Acta Biomaterialia 31 (2016) 134–143

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Hybrid hydrogel-aligned carbon nanotube scaffolds to enhance cardiac differentiation of embryoid bodies

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ARTICLE INFO

Article history:
Received 14 July 2015
Received in revised form 20 November 2015
Accepted 23 November 2015
Available online 24 November 2015

Keywords:
Carbon nanotubes
Cardiac differentiation
Electrical stimulation
Embryoid body
Hydrogel

ABSTRACT

Carbon nanotubes (CNTs) were aligned in gelatin methacryloyl (GelMA) hydrogels using dielectrophoresis approach. Mouse embryoid bodies (EBs) were cultured in the microwells fabricated on the aligned CNT-hydrogel scaffolds. The GelMA-dielectrophoretically aligned CNT hydrogels enhanced the cardiac differentiation of the EBs compared with the pure GelMA and GelMA-random CNT hydrogels. This result was confirmed by Troponin-T immunostaining, the expression of cardiac genes (i.e., Tnnt2, Nkx2-5, and Actc1), and beating analysis of the EBs. The effect on EB properties was significantly enhanced by applying an electrical pulse stimulation (frequency, 1 Hz; voltage, 3 V; duration, 10 ms) to the EBs for two continuous days. Taken together, the fabricated hybrid hydrogel-aligned CNT scaffolds with tunable mechanical and electrical characteristics offer an efficient and controllable platform for electrically induced differentiation and stimulation of stem cells for potential tissue regeneration and cell therapy applications.

Statement of significance

Dielectrophoresis approach was used to rapidly align carbon nanotubes (CNTs) in gelatin methacryloyl (GelMA) hydrogels resulting in hybrid GelMA-CNT hydrogels with tunable and anisotropic electrical and mechanical properties. The GelMA-aligned CNT hydrogels may be used to apply accurate and controllable electrical pulses to cell and tissue constructs and thereby regulating their behavior and function. In this work, it was demonstrated that the GelMA hydrogels containing the aligned CNTs had superior performance in cardiac differentiation of stem cells upon applying electrical stimulation in contrast with control gels. Due to broad use of electrical stimulation in tissue engineering and stem cell differentiation, it is envisioned that the GelMA-aligned CNT hydrogels would find wide applications in tissue regeneration and stem cell therapy.

1. Introduction

The capability of stem cells to undergo unlimited self-renewal and differentiate into multiple cell types has prompted their widespread application in tissue engineering (TE) and cell therapy fields [1]. The stem cell microenvironment has a major effect on stem cell

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http://dx.doi.org/10.1016/j.actbio.2015.11.047
1742-7061/

renewal and differentiation. This microenvironment, termed the niche, was first described by Schofield [2]. The stem cell niche is composed of extracellular matrix (ECM) components, soluble factors, and supportive cells, which provide spatiotemporal signals to stem cells to direct their fate. Creating biomimetic stem cell niches *in vitro* is crucial for controlling stem cell behavior in therapeutic applications [3]. Therefore, the design and fabrication of biomaterials and tools to mimic various aspects of the stem cell niche to control stem cell behavior are an interesting area of research. In particular, the precise control of stem cell differentiation and fate needs to be further achieved [4].

Carbon nanotubes (CNTs) have recently attracted significant attention in biological applications ranging from bioimaging [5], drug delivery [6], biosensing [7], cancer therapy [8], and TE scaffolding [9]. This recent interest in CNTs arises from their outstanding chemical, mechanical, electrical, and optical properties [10]. Incorporating CNTs into TE scaffolds leads to enhanced scaffold flexibility, strength, and electrical conductivity. For example, adding CNTs to gelatin methacryloyl (GelMA) hydrogel scaffolds increases their Young's modulus and electrical conductivity without significantly affecting gel porosity [11]. GelMA is a biocompatible, biodegradable, and photocrosslinkable hydrogel that is suitable for culturing different cell types and fabricating various tissues [12]. However, the relatively poor electrical conductance of this gel limits its application in the regulation of electro-active cell behaviors and electrical stimulation (ES) of cell and tissue constructs [13]. Here, hybrid GelMA-CNT hydrogels with tunable electrical and mechanical characteristics were used as scaffolds to culture and electrically regulate the cardiac differentiation of mouse embryoid bodies (EBs).

Micro- and nanoscale technologies have widely been used in biomedicine [14]. They can be used to precisely fabricate biomaterials or cellular structures that mimic the complex architecture of native biological constructs. Dielectrophoresis (DEP) is one such useful and versatile technology. DEP is based on particle polarization and manipulation in a medium by applying a non-uniform electric field [15]. For example, we recently reported the use of DEP for the rapid formation of three-dimensional (3D) EBs in GelMA hydrogel [16]. It was possible to fabricate 3D EBs of varying sizes and shapes using a high-throughput approach. The DEP method was also employed to align CNTs in GelMA gels [17,18]. The hybrid GelMA-aligned CNT scaffolds showed better performance in the generation of functional and contractile skeletal muscle myofibers in contrast with pure GelMA and GelMA-random CNT scaffolds.

Here, DEP was utilized to fabricate hybrid GelMA-aligned CNT gels. The mechanical and electrical properties of these gels were measured and compared against pure GelMA and GelMA-randomly dispersed CNT hydrogels as the control samples. We then used the GelMA-aligned CNT hydrogels to support the cardiac differentiation of EBs in response to ES. The efficiency of the GelMA hydrogel containing the aligned CNTs and control hydrogels (i.e. pure GelMA and GelMA-randomly dispersed CNT gels) in supporting the cardiac differentiation of EBs was determined by using gene and protein expression analyses and beating activity of the differentiated cells.

2. Materials and methods

2.1. Materials

The following materials were used: developer (MF CD-26; Shipley Far East, Japan); photoresist (S1818; Rohm and Haas, USA); SU-8 3050 and SU-8 developers (MicroChem, USA); methacrylic anhydride, gelatin type A from porcine skin, trichloro (1H,2H,2H-perfluorooctyl)silane, 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), and penicillin/streptomycin (P/S) (Sigma–Aldrich Chemical, USA); multi-walled CNTs (Hodogaya Chemical, Japan); 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propa none (Irgacure 2959; Ciba Chemicals, Japan); fetal bovine serum (FBS; Bioserum, Japan); and indium tin oxide (ITO) glass slides (Hiraoka Special Glass, Japan).

2.2. Fabrication of interdigitated array of ITO (IDA-ITO) electrodes and SU-8 microstamp

The IDA-ITO electrodes and the SU-8 microstamp were made on a glass slide (Matsunami, Japan) using photolithography and chemical etching techniques [13]. The glass slides were cleaned using plasma oxygen prior to the photolithography. To fabricate the IDA-ITO electrodes, S1818 and MF CD-26 were used as the positive photoresist and developer, respectively. SU-8 3050 (photoresist) and SU-8 (developer) were used to fabricate the SU-8 microstamp. The etchant solution was a mixture of HCl, HNO_3, and H_2O in a volume ratio of 4:1:5. The etching was performed for 120 min under stirring. The photoresist was removed with acetone.

2.3. Chemical functionalization of the CNTs

A controlled acid treatment process was used to functionalize the multi-walled CNTs. In brief, the CNTs were refluxed in 68 wt % HNO_3 and 98 wt% H_2SO_4 (volume ratio 1:3) at 110 °C for 20 min. After washing with pure water on a 1.2 μm membrane, an aqueous dispersion of the CNTs was prepared using probe sonication. As shown in our previous study [18], the zeta potential of the CNTs was ~−40 mV at a pH of ~4.1. In addition, the CNTs had a high purity, which was confirmed by Raman spectroscopy and microscopy.

2.4. Synthesis of the GelMA prepolymer

Gelatin (6 g) and methacrylic anhydride (12 mL) were dissolved in Dulbecco's phosphate-buffered saline (DPBS) (60 mL) at 50 °C for 1 h. The degree of gelatin modification was ~80%. The mixture was dialyzed against pure water using a 12–14 kDa dialysis membrane at 40 °C for 1 week. The mixture was then lyophilized for 1 week. Photoinitiator (1% (w/v); Irgacure 2959) was added to the 10% (w/v) GelMA prepolymer in pure water at 60 °C to obtain the GelMA prepolymer solution.

2.5. Dielectrophoretic alignment of the CNTs in GelMA gels

The interdigitated electrodes were subjected to plasma oxygen treatment followed by methacrylation using TMSPMA under vacuum for 2 h to obtain good adhesion between the electrodes and the GelMA hydrogels. The SU-8 microstamp was treated using tri chloro(1H,2H,2H-perfluorooctyl)silane to avoid attachment to the GelMA hydrogel. A chamber was created for the DEP experiments by mounting the SU-8 microstamp on the electrodes. The thickness of the chamber was 200 μm (Fig. 1B). The 10% (w/v) GelMA prepolymer solution was mixed with the CNT aqueous solution at a ratio of 1:1 to obtain a final GelMA concentration of 5% (w/v). The GelMA-CNT prepolymer was sonicated for 15 min to obtain a homogeneous mixture. The GelMA-CNT prepolymer was then injected into the chamber. A function generator (Hioki 7075, Hioki, Japan) applied an electric field (frequency, 1 MHz; voltage, 20 V) to horizontally align the CNTs in the GelMA prepolymer. The GelMA gels containing the aligned CNTs were then crosslinked with UV (Hayashi UL-410UV-1, Hayashi Electronic Shenzen, Japan) for 150 s. After crosslinking, the SU-8 microstamp was removed from

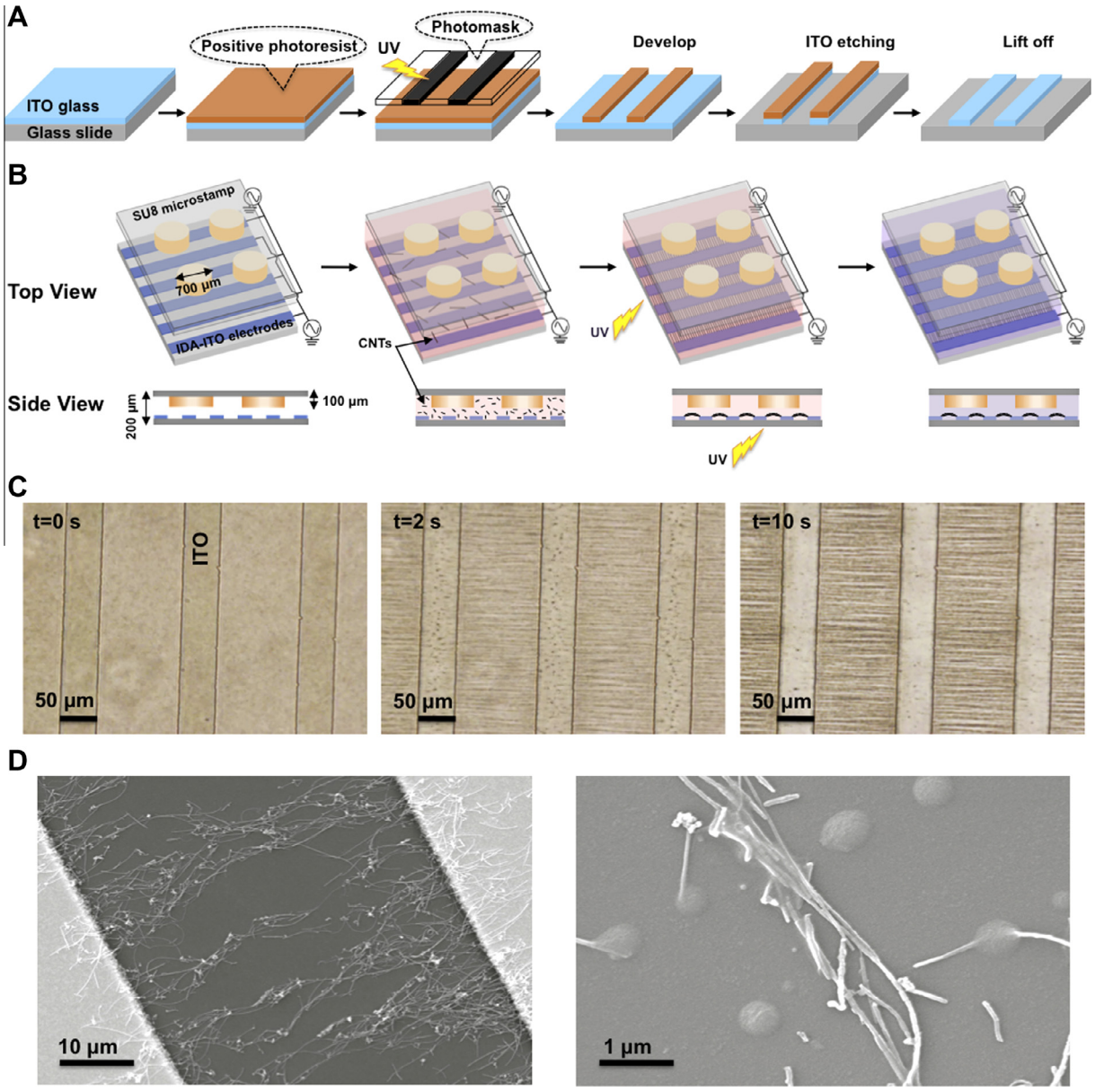

Fig. 1. Fabrication of the IDA-ITO device and its use for the dielectrophoretic alignment of CNTs in GelMA hydrogels. (A) Schematic of the IDA-ITO fabrication. The S1818 photoresist was spin-coated on the ITO glass slide at 5000 rpm for 3 min. The photoresist was cured by applying UV under a photomask. The MF CD-26 was used for the development of micropatterns for 10 min. The etchant solution was used to reveal the ITO micropatterns on the glass slide. This solution was a mixture of HCl, HNO_3, and H_2O in a volume ratio of 4:1:5. The etching was performed for 120 min under stirring. (B) CNT alignment in GelMA prepolymer under DEP force (voltage, 20 V; frequency, 1 MHz). Highly aligned CNTs were obtained in less than 1 min. The GelMA prepolymer was then crosslinked to maintain the CNT alignment in the gel after cutting off the DEF force. (C) Phase images of CNT alignment procedure at different times. (D) SEM images of dielectrophoretically aligned CNTs showing highly aligned CNTs between ITO electrode bands.

the IDA-ITO electrodes, and the gel with microwells remained on the IDA-ITO electrodes.

2.6. Mechanical characterization of pure GelMA and GelMA-CNT hydrogels

An atomic force microscopy (AFM) method was used to obtain the force–deformation curves of the gels as described elsewhere [18]. A MultiMode 8 AFM (Bruker, USA) was used. A colloidal probe (1.0 μm radius, Novascan Technologies, USA) was used in the AFM cantilever. The thermal noise method was employed to determine the cantilever spring constant [19]. The stiffness of the gels was calculated using the force–deformation curves, taking into account the Derjaguin, Muller, and Toporov (DMT) fitting equation [20]. The DMT model presumes that the contact mechanics between the AFM probe and the underlying material is Hertzian contact, and only adhesion forces between the two surfaces are considered. The gel prepolymer was confined between one TMSPMA-treated glass slide and one untreated glass slide, UV crosslinked, and then obtained on the treated glass slide. Therefore, the compact gels with smooth surface and low porosity were obtained for the AFM measurements for which the Hertzian contact model was valid. The reported stiffness values are the average of five independent measurements.

2.7. Electrical characterization of pure GelMA and GelMA-CNT hydrogels

The current–voltage (I–V) curves were obtained at ambient temperature using a two-probe station (H19S00556, HiSOL, Japan).

The currents were recorded by sweeping the voltage from 0 to 5 V. The cables of the probe-station were connected to the interdigitated electrodes to obtain reproducible and accurate conductivity measurements. The impedance values were measured using a CompactStat Potentiostat (CompactStat, Ivium Technologies, Netherlands) with IviumSoft software. The frequency was altered from 0.2 to 5 Hz, and the perturbation amplitude was 25 mV.

2.8. Cell culture

129/SVE-derived mouse stem cells (DS Pharma Biomedical, Japan) at a passage number between 5 and 20 were used in these experiments. The cells were kept in an undifferentiated state in serum-free media (DS Pharma Biomedical, Japan) containing 1000 U/mL mouse leukemia inhibitory factor (Millipore, USA), 1 mM β-mercaptoethanol (Millipore, USA), and 1% P/S. The cells were cultured on gelatin (0.1%; Millipore, USA)-coated flasks at 37 °C and 5% CO_2. The cell culture medium was replenished every day. The EBs were generated using the conventional hanging drop technique with 500 cells per drop. The cardiac differentiation was induced using differentiation medium (10% FBS, 1% P/S, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM non-essential amino acid solution, and 1 mM sