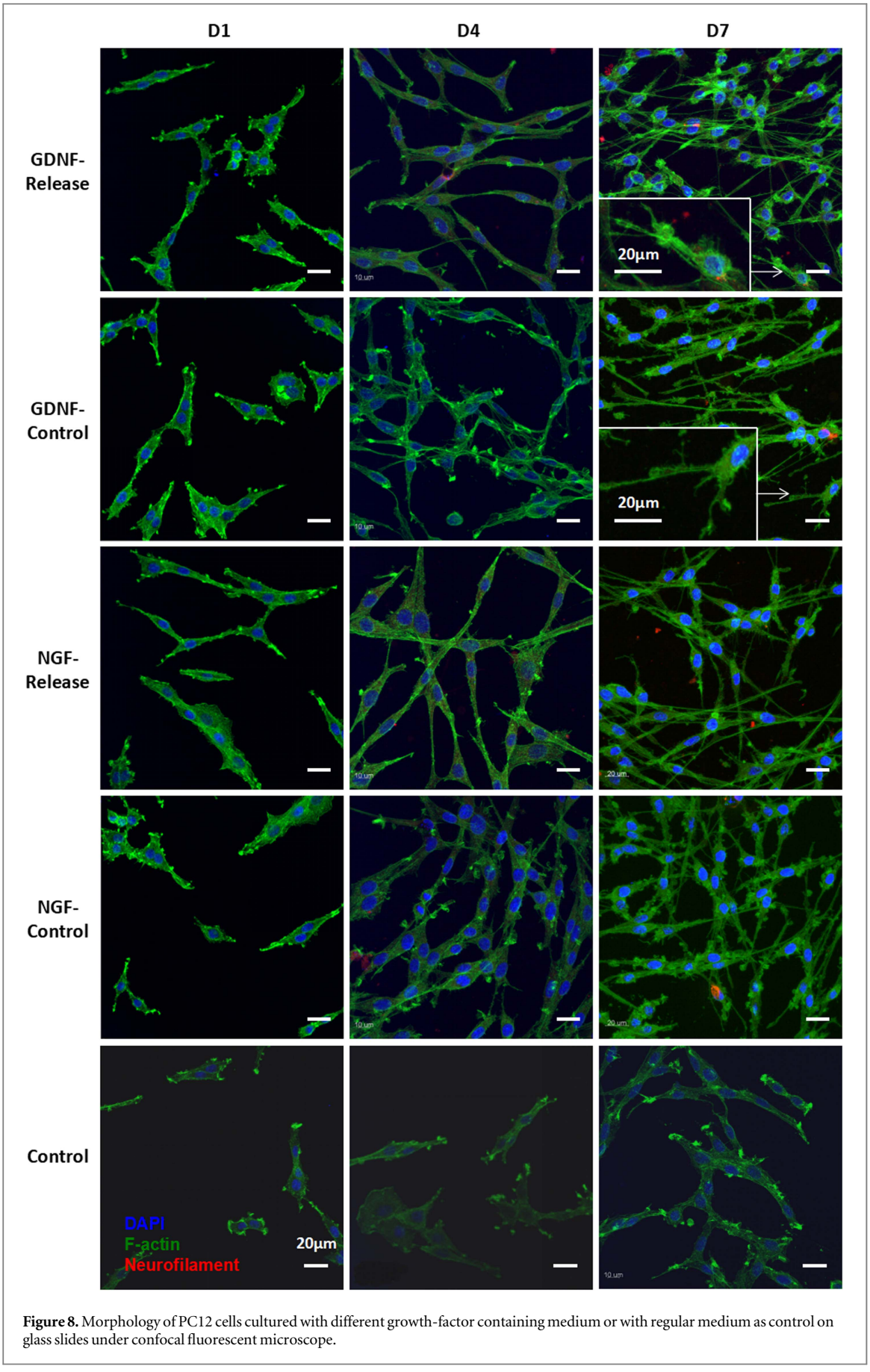

Figure 8. Morphology of PC12 cells cultured with different growth-factor containing medium or with regular medium as control on glass slides under confocal fluorescent microscope.

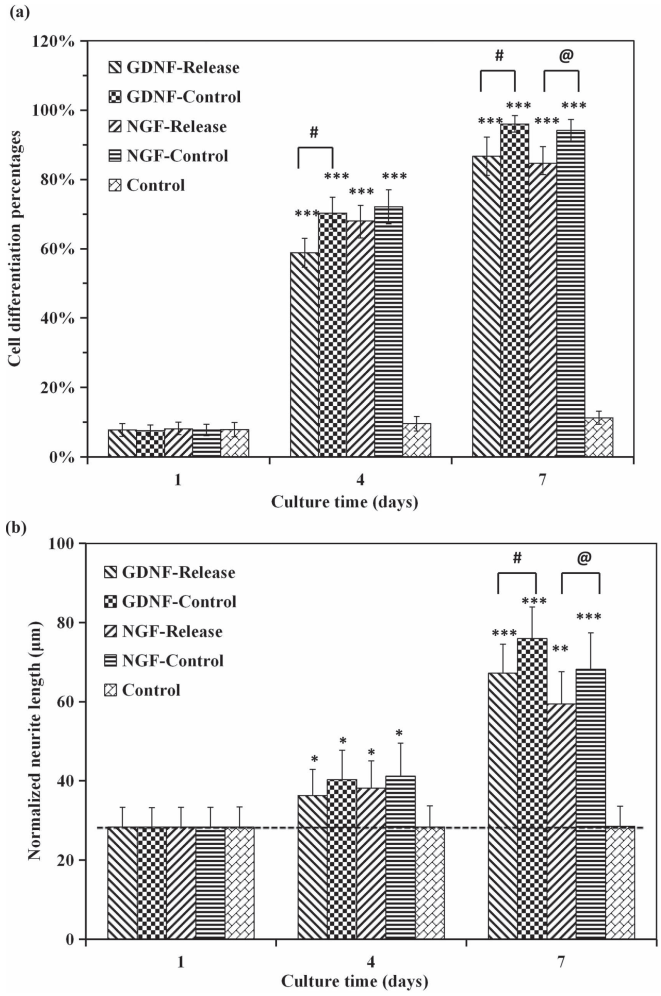

Figure 9. Bioactivity assessment of released NGF and GDNF comparing with virgin GFs: (a) differentiation percentages of PC12 cells, (b) normalized neurite length of PC12 cells. (*, **, ***) Cell differentiation percentage and normalized neurite length in groups with GFs statistically different than that in control group for respective culture days (*$= p < 0.05$, **$= p < 0.01$, ***$= p < 0.005$); (#) cell differentiation percentage and normalized neurite length in GDNF-release groups statistically different than that in GDNF-control group for respective culture days (#$= p < 0.05$); (@) cell differentiation percentage and normalized neurite length in NGF-release groups statistically different than that in NGF-control group for respective culture days (@$= p < 0.05$).

monocomponent GDNF/PLGA scaffolds in G1 group maintained fibrous structure and mainly underwent the process of fiber swelling and slight fiber fusion, followed by fiber erosion. In the subsequent 2 weeks, the scaffolds degraded much faster with scarcely any intact fiber being observed, generating large pores and abundant fragments. In comparison, monocomponent GDNF/PLGA scaffolds in G2 group mainly underwent the process of fiber swelling, fiber recoiling, fiber fusion and erosion; and fibrous structure could be hardly noticed after 4 weeks. Monocomponent NGF/PDLLA scaffolds behaved dramatically differently, which underwent the process of fiber swelling and slight fiber fusion and remained intact. In bicomponent scaffolds, GDNF/PLGA and NGF/PDLLA components could be easily differentiated. At long degradation times, GDNF/PLGA fibers lost fibrous morphology while NGF/PDLLA fibers still maintained fibrous morphology.

4. Discussion

Repairing severe peripheral nerve defects necessitates the development of superior nerve guide conduits. Scaffold-based nerve guide conduits loaded with GFs have attracted great attention [20, 25, 26]. Sustained delivery of GFs from elaborately designed scaffolds would be beneficial considering the short half-life of GFs. For nerve tissue regeneration, NGF and GDNF have been widely employed owing to their potent effect on neural responses and cell fate. In the current investigation, NGF and GDNF were incorporated in bicomponent fibrous scaffolds through DSDP-ES and sustained and controlled release of both GFs was achieved.

4.1. Structure and properties of mono- and bicomponent scaffolds

Electrospinning and as-spun scaffolds have been widely employed in tissue engineering due to structural similarity

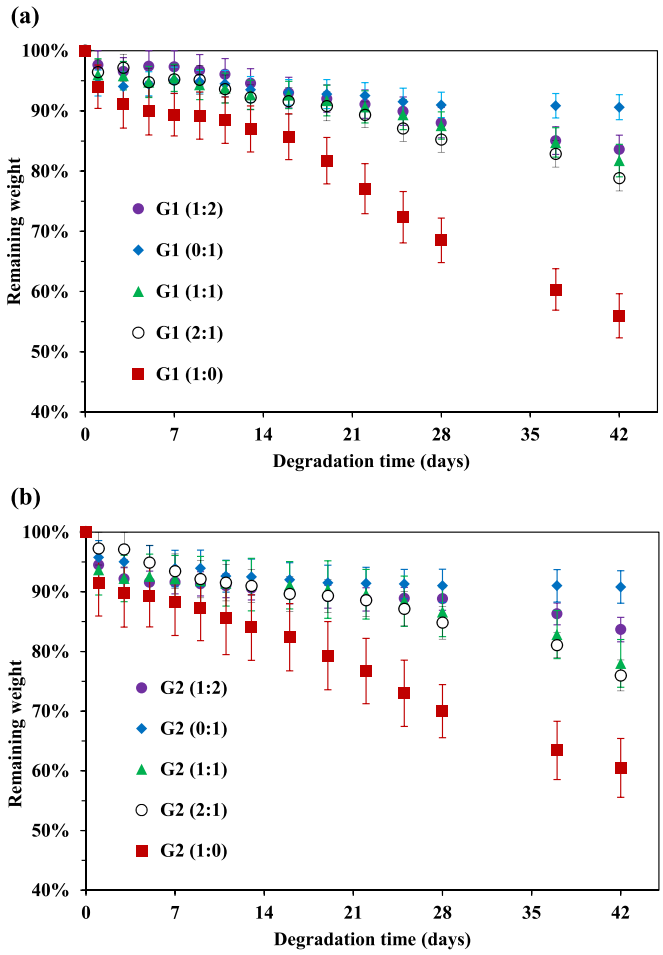

Figure 10. Weight loss of mono- and bicomponent scaffolds during 42 day degradation tests: (a) G1 group, (b) G2 group.

of as-spun scaffolds with ECM of human body tissues, excellent cell response and capability of delivering functional biomolecules in a controlled manner. Scaffolds with different morphology and structures for diverse applications can be achieved through various electrospinning techniques. Core–shell structured fibers produced by emulsion electrospinning can be employed as delivering vehicles of subtle biomolecules, such as GFs. In the current study, robust GDNF/PLGA and NGF/PDLLA electrospun fibers with smooth surface were made. The formation of beadless fibers indicated process stability during emulsion electrospinning. Core–shell structure in fibers was confirmed in both GDNF/PLGA fibers and NGF/PDLLA fibers (figure 2(b)) with discontinuous growth factor-contained water phase pockets being the core and polymer matrix forming the shell. The evolution and formation of core–shell structure could be explained by the 'evaporation and stretching induced de-emulsification' mechanism which was proposed by Xu *et al* [14]. During emulsion electrospinning, the spherical emulsion droplets were first stretched into elliptical shape in the fiber direction. The elliptical droplets could break up into smaller water droplets due to Rayleigh/capillary instability, depending on viscosity difference and interfacial tension, etc. Due to faster evaporation of solvent than water, the viscosity of polymer solution increased more rapidly than that of water droplets. The viscosity gradient from the surface to the center resulted in the inward movement of emulsion droplets. Thus, the coalescence of water droplets towards the central region occurred along with the solidification of shell layer, resulting in a core–shell structure for fibers. Interestingly, the core–shell structure in the aforementioned two types of fibers looked different. A distinctive phase boundary between the core and the shell was delineated in NGF/PDLLA fiber. However, ambiguous phase boundary could only be observed in GDNF/PLGA fiber. This phenomenon might be attributed to different hydrophilicity/hydrophobicity of polymer matrix. It was well known that PDLLA was more hydrophobic than PLGA, leading to more apparent phase-separation during de-emulsification. The features of core–shell structure including core to shell ratio and core continuity could be influenced by a variety of process parameters and would significantly affect the release profile of incorporated biomolecules.

Concentration of polymer solution was one of the key electrospinning parameters and utilized herein to control the diameter of as-spun fibers. Higher concentration of polymer solution would result in thicker fiber diameter due to difficulty in jet stretching during electrospinning caused by higher viscosity. Monocomponent fibers with distinctive average fiber

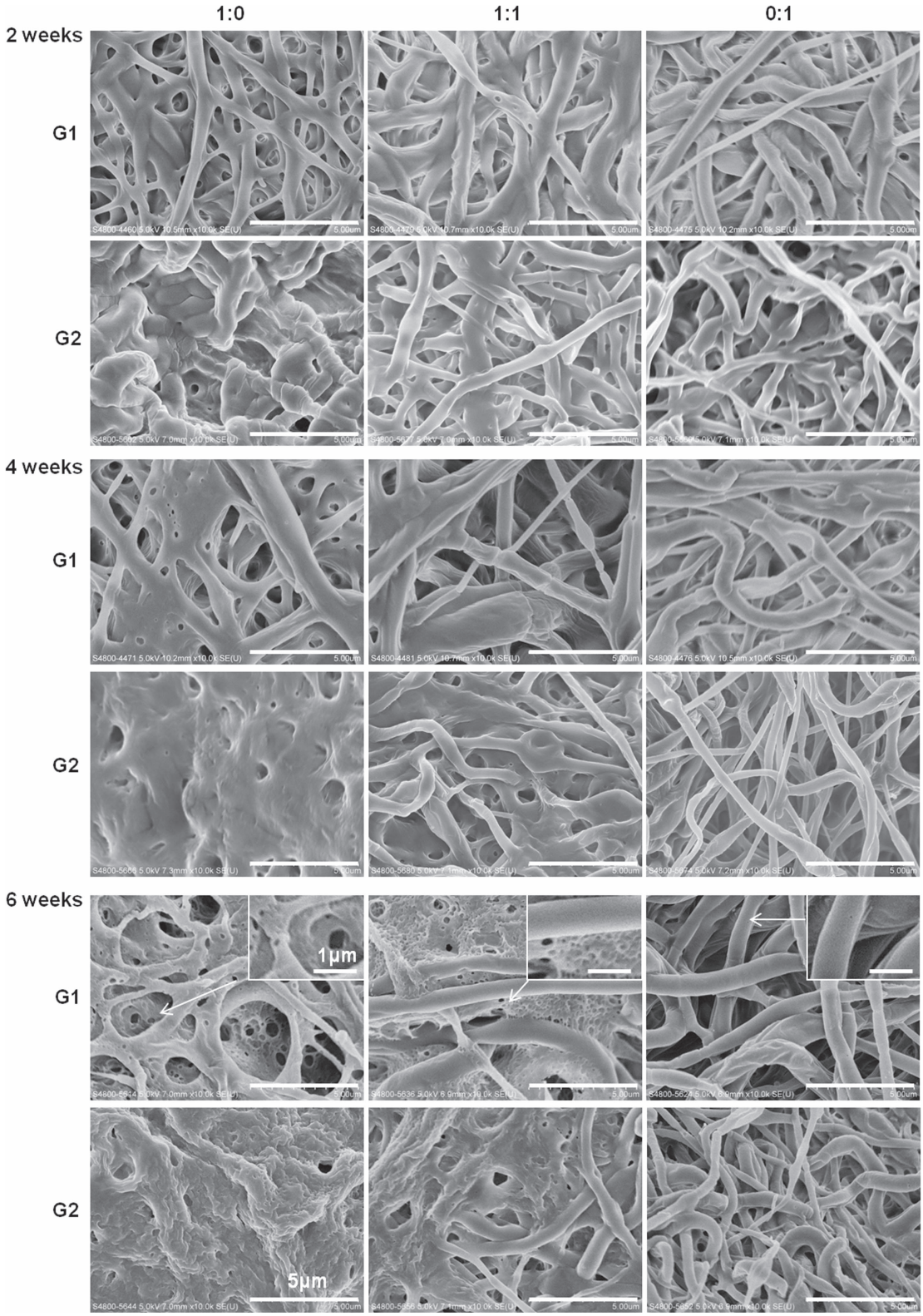

Figure 11. SEM images of scaffold samples collected after 2, 4 and 6 weeks of degradation.

diameter were obtained by using different polymer concentration for both PLGA and PDLLA. Bicomponent scaffolds with varying component ratios were constructed using these monocomponent fibers. The average fiber diameter of bicomponent scaffolds could be assumed as a weighted average of each component. The result in figure 3 showed that the average fiber diameter decreased approximately proportionally with the increase of GDNF/PLGA fiber to NGF/PDLLA fiber component ratio, which correlated well with the assumption. The feasibility of utilizing bicomponent strategy to produce multifunctional electrospun scaffolds has been demonstrated by quite a few studies. Wang *et al* fabricated multifunctional

electrospun scaffolds using PLGA and PDLLA for bone tissue engineering [22]. Bicomponent scaffolds with controlled and tunable mass ratios of rhBMP-2/PDLLA fibers to Ca–P/PLGA fibers were achieved by employing DSDP-ES and multiple syringe strategy. Bonani *et al* also used similar strategy and constructed gradient micropatterns of electrospun fibers through programming the deposition of poly (ε-caprolactone) (PCL) and PLGA fibers and achieved spatiotemporal release of biomolecules incorporated [27]. Their results indicated that the produced scaffolds had a closely matching composition as designed and spatiotemporal release of biomolecules was obtained accordingly. Based on these studies and also work in this project, it may be concluded that designed composition of bicomponent scaffolds was effectively achieved by employing DSDP-ES. The component ratio and average fiber diameter of bicomponent scaffolds herein could be controlled through DSDP-ES.

The monocomponent GDNF/PLGA scaffolds had much lower WCA than monocomponent NGF/PDLLA scaffolds, which could be attributed to less hydrophobicity of PLGA polymer and involvement of surfactants. The glycolic acid group of PLGA and amphiphilic surfactants partially exposed on surface of fibers could improve the spread and ingression of water and hence improved the wettability of scaffolds. The wettability of bicomponent scaffolds was affected by the NGF/PDLLA fiber content. The results showed that PDLLA fibers played a dominant role in wettability of bicomponent scaffolds as all of them had similar wettability to the monocomponent PDLLA electrospun scaffolds. The similar phenomenon was observed by other research group [28]. They fabricated electrospun hybrid scaffolds composed of PCL fibers and gelatin fibers and found that the wettability of hybrid scaffolds was affected by PCL content due to the high hydrophobicity of PCL. The produced hybrid scaffolds had similar wettability to electrospun PCL mats. The wettability of electrospun scaffolds is a complicated issue and hard to define to some extent due to their surface roughness and void space occupied by air. However, it plays an important role in cell-scaffold interaction including cell adhesion and proliferation. There is no general consent about the optimum wettability as specific wettability might be favored by different cell types. The wettability of scaffolds might even play diverse roles in cell response. Hakamada *et al* fabricated electrospun scaffolds using biodegradable polymer poly (γ-benzyl-L-glutamate) and modified their wettability through alkali-treatment [29]. They found that an increase in wettability of scaffolds decreased adhesion of endothelial cells but increased cell spreading.

It was found that the UTS, EM and EB increased with the increase of GDNF/PLGA fiber to NGF/PDLLA fiber component ratio in the bicomponent scaffolds. The result revealed that PLGA was tougher and more ductile than PDLLA, which was consistent with existing knowledge. It was believed that flexibility of glycolic acid chain segment in polymer backbone influenced the crystallinity and improved the mechanical properties of PLGA fibers, while the rigidity of lactic acid chain segment and racemization in PDLLA polymer backbone compromised their mechanical properties. Fiber diameter also played an important role in mechanical properties of fibrous scaffolds. Thinner fiber diameter resulted in lower UTS, EM and less EB as shown in figure 5. These results demonstrated that the bicomponent strategy combining with control of fiber diameter was an effective way to produce fibrous scaffolds with tunable mechanical properties. Moreover, the UTS and EM were similar with or of the same order of magnitude from those of natural soft tissues as well as those of organs consisted of soft connective tissues, suggesting that produced fibrous scaffolds were promising for soft tissue regeneration.

4.2. Encapsulation of GFs and their *in vitro* release

Combination of transient and prolonged delivery of GFs is normally desirable for short- and long-term peripheral nerve regeneration [30, 31]. Electrospinning has been employed as a versatile technique to fabricate delivery vehicles for various drugs and biomolecules [32, 33]. Concerns about potential denaturation of subtle biomolecules such as proteins and growth factors due to hydrophobic interaction with organic solvent in conventional electrospinning have advanced the development of coaxial electrospinning and emulsion electrospinning. Electrospinning of water-in-oil emulsions is particularly advantageous for the delivery of hydrophilic drugs and proteins over other techniques. For example, delivery of hydrophilic drugs and proteins by microspheres fabricated by double emulsion methods often renders relatively low EE. During emulsion electrospinning, hydrophilic proteins are preserved in water phase core and their bioactivity could be retained to a large extent. The EE of NGF and GDNF in electrospun fibrous scaffolds reached to an average of 86.6 $\pm$ 1.0% and 80.7 $\pm$ 1.6%, respectively.

In this investigation, by incorporating GFs into polymers with different degradation rates and tuning component ratios in bicomponent scaffolds through DSDP-ES, dual and controlled release of NGF and GDNF was achieved. Following an initial burst release in the first 24 h, sustained release of NGF and/or GDNF from corresponding fibers was found during the release period. The initial burst release was attributed to the growth factor-contained minute reservoirs formed on or close to the surface of fibers during emulsion electrospinning. As discussed in the evolution of core–shell structure, the majority of stretched water droplets moved inward coalesced in the central region and formed the water-phase core. However, a minority of water droplets were detained while the outer layer solidified due to rapid evaporation of

organic solvent. These tiny water droplets dwelling on or near the surface of fibers served as reservoir of growth factors and rendered initial burst release through diffusion when the scaffolds were immersed in testing liquid. After the initial burst release, sustained release was obtained which was attributed to either diffusion of growth factor from interior of fibers to immersion liquid through the polymer matrix of fibers or a combination of diffusion and polymer matrix degradation.

Comparing the release profiles including cumulative release amount and cumulative release percentage of growth factors, it could be seen that biomedical polymers with different wettability and degradation rates could be leveraged to tailor the release behavior of incorporated growth factor. NGF and GDNF were herein incorporated into PDLLA fibers and PLGA fibers, respectively, to obtain different release profile as PLGA was less hydrophobic and had much faster degradation rate than PDLLA. The release rate of GDNF was therefore much faster than that of NGF. Average diameter of electrospun fibers in scaffolds was also a useful control variable to manipulate the release profile of growth factors including the initial burst release and subsequent sustained release. It was not difficult to speculate that disparate mechanisms might dominate the release of respective growth factor over different time periods. Both diffusion and matrix erosion contributed to release kinetics of protein from biodegradable polymer matrix such as PLGA and PDLLA. With the consideration of surface erosion and bulk erosion of biodegradable polymer matrix in different situations, polymer degradation would become more complicated [34, 35]. Although there is no perfect model for modeling the release behavior of NGF or GDNF for bicomponent scaffolds, the results in this study did demonstrate that concurrent and sustained release of NGF and GDNF with tunable amounts and ratios could be achieved.

4.3. Bioactivity of incorporated GFs

There is concern about bioactivity loss of biomolecules in electrospun fibers due to their potential deactivation and denaturation which might occur during solution preparation, electrospinning at high voltage, freeze-drying and sample sterilization [6, 36, 37]. Quite a few reports demonstrated that protein released from electrospun scaffolds could induce various cellular responses, indicating that protein activity was at least partially preserved during electrospinning [5, 38, 39]. Emulsion electrospinning and coaxial electrospinning techniques could help preserve the bioactivity of vulnerable biomolecules and provide delivery of these biomolecules in a controlled manner [40–43]. In this study, the bioactivity of GDNF and NGF released from GDNF/PLGA fibers and NGF/PDLLA fibers, respectively, were assessed on a culture-to-culture basis. Both released growth factors induced much neurite outgrowth and neural differentiation of PC12 cells (figures 8 and 9), although much lower concentration of NGF was used. The degree of neural differentiation and neurite outgrowth of PC12 cells induced by released GDNF and NGF both decreased as compared to those induced by virgin GDNF and NGF, indicating that there was partial bioactivity loss for both GDNF and NGF released from GDNF/PLGA fibers and NGF/PDLLA fibers, respectively. However, the degree of neural differentiation induced by released GDNF and NGF only decreased 9.7% and 10.1% as compared with that induced by virgin GDNF and NGF, respectively, at day 7. These results revealed that the bioactivity of GDNF and NGF in GDNF/PLGA fibers and NGF/PDLLA fibers, respectively, were both preserved to a very large extent, indicating that emulsion electrospun fibers were robust delivery vehicles for hydrophilic proteins.

4.4. *In vitro* degradation of mono- and bicomponent scaffolds

Polyesters such as PLGA and PDLLA have been widely employed for controlled release vehicles owing to their excellent biocompatibility and biodegradability. The monomers generated during hydrolytic degradation of PLGA and PDLLA are easily metabolized. *In vitro* degradation of polymer-based scaffolds could be influenced by a variety of factors, including polymer properties, scaffold structure, immersed medium environment, etc. In this investigation, these two types of polymer with different degradation rates (1–2 months for PLGA, 12–14 months for PDLLA) were employed to construct fibrous scaffolds with the aim of modulating the degradation behavior of scaffolds and hence release behavior of incorporated GFs. Electrospun scaffolds with various component ratios were fabricated taking the advantage of DSDP-ES and multiple syringe strategy as described before. As shown in figure 10, the weight loss of PDLLA monocomponent scaffolds was small as compared to the rest of tested scaffold samples. Weight loss of all scaffolds containing PLGA component increased as the degradation test proceeded, which also augmented with the increase of component ratio of GDNF/PLGA fiber to NGF/PDLLA in bicomponent scaffolds. The scaffolds composed of fibers with different diameters exhibited similar degradation profiles except monocomponent GDNF/PLGA scaffolds. Weight loss rates were nearly constant within first 2 weeks for GDNF/PLGA scaffolds in both G1 and G2 groups, although the weight loss rate of G2 scaffolds was slightly higher than that of G1 scaffolds. Within 2 to 4 weeks, an unconspicuous increase of weight loss rate was noticed in G2 group. In comparison, a sharp increase of weight loss rate was observed in G1. The above results allowed one to affirm that fibrous NGF/PDLLA scaffolds produced by emulsion electrospinning did not degrade during the 42 day experiment considering the much longer

degradation period of PDLLA. Hydrolytic degradation of fibrous scaffolds based on PLGA was confirmed and autocatalytic hydrolysis was believed to contribute in light of the evidence of degradation acceleration. Different mechanisms of degradation were thought to dominate the degradation behavior of PLGA scaffolds with different fiber diameters.

Morphology of fibrous scaffolds collected at different times of immersion in PBS as shown in figure 11, was studied to help further investigate the degradation mechanism. Within first 2 weeks, fibers became swollen and fused for both GDNF/PLGA and NGF/PDLLA scaffolds. Due to its better hydrophilicity, the swelling and fusion phenomenon was more obvious in PLGA scaffolds than PDLLA scaffolds. Water took over the air void when samples were impregnated in immersion medium. The morphology of scaffolds in terms of pore closure and fibrous network evolved dynamically with the fiber swelling and welding due to fiber relaxation and polymer chain mobility. After 2 weeks, the fibrous morphology of PDLLA scaffolds barely changed. Even after 6 weeks, the integrity of PDLLA fibers remained intact, although very small amount of nanopores were observed on the surface of a few fibers (G1 (0:1)). The observation was completely different for PLGA fibrous scaffolds. After 2 weeks, abundant pore closure and network collapse were observed with a gradual disappearance of fibrous morphology, which was more obvious in scaffolds with thinner fibers. After 6 weeks, the fibrous morphology was completely lost, generating fiber fragments and many micro- and nanopores in the scaffold (G1 (1:0)). In sharp contrast, a rough membrane was produced without any retrievable fiber in scaffold G2 (1:0).

It is well known that more surface area and hence more reaction sites will promote the hydrolysis of PLGA which can also be auto-catalyzed by its carboxylic terminal degradation products. The enrichment of acidic degradation products-oligomers locating well within fiber matrix could exist, leading to bulk erosion, as only soluble oligomers near the surface are able to leach out. As discussed in the evolution of core–shell structure in emulsion electrospun fibers, the water phase in emulsion electrospun fibers tended to coalesce inwards, leaving a shell layer of polymers. The formed polymer shell could provide a barrier for the oligomers to leach out. Thinner fibers with less solid polymer shell provided less resistance for the diffusion of oligomers and experienced alleviated bulk erosion. These might help explain the differences in degradation profiles and morphology in G1 (1:0) and G2 (1:0) scaffolds. More reaction sites due to more surface area of G2 (1:0) scaffolds produced a faster degradation rate as compared to G1 (1:0) scaffold in the first phase of degradation. The autocatalytic hydrolysis during bulk erosion of G1 (1:0) scaffold greatly accelerated its degradation rate in the second phase of degradation. The degradation mechanisms would significantly influence the release profile of incorporated biomolecules.

5. Conclusions

Mono- and bicomponent electrospun fibrous scaffolds were fabricated through emulsion electrospinning and DSDP-ES technique. The discontinuous core–shell structures were formed in both NGF/PDLLA fibers and GDNF/PLGA fibers, with growth factor-contained water phase being the core. Both NGF and GDNF were successfully incorporated with relatively high EE above 80%. Bicomponent scaffolds with different fiber component ratios were constructed. The study demonstrated that *in vitro* release of GFs and *in vitro* degradation of scaffolds could be tuned by varying factors including polymer matrix, fiber diameter and fiber component ratio. The *in vitro* release and degradation mechanisms were also thoroughly investigated, suggesting that diverse degradation mechanisms might dominate the degradation behaviors of different systems and consequently influence the release behaviors of GFs incorporated. We believe the bicomponent scaffolds, capable of providing sustained and tunable release of NGF and GDNF and adjustable degradation profile, will benefit the optimization of the synergetic effect between NGF and GDNF and hold great promise in peripheral nerve tissue repair.

Acknowledgments

This work was supported by the Hong Kong Research Grants Council through a GRF grant (HKU 718109E). Assistance provided by M Wang's group and technicians in Department of Mechanical Engineering, HKU, is acknowledged.

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