Biomaterials 35 (2014) 6268–6277

Contents lists available at ScienceDirect

Biomaterials

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

Myotube formation on gelatin nanofibers – Multi-walled carbon nanotubes hybrid scaffolds

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ARTICLE INFO

Article history:
Received 9 February 2014
Accepted 6 April 2014
Available online 13 May 2014

Keywords:
Myotube
Gelatin fibers
Electrospinning
Carbon nanotubes
C2C12

ABSTRACT

Engineering functional muscle tissue requires the formation of densely packed, aligned, and mature myotubes. To enhance the formation of aligned myotubes with improved contractibility, we fabricated aligned electrospun gelatin multi-walled carbon nanotubes (MWNTs) hybrid fibers that were used as scaffolds for the growth of myoblasts (C2C12). The MWNTs significantly enhanced myotube formation by improving the mechanical properties of the resulting fibers and upregulated the activation of mechanotransduction related genes. In addition, the fibers enhanced the maturation of the myotubes and the amplitude of the myotube contractions under electrical stimulation (ES). Such hybrid material scaffolds may be useful to direct skeletal muscle cellular organization, improve cellular functionality and tissue formation.

1. Introduction

Skeletal muscle tissue engineering (SMTE) aims to fabricate or to regenerate muscle tissues that have been damaged or lost some of their intrinsic functionalities [1]. SMTE also has potential applications in cell-based assays [2,3], biorobotics [4], biosensing [5], and energy harvesting [6]. To engineer a functional skeletal muscle tissue, myoblasts must be aligned and cultured on a scaffold to mimic the natural microenvironment [7]. In vivo, the cellular environment comprises the extracellular matrix (ECM), the neighboring cells, and various growth factors and signaling molecules. The ECM provides mechanical support to cells and spatiotemporally regulates many of the biochemical signals needed for cell proliferation, migration, differentiation, and tissue organization [1]. The ECM is a three-dimensional (3D) highly hydrated viscoelastic network formed by various proteoglycans, glycosaminoglycans, and fibrillar proteins with nanoscale structures, which determine the cell–matrix interactions. For example, collagen fibrils, which are one of the most important components of the ECM, are approximately dozens of micrometers long and between 260 and 410 nm wide [8]. One approach to align cells and to mimic the ECM *in vitro* is to use aligned nanofibers [9]. Numerous synthetic or natural polymers have been electrospun into nanofibrous scaffolds

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http://dx.doi.org/10.1016/j.biomaterials.2014.04.021
0142-9612/

for tissue engineering applications [10]. Among them, gelatin, which is derived from native collagen by acidic or basic partial hydrolysis, has been extensively studied for its biodegradable, biocompatible and cell supportive properties [11]. Gelatin is also well suited for tissue engineering applications because of its biodegradability, biocompatibility, and low cost [11]. Although hydrogels such as collagen and its denatured form, gelatin, offer an excellent environment for cellular growth, they usually exhibit weak mechanical properties and poor electrical conductivity. Such properties limit the application of gelatin hydrogels in regulating the cellular activity of electroactive cells such as skeletal muscle cells [12]. Recent studies using hybrid-hydrogels have demonstrated that the inclusion of nanomaterials can improve the mechanical, biological, and electrical properties of these biomaterials [13]. We previously demonstrated that the inclusion of carbon nanotubes (CNTs) in methacrylated gelatin (GelMA) hydrogels improved the mechanical properties of the hydrogel and conductivity of the hydrogels [14]. However, such hydrogels did not contain the nanoscale architecture of natural ECM, which may hinder bioactivity. Therefore, combining the nanotopographical cues of aligned gelatin fibers, the viscoelasticity and hydration of a gelatin hydrogel, and the thermal, mechanical and electrical properties of MWNTs may improve SMTE.

In this report, we investigated MWNT-gelatin hybrid fibers in myotube formation by comparing 20% gelatin fibers with 20% gelatin fibers combined with 0.5 mg/ml and 5 mg/ml MWNTs. These fibers were fabricated by electrospinning gelatin solutions in pure water with or without MWNTs by warming these polymer solutions through a glass chamber. The resulting nanofibers were characterized by field emission scanning electron microscopy (FE-SEM). In addition, the effects of the gelatin concentration, the voltage used during electrospinning, the temperature of the polymer solutions, and the crosslinking of the fibers by glutaraldehyde (GTA) were studied prior to use in cell culture.

2. Materials and methods

2.1. Materials

Type A gelatin from porcine skin, and penicillin/streptomycin (P/S) were purchased from Sigma–Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM), essential amino acids solution (MEM), non-essential amino acids solution (MEM-NEAA), Dulbecco's phosphate buffer saline (DPBS) and horse serum (HS) were purchased from Gibco (USA). Fetal bovine serum (FBS) was purchased from BioWest (USA). Hepes was purchased from Dojindo (Japan). GTA was purchased from Kanto-Chemical (Tokyo). The 35-mm dishes were purchased from Greiner Bio-One (Germany). The electrospinning set-up consisted of a DC high voltage power supply (Max Electronics, AMK-30 K06PBX, Tokyo), a syringe pump (World Precision Instruments, Aladdin syringe pump, Sarasota, FL), and a glass chemical cooler (on request).

2.2. Gelatin fiber fabrication

Gelatin was dissolved in Mili-Q water at 60 °C and loaded in a 1 ml plastic syringe, which was placed in a glass chamber that was used as warmer and has been custom-designed to fit a 1 ml syringe (Terumo/SS-01T). A 20-gauge steel needle (Hoshiseido, Tokyo) was used as spinneret. To warm the needle, it was fixed through the bottom of a 1.5 ml tube (Eppendorf/0030 125150), which was used as a reservoir, using a drop of polydimethyl siloxane (PDMS) and cured at 70 °C for 90 min (Fig. 1A). The gelatin solution was delivered at a flow rate of 10 μl/min, and the gelatin fibers were collected under a tension of 18 kV on aluminum foil, which was used as a counter electrode and placed 8 cm from the spinneret.

2.3. Crosslinking of gelatin fibers and sterilization

The gelatin fibers were taped in 2 ml dishes and crosslinked overnight in GTA vapor. The fibers were then rinsed in Mili-Q water for 2 days at room temperature. Before their use in experiments, the fibers were sterilized under UV light on a clean bench for 10 min.

2.4. Characterization of the gelatin nanofibers

The morphology of the gelatin fibers was examined by FE-SEM after 30 s of tungsten metallization in a sputter coater (JEOL/JFC1600/Tokyo) at 10 mA. The fiber diameters were measured from FE-SEM images with the AxioVision Rel. 4.8 software package by analyzing 100 individual fibers.

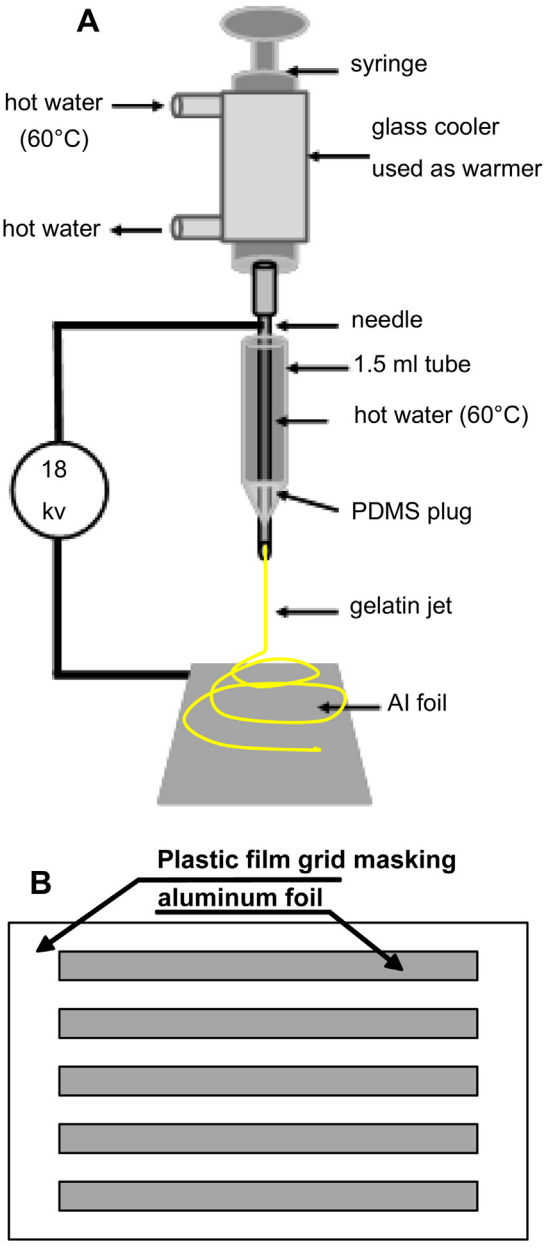

Fig. 1. Schematic of the electrospinning set up. (A) Glass chamber was used to warm the syringe, and a 1.5 ml Eppendorf tube was fixed on the needle to warm the needle at 60 °C. (B) Schematic showing the counter electrode used to produce aligned gelatin fibers.

2.5. Cell culture and cell seeding

Murine C2C12 myoblast cells (between passages 5 and 8) from American Type Culture Collection (ATCC) were cultured in DMEM supplemented with 10% FBS, 1% P/S, and 20 mM Hepes in a humidified incubator at 37 °C and 5% CO_2. At ~70% confluency, the cells were harvested by trypsinization, sub-cultured, or used for experiments. A differentiation medium composed of DMEM supplemented with 2% HS, 1% P/S, and 20 mM Hepes was used to induce myotube formation. Cells were seeded on the fibers at 1×10^5 cells/cm^2 and left for 30 min in the incubator at 37 °C to attach. The fibers were then gently rinsed to remove the non-adherent cells; 2 ml of warm culture medium was added to begin the cell culture.

2.6. Myotube length quantification

Myotubes were fixed in 4% (w/v) paraformaldehyde for 15 min, permeabilized with 0.3% (v/v) Triton X-100 for 5 min, blocked with 5% bovine

serum albumin (BSA) in DPBS for 20 min at 37 °C, then incubated at 4 °C overnight with mouse monoclonal anti-fast skeletal myosin IgG antibody (Abcam, code ab-7784, 1:1000 dilution in DPBS with 0.1% BSA). Next day, the samples were stained with Alexa Fluor 488-conjugated goat anti mouse IgG antibody (Invitrogen, code A11001, 1:1000 dilution in DPBS with 0.1% BSA) and incubated at 37 °C for 60 min. Myotubes, defined as multinucleated cells containing at least 3 cell nuclei, were quantified on day 4 of culture in the differentiation medium. The myotube length was determined using the AxioVision Rel. 4.8 software package.

2.7. Electrical stimulation

Surfaces of aligned 20% gelatin fibers with or without MWNTs and with a dimension of 1 cm × 0.5 cm were taped in a 6-well plate (Greiner Bio-one, code 657-160). After 1 day in growth medium and 4 days in differentiation medium, the cultures were subjected to 2 days of electrical pulse stimulation of 5 V at a frequency of 1 Hz and for 1 ms in stimulation medium (DMEM supplemented with 2% HS, 1% P/S, 20 mM HEPES, 1% MEM-NEAA, and 2% MEM) via an electronic stimulator (Ion Optix, C-pace EP) and 6 pairs of carbon electrodes (Ion Optix) with an electrode gap of 1.8 cm.

2.8. TEM Measurements

On a TEM grid, 20% gelatin fibers with MWNTs (0.5 mg/ml and 5 mg/ml) were electrospun for 20 s. Microstructure characterization was performed using a transmission electron microscope (JEOL JEM-2100F).

2.9. Raman spectra

A micro-Raman spectrometer (Renishaw InVia RM 1000) with an excitation laser wavelength of 632.8 nm was used to perform Raman measurements. The laser power was set at a low value to avoid sample damage.

2.10. Free-standing substrate of aligned gelatin fibers

To fabricate a counter electrode for electrospinning with a thermoresponsive sacrificial layer, a 100 mg/ml solution of poly(N-isopropylacrylamide) (pNIPAM) in 2-propanol was spin coated at 4000 rpm for 40 s on a plastic grid featuring alternating plastic bands (8 mm × 5 mm) and empty lines. This coated plastic grid was then heated at 100 °C for 90 s to remove excess solvent and was then taped on aluminum foil to create alternating aluminum and plastic bands, which served as a counter electrode to fabricate aligned nanofibers. After deposition of the gelatin fibers with or without MWNTs, the fibers were subjected to crosslinking in GTA vapor overnight, incubated for 1 day at 37 °C with warm water (37 °C) to remove any GTA residues, and sterilized for 15 min under the UV light of a clean bench before use in cell culture.

2.11. AFM Measurements

To determine the mechanical properties of hybrid fibers, nanomechanical mapping was performed with 20% gelatin with or without MWNTs (0.5 mg/ml and 5 mg/ml) in MilliQ water using atomic force microscopy (AFM) and based on force–distance curve measurements as described in our previous works [15,16]. The nanomechanical mapping was operated in force volume mode, in which force–distance curves were collected over randomly selected surface areas at a resolution of 64 × 64 pixels. All the measurements were performed on a Bruker MultiMode™ AFM with a NanoScope V controller under water. Cantilevers (OMCL-TR800PSA-1) were purchased from Olympus. The spring constant of the cantilever was determined using the thermal noise method [17]. The Derjaguin, Muller, and Toporov (DMT) theory of contact mechanics was adopted to analyze the force–distance curves that yielded the mapping of the Young's modulus on the surface of the hydrogels [18].

2.12. qRT-PCR

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on a Bio-Rad MYiQ2 two-color real-time PCR machine. Total RNA was extracted from C2C12 cells that had been cultured 1 day in growth medium, followed by 4 days in differentiation medium on 20% gelatin fibers with or without MWNTs. The isolated RNA was then subjected to reverse transcription (RT) and the complementary DNA (cDNA) was amplified by quantitative polymerase chain reaction (qPCR) in one-step, which was performed in 4 replicates using the SuperScript III Platinum SYBR green One-step kit (Invitrogen) and primers (listed in Table TS4 with PCR conditions). Gene expression was quantified by calculating $2^{-\Delta CT}$ values, where CT represents the cycle number at which an arbitrary threshold is reached and ΔCT = (CT targeted gene − CT GAPDH). The gene expression analysis was repeated at least 2 times.

3. Results and discussion

3.1. Production and characterization of gelatin fibers

Among the numerous natural or synthetic polymers that have been electrospun into nanofibrous scaffolds, gelatin has been extensively studied due to its cellular compatibility [11]. However, organic solvents such as hexafluoroisopropanol (HFIP) [19] or trifluoroethanol (TFE) [20] are often used during the fabrication of gelatin nanofibers. These solvents are cytotoxic and are difficult to completely remove due to their strong interactions with gelatin [11]. To overcome this issue, the use of less toxic solvents has been continuously researched. Different acidic solutions such as acetic acid [21] or formic acid [22] have been investigated as less toxic alternatives to organic solvents, but acidic environments induce unwanted partial degradation of gelatin. Mixtures such as water/alcohol/salt or water/acid/alcohol [23], and more recently, ethanol/phosphate buffer [11] have also been proposed. So far, the use of water alone as solvent was believed to be difficult due to the gelation of gelatin at room temperature. To address this problem, the electrospinning of gelatin in water has been performed by placing the whole apparatus set-up in a warmed enclosure [24]. In our study, we developed a method to warm the gelatin solution in pure water based on the use of a glass cooler as a warmer (Fig. 1A). This new set-up allowed us to easily fabricate gelatin fibers at low and elevated temperatures (40–70 °C).

Fig. S1 presents the FE-SEM images of gelatin fibers obtained by electrospinning gelatin dissolved in water at different concentrations (10%, 15%, 20%, and 25% w/v) and two applied voltages (15 kV or 18 kV) at 60 °C. Due to the viscosity, charge density, and surface tension conditions of the 10% gelatin solution, the Rayleigh instabilities of the jet polymer solution were important and favored the formation of beads during electrospinning [25]. However, at 15% and 20% gelatin, long and smooth gelatin fibers were produced. At 25% gelatin, the produced fibers started to branch and did not appear homogeneous in shape and diameter. Accordingly, the diameter distribution curve for 25% gelatin (Fig. S2) revealed a large distribution of the fiber diameters leaning toward large diameters. Gelatin solution electrospun at 18 kV and 15 kV, had diameters from 250 to 900 nm and 300–600 nm, respectively. In contrast, the diameter distribution curves at 15% gelatin revealed a narrow fiber diameter distribution where most of the fibers fell into 250–300 nm range. At 20% gelatin, equivalent proportions of fibers with diameters of 250–450 (18 kV) or 300–500 nm (15 kV) were produced, as demonstrated by the diameter distribution graphs. At 15% and 20% gelatin, the difference in fiber diameters between gelatin electrospun at 15 kV and 18 kV was not marked. However, at 18 kV, the polymer jet ejection speed became more important, and the fibers were straighter, whereas the diameter distribution curves were more compact, with smaller diameters. In agreement with the results of previous studies, we observed that the gelatin fiber diameters increased with increasing gelatin concentrations (Table TS1, TS2) [26].

Fig. S3 shows the effects of temperature on the morphology and diameter of 20% gelatin fibers. In our system, it was still possible to produce gelatin fibers at 40 °C. However, the process was complicated by high viscosity and most of the fibers appeared branched with a large distribution of diameters of 200–700 nm with a peak at 300–350 nm. At 50 °C, the viscosity of the aqueous solution of 20% gelatin was reduced, and therefore, the production of smooth and long fibers was eased. The distribution curve of the diameters was the narrowest, 250–500 nm with a peak at 300–350 nm. At 60 °C, the production of gelatin fibers was easy, indicating that this temperature should be adopted for electrospinning. The fiber diameters ranged from 200 to 500 nm and centered mainly at 300–

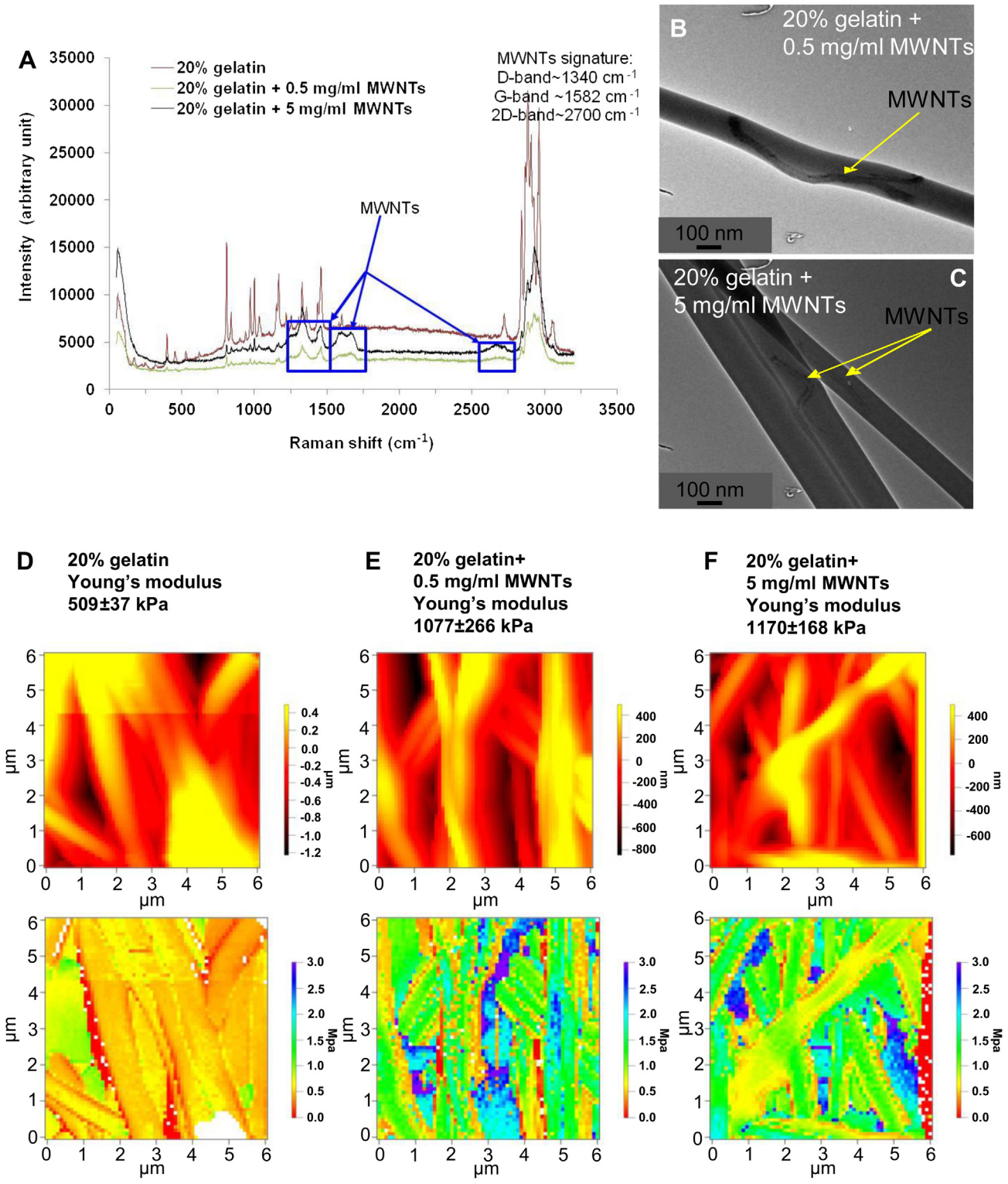

Fig. 2. Inclusion of MWNTs in 20% gelatin fibers: Raman spectra for 20% gelatin fibers, 20% gelatin fibers with 0.5 mg/ml MWNTs and 20% gelatin fibers with 5 mg/ml MWNTs (A). TEM images showing the encapsulation of MWNTs into 20% gelatin fibers with 0.5 mg/ml MWNTs (B) and 5 mg/ml MWNTs (C). Young's modulus evaluation by AFM measurement in water on 20% gelatin fibers (D), 20% gelatin fibers with 0.5 mg/ml MWNTs (E) and 20% gelatin fibers with 5 mg/ml MWNTs (F). Topographical mapping (top) and Young's modulus mapping (bottom).

400 nm. At 70 °C, the viscosity of the gelatin solution was yet lower, and the production of fibers was very easy. The distribution curve of the diameters was also centered at 300–400 nm with equivalent proportions of fibers at 300–350 nm and 350–400 nm.

3.2. Crosslinking of gelatin fibers

Gelatin is a natural bioresorbable polymer with many functional groups with tailorable physico-chemical properties. However,

electrospun gelatin is water soluble and must be crosslinked [20]. Several methods have been employed to crosslink gelatin fibers to maintain their structural integrity and to avoid rapid degradation in aqueous solutions. Thus, the use of GTA vapor at room temperature is a common technique to crosslink collagen and gelatin fibers [20]. However, high concentrations of GTA are cytotoxic [27], and thus, a low concentration of GTA must be used, and the fibers must be washed before use with cells [28]. Glyceraldehyde, has also been used for crosslinking gelatin fibers; the resulting crosslinked gelatin was well tolerated in vivo [29]. Glucose has also been investigated as a crosslinker because the aldehyde group of the glucose can react with the amino groups of gelatin [28]. In addition, genipin (GIP), which is derived from geniposide extracted from *Gardenia jasminoides*, reacts with free amino groups on proteins and is significantly less toxic than GTA when used as a natural crosslinker of gelatin [21]. Procyanidins (PA) which are a type of flavonoid that are widely present in plants have also been used for crosslinking gelatin scaffolds developed as bone substitutes [30]. Carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is a zero-length type crosslinker with low cytotoxicity that can be used in combination with N-hydroxysuccinimide (NHS) to crosslink gelatin fibers [24]. In our study, we used exposure to GTA vapor for 24 h to crosslink electrospun gelatin fibers, followed by their rinse in water for 2 days. Indeed, among the different methods of crosslinking, GTA is the fastest and strongest crosslinker of gelatin [31]. In addition, it has been shown that the use of GTA vapor reduced considerably the cytotoxicity of GTA treatment and that residual uncrosslinked GTA molecules could then be removed by short time step of rinsing [32,33]. The FE-SEM images presented in Fig. S4 demonstrate that the gelatin fibers lost their cylindrical shape and flattened slightly after the crosslinking step with GTA due to the presence of water moisture in the GTA vapor, in agreement with other studies [20].

3.3. Inclusion of MWNTs in gelatin fibers and stiffness characterization

The inclusion of nanomaterials such as MWNTs into gelatin fibers may be beneficial to muscle tissue formation by improving the electrical and mechanical properties of the fibers upon their interaction with cells. Two different concentrations of MWNTs (0.5 mg/ml and 5 mg/ml) in water were sonicated for 30 min, then mixed with an aqueous 40% gelatin solution to prepare two different 20% gelatin-MWNT solutions, which were electrospun. Fig. 2A presents the Raman spectra and transmission electron microscopy (TEM) images of the 20% gelatin fibers with 0.5 and 5 mg/ml MWNTs. The Raman spectra featured characteristic bands of MWNTs at 1340 cm^{-1} (D-band), 1582 cm^{-1} (G-band), and 2700 cm^{-1} (2D band) [34]. The TEM images confirmed the encapsulation of MWNTs in gelatin fibers (Fig. 2B, C). The proportion of gelatin fibers containing MWNT inclusions increased with increasing MWNT concentration. The diameters of the gelatin fibers and MWNTs were approximately 296 nm and 50 nm, respectively, whereas the presence of MWNTs may also deform the gelatin fibers.

The stiffness of a material affects myotube formation and maturation [35]. The viscoelastic properties and high water content of gelatin scaffolds favor cell proliferation and maintenance, but offer weak mechanical support. Previously, we have shown that the inclusion of MWNTs reinforced hybrid CNT-GelMA hydrogels [14]. We proposed the same reinforcement approach with the inclusion of MWNTs into electrospun gelatin. We used AFM measurements of 20% gelatin fibers with 0 mg/ml, 0.5 mg/ml, and 5 mg/ml MWNTs to evaluate the microscopic surface properties of the fibers and create an AFM nanomechanical map, from which the Young's modulus of the material could be extracted. The results in Fig. 2(D–F) demonstrated an increase in Young's modulus in the presence of MWNTs from 509 ± 37 kPa to 1077 ± 266 kPa and 1170 ± 168 kPa for gelatin nanofibers with 0 mg/ml, 0.5 mg/ml, and 5 mg/ml MWNTs, respectively. These Young's modulus values are lower than those usually reported in the literature for gelatin fibers. For example, gelatin/PCL fibers that were electrospun had Young's modulus values of 105 MPa and 30.8 MPa for gelatin and gelatin/PCL fibers, respectively [36]. In another study, Young's modulus of 46.5 MPa was measured for uncrosslinked gelatin fibers and 424.7 MPa for gelatin fibers that were crosslinked by GTA vapor for 3 days, dried for 2 h in a fume hood, and hardened at 100 °C in an oven for 1 h to remove any residual GTA [20]. This 10-fold improvement of the Young's modulus after crosslinking was attributed to point-bonded structures between the gelatin fibers. However, these measurements were performed on bulk material under dried conditions, as a function of the crosslinking time and method. In the present study, the Young's modulus was measured locally using force volume mode in wet conditions after one night of fiber crosslinking in GTA vapor without the drying or hardening step, resulting in lower Young's modulus values. As a more appropriate comparison, we previously measured Young's modulus values in water of 21 and 12 kPa for a GelMA hydrogel slide with or without the inclusion of CNTs [37]. However, GelMA is a relatively soft hydrogel, and the crosslinking step was performed using UV light for 150 s. While we observed a net increase in the Young's modulus, when gelatin fibers with MWNTs were compared to those without them, the difference in the Young's modulus between gelatin fibers with 0.5 and 5 mg/ml MWNTs was not as marked. This observation may be explained by the TEM analysis, which revealed that the proportion of gelatin fibers with MWNT inclusions increased with increasing MWNT concentration rather than the number of MWNTs pieces included by fibers.

3.4. Cell alignment and viability

A classical electrospinning method to obtain aligned fibers is to use a rotating mandrel as the counter electrode [38]. In our study, we used an array of parallel electrodes as the counter electrode. This set-up enables the formation of aligned fibers over the electrode gaps, particularly for short time deposition (15 min duration) when two adjacent aluminum bands are fully attractive and not yet covered by polymer fibers. We fabricated a counter electrode by covering aluminum foil with a plastic film grid featuring alternating aluminum and plastic bands of 8 cm × 0.5 cm (Fig. 1B). After electrospinning an aqueous solution of 20% gelatin with or without 0.5 mg/ml or 5 mg/ml MWNTs, we harvested the aligned fibers formed by the creation of a bridge over a plastic band under the electrical attraction of two neighboring aluminum bands (Fig. 3A). When C2C12 cells were seeded on these aligned fibers, we observed alignment of the cells within 1 h (Fig. 3(B, C)). Our results demonstrated that the topographical constraint generated by the aligned fibers induced the alignment of C2C12 by contact guidance. This cell alignment is important for SMTE because it is a pre-requisite for myotube formation [39].

The potential cytotoxicity of single-walled carbon nanotubes (CNTs) and MWNTs has been the subject of extensive research [40]. Some reviews, have suggested that CNTs and their derivatives are cytotoxic when dispersed in suspension in the culture medium and are non-toxic when immobilized in a matrix or on a culture dish [41]. In our study, C2C12 were cultured for 2 days on 20% gelatin fibers with 0, 0.5, and 5 mg/ml MWNTs and the cytotoxicity of the fibers was evaluated using a live (green fluorescence)/dead (red

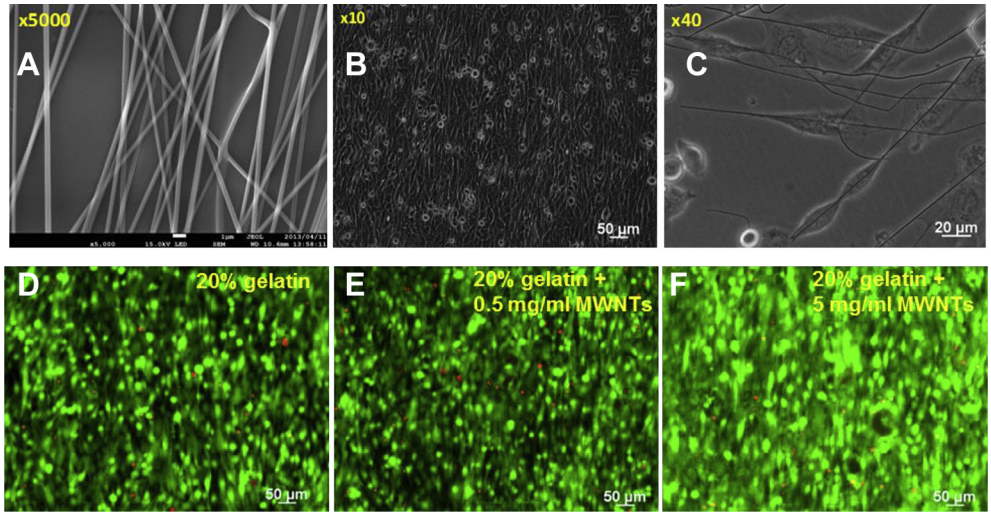

Fig. 3. Photos of aligned gelatin fibers taken by FE-SEM (A). Phase contrast image of myoblast alignment induction by aligned fiber (B). Close up image of cell alignment with fiber (C). Cell viability on gelatin fibers with or without MWNTs. Fluorescence microscopy image of C2C12 (live (green)/dead (red)) at day 2 of culture on 20% gelatin fibers (D), 20% gelatin fibers with 0.5 mg/ml MWNTs (E) and 5 mg/ml MWNTs (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fluorescence) assay. As shown in Fig. 3(D–F), induction of cytotoxicity by MWNTs was not observed and the cell viabilities of three cultures were similar.

3.5. Myotube formation

Serum starvation is a well known method to induce the differentiation of myoblasts into myotubes [39]. As shown in Fig. 4(A–C) myotube aligned along the linear topography of the fibers. We previously showed that the average length of the myotubes that formed in micropatterned lines of a GelMA hydrogel after 7 days in differentiation medium was around 156 μm [42]. In this study, we observed that the myotubes formed on gelatin fibers after 4 days in differentiation medium also had an average length of ~150 μm (Fig. 4D). Thus, myoblast differentiation was more rapid when the myoblasts were cultured on gelatin fibers. Notably, we observed a significant increase in myotube length when MWNTs were integrated in the nanofibers, with lengths of 267 and 630 μm for 0.5 mg/ml and 5 mg/ml MWNTs, respectively. This increase in myotube length with increasing MWNT inclusion in a polymer was also observed by Sirivisoot and Harrison, who used polyurethane fibers with different concentrations of MWNTs [43].

Electrical stimulation (ES) can be used to induce myotubes to mature [44]. ES improves myoblast differentiation by increasing the speed and the rate of myotube formation [45] and enhancing myotube striation and contractibility [46]. We have shown in a previous study a 42% increase in myotube length after 2 days of culture with ES when myotubes were cultured on micropatterned lines of GelMA [42]. Similarly, in this study, we observed a 45% enhancement of the myotube length, after 2 days of culture with ES (Fig. 4E) when myotubes were cultured on gelatin fibers. The myotube length also increased by 42% and 13% after ES, to 459 and 724 μm when the myotubes were cultured on 0.5 mg/ml and 5 mg/ml MWNTs, respectively. Similarly, Ku et al. reported synergistic effects on myoblast differentiation by cell alignment with polycaprolactone–polyaniline (PCL-PANI) nanofibers and electrical conductivity [47]. It is interesting to highlight that several studies have reported enhancement of myotube formation when myoblasts were cultured on an electroconductive polymer without any electrical stimulation [48,49]. The benefit of the myotube formation gained under ES is therefore proposed as an additional effect.

3.6. Myotube contraction

Adult muscle are composed of different fiber types listed following their speeds of contraction and relaxation into slow-twitch fibers (type 1) and fast-twitch fibers (type 2, subdivided into 2A, 2X, and 2B), which have a slow or fast myosin heavy chain (MHC) isoforms [50]. Intermediate fibers (e.g. 1 + 2A, 2A + 2X, and 2X + 2B) are also present giving rise to a continuous spectrum of fibers [51]. This fiber type composition and its maintenance are under the control of nerve activity through the action of calcineurin [52]. In denervated muscle, the muscle inactivity induces muscle atrophy and switch from slow fiber type to fast fiber type (1 toward 2A, then 2X then 2B). In the opposite, muscular activity induces a switch from fast fiber type to slow fiber type (2B toward 2X then 2A then 1) [53]. Similar changes can be obtained by using ES. Low frequency (until 20 Hz) mimics the firing pattern of slow motor neuron and promotes the formation of slow fiber type (type 1). High frequency (50–150 Hz) mimics the firing pattern of fast motor neuron and promotes the formation of fast fiber type (type 2) [53]. Several patterns of ES exist in the literature. Among them the use of a continuous stimulation at low voltage and 1 Hz is frequent [54] and has shown the induction of slow fiber type [55] with higher secretion of ECM [56], while avoiding exhausting the myofibers with the formation of a tetanus state, and the myofiber detachment from the substrate as observed under high frequency. To evaluate the maturation of myotubes after 4 days in differentiation medium, we stimulated different culture systems with electrical pulses (5 V, 1 Hz, 1 ms duration) during 2 days and analyzed the contractibility of the myotubes from videos. Fig. 5 presents the contractibility analysis of myotubes formed on 20% gelatin, 20% gelatin with 0.5 mg/ml MWNTs, and 5 mg/ml MWNTs fibers, respectively.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2014.04.021.

We observed higher maturation and contractibility of myotubes with increasing MWNT concentrations. One of the movies presented in the supplementary section, shows an example of myotube cultured on 20% gelatin fibers with 0.5 mg/ml MWNTs with a

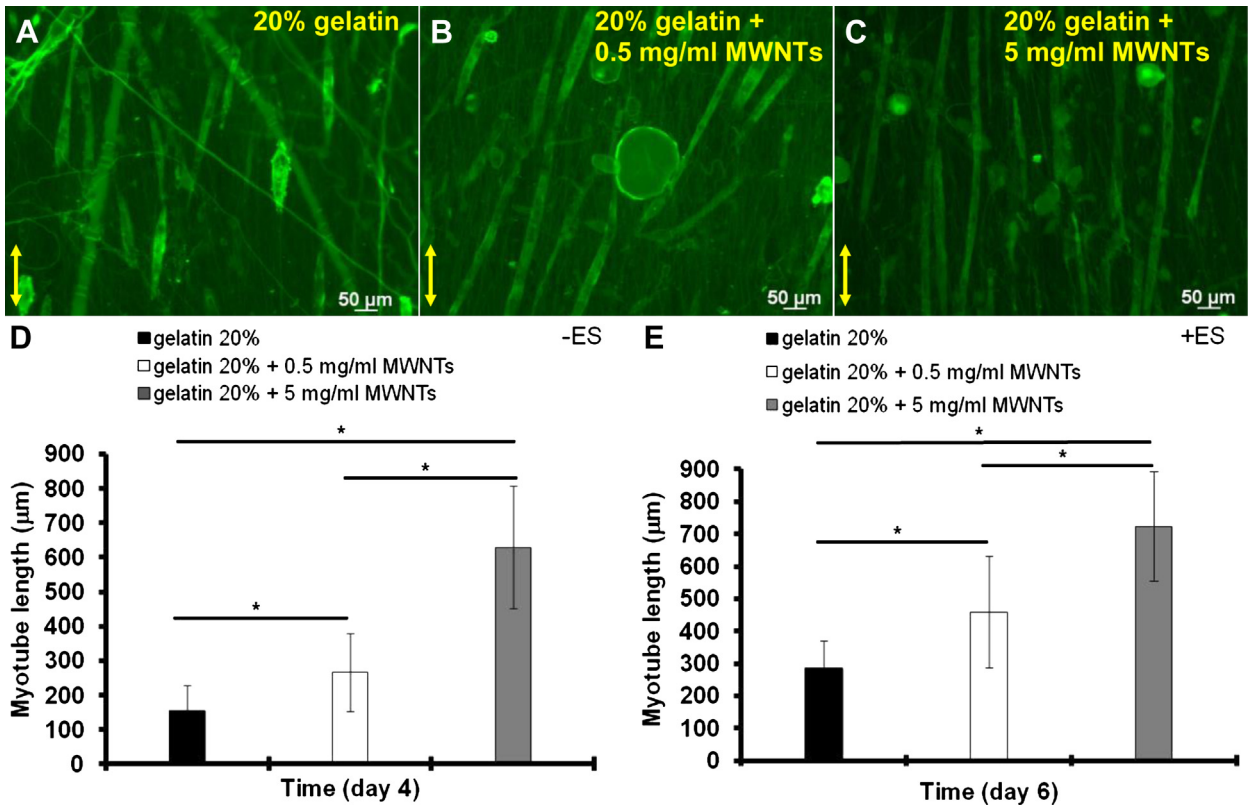

Fig. 4. Fluorescence microscopy images of myotubes (stained with anti MYO-32 antibody and revealed by Alexa Fluor 488 antibody) cultured on electrospuns of aligned 20% gelatin (A), 20% gelatin with 0.5 mg/ml MWNTs (B) and 20% gelatin with 5 mg/ml MWNTs (C). Myotube length quantification at day 4 in differentiation medium without electrical stimulation (D) and with 2 additional days under electrical stimulation (E) (* = $p < 0.01$).

high level of maturation, as indicated by its continued contraction even after ES was OFF for more than 10 min. Other movies, reveal a higher proportion of contractile myotubes or a higher contraction amplitude when the myotubes where cultured on 20% gelatin fibers with 5 mg/ml MWNTs.

3.7. Mechanism of action of MWNTs on C2C12

The inclusion of MWNTs in 20% gelatin fibers may improve myotube formation by increasing the conductivity of the matrix or the Young's modulus or by enhancing the cell–material interactions [43,57]. The fabrication of "electrically conductive hydrogels" via the inclusion of conductive materials such as polymers (PEDOT, PANI) or CNTs inside a hydrogel matrix is an interesting approach for engineering muscle tissue [58,59]. Inclusion of these materials has been shown to improve the electrical conductivity of the material, thereby favoring the development of electroactive cells such as muscle cells [43]. We previously enhanced the electrical conductivity of a GelMA hydrogel slide by loading MWNT pieces that we aligned using the dielectrophoresis technique [37]. However, such bulk electrical conductivity improvement is usually difficult to observe, when MWNT pieces are randomly entrapped within the nonconductive hydrogel matrix at relatively low concentrations [57]. Thus, we performed

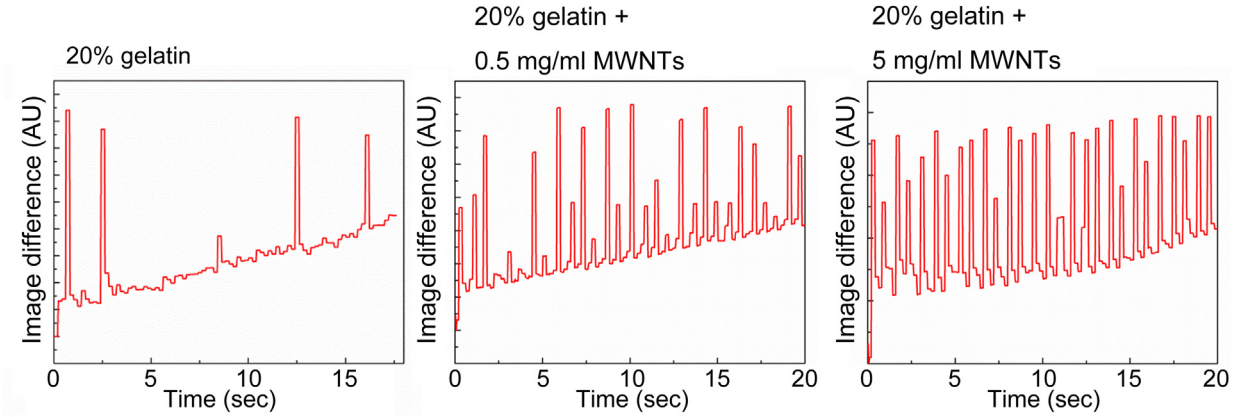

Fig. 5. Myotube contraction analysis after 4 days in differentiation medium followed by 2 additional days with electrical stimulation (5 V, 1 Hz, 1 ms duration).

conductivity measurements using the Van der Pauw method on 20% gelatin fibers with 0 mg/ml, 0.5 mg/ml, and 5 mg/ml MWNTs. The results showed that the resistivity of the different samples tested were $\geq$10 GΩ and thus the fibers with or without MWNTs exhibited insulating behavior (Table TS3). The presence of MWNTs at the concentrations used did not improve the bulk fiber conductivity because the MWNTs were not linked to each other, breaking the charge displacement path. However, conductive atomic force microscopy (C-AFM) measurements have demonstrated that the inclusion of conductive nanomaterials in a hydrogel may generate local conductivity [60].

The inclusion of nanomaterials into a polymeric matrix usually improves cell–material interactions and fosters tissue formation [61]. Fig. 6 shows the relative gene expression in C2C12 of genes related to cell attachment and myogenesis after culture on 20% gelatin fibers with 0, 0.5 and 5 mg/ml MWNTs for 1 day in growth medium and 4 days in differentiation medium. The β1 mRNA expression level was similar for cells cultured on 20% gelatin fibers with or without MWNTs. Integrins, which are heterodimeric proteins consisting of an α and a β subunits, enable adhesion of cells to the ECM. The β1 subunit is involved in the primary myoblast fusion (myoblast–myoblast fusion) [62]. Since in our study MWNTs were wrapped by gelatin, the attachment of the cells to the β1 subunits is probably similar whatever the fiber types, resulting in a similar expression of β1 mRNA. However, we observed a significant increase in focal adhesion kinase (FAK) mRNA expression when myoblasts were cultured on 20% gelatin fibers with MWNTs. FAK is a key cytoplasmic tyrosine kinase involved in myoblast fusion that transmits the integrin-mediated signal and undergoes autophosphorylation after activation [63]. FAK can be activated through several ways and notably via different integrin subunits [64,65]. The results observed here show that in addition to the nature of the nanofibers, C2C12 also detect the difference in the nanofiber stiffness, since the presence of MWNTs increased their Young's modulus. This is in correlation with other studies, which have shown that for a same chemical environment, focal adhesions are regulated by substrate flexibility and that there are increased amount of phosphotyrosine at adhesion sites on firm substrates compared to flexible substrates [66,67]. This upregulation of FAK mRNA expression is correlated with myoblast differentiation since myogenin mRNA expression is also upregulated on gelatin fibers with MWNTs and several studies have shown that FAK is one of the components regulating C2C12 differentiation [63,65]. This finding correlates with the enhancement of myotube formation we observed in the present study because myogenin is one of the 4 myogenic regulatory factors (MRFs), which also include MyoD, Myf-5, and MRF4, and plays a key role in the entry of myoblasts into the differentiation program [68]. Moreover, we observed a significant decrease in Id1 mRNA expression when cells were cultured on 20% gelatin fibers with MWNTs. Id (for inhibitor of DNA binding) is a helix-loop-helix (HLH) protein that lacks the basic motif involved in DNA binding. In mice, the Id protein family has three members, Id1, Id2, and Id3. Id1 is highly expressed in proliferative cells and inhibits myogenesis by forming heterodimers with E proteins, impairing their binding with MyoD and activation of myogenesis [69]. Taken together, these gene expression results indicated an increased activation of myoblast differentiation when C2C12 are cultured on 20% gelatin fibers with MWNTs, in agreement with the myotube formation enhancement we observed in this study.

3.8. Free-standing aligned gelatin fibers band

Isolation of free-standing gelatin fibers could substantially aid in the development of SMTE constructs and implantable scaffolds. C2C12 cells were cultured on a whole plastic band (8 mm × 5 mm) coated by *p*(NIPAM) and covered with 20% gelatin with or without

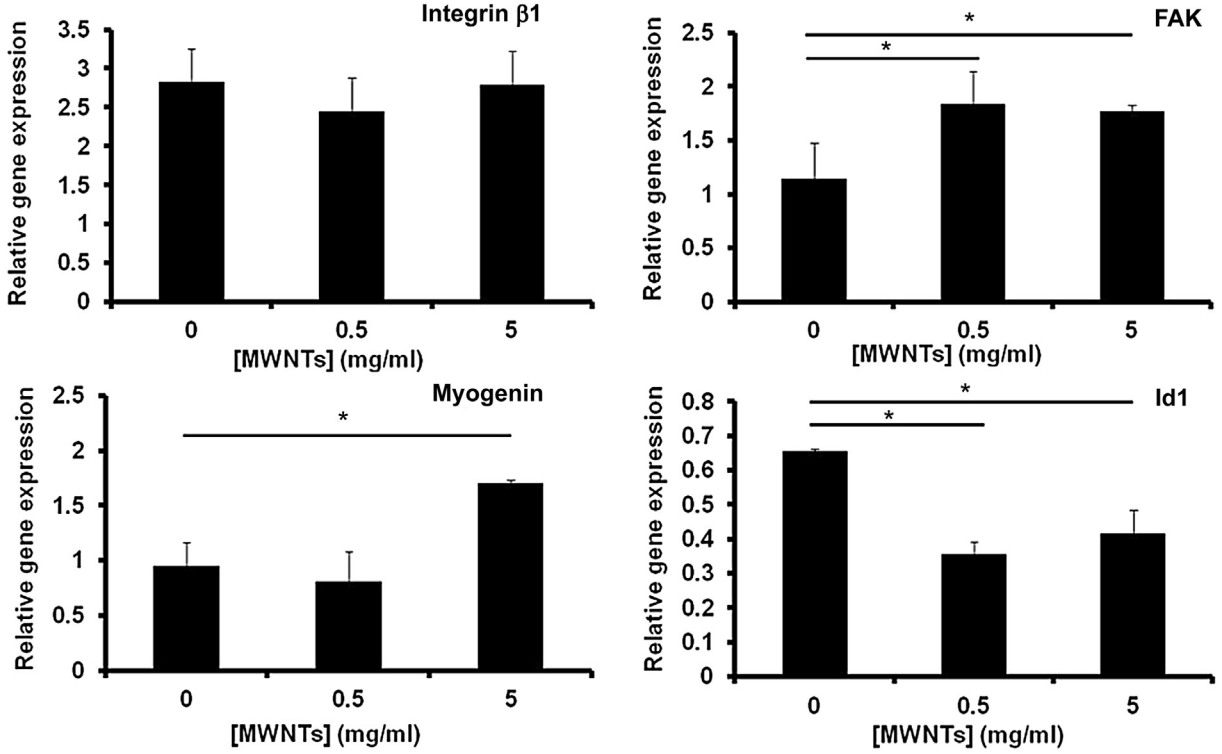

Fig. 6. Genes expression analysis of C2C12 cultures on 20% gelatin fibers with or without MWNTs (0.5 mg/ml and 5 mg/ml) after 4 days in differentiation medium. Results were normalized to GAPDH (* = $p < 0.05$).

MWNT aligned fibers in warm culture medium (37 °C) at a temperature higher than the lowest critical solution temperature (LCST = 32 °C) of the *p*(NIPAM). After 1 day in growth medium and 4 days in differentiation medium, aligned myotubes were formed along the fiber band, which can be released from its plastic substrate by lowering the temperature below the LCST (Fig. 7). This type of free-standing film therefore enables the harvesting of myotubes. The fiber band can also be rolled on itself or on substrates to develop 3D culture applications or tubular structures [70].

4. Conclusions

In summary, we showed that the presence of MWNTs in gelatin fibers improved the mechanical properties of the fibers by increasing the Young's modulus. This stiffness enhancement triggered differently the activation of the mechanotransduction, notably by up regulating the gene expression of FAK and myogenin, which contributed to the enhancement of myotube formation and maturation we observed in C2C12 cultures on gelatin fibers with MWNTs. The combination of the topographical constraints imposed by the linear shape of the aligned gelatin fibers, the cellular-activity-supporting properties of gelatin, and the mechanical properties of the MWNTs, synergized, and improved myoblast alignment and differentiation to generate functional myofibers. In addition, we showed that such hybrid-hydrogel nanofiber scaffold could be harvested to develop 3D culture applications or tubular structures. In addition, we developed a method for gelatin fiber production by electrospinning, where gelatin solution in pure water with or without MWNTs is warmed using a glass chamber.

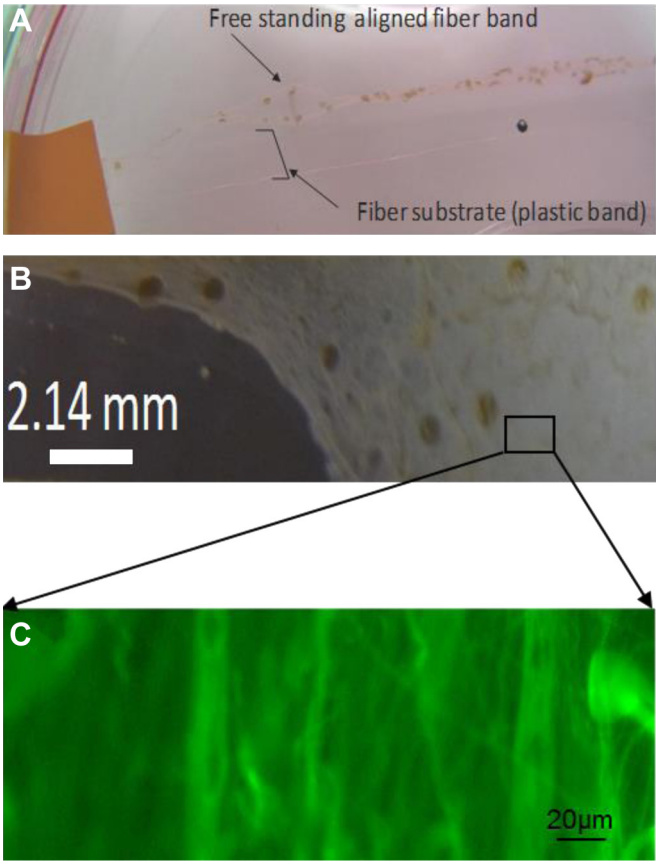

Fig. 7. Free standing film fabrication of 20% gelatin with 0.5 mg/ml MWNTs (A) and photo (B) in fluorescence microscopy of aligned myotubes stained with anti MYO-32 antibody and revealed with Alexa fluor 488 antibody (C).

Acknowledgments

This work was supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan. The authors thank Dr. Akichika Kumatani for evaluating the electrical conductivity of the gelatin fibers with or without MWNTs and Dr. Haixin Chang for providing MWNTs. In part, this project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, under grant number 18-130-1434-HiCi. The authors, therefore, acknowledge technical and financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.04.021.

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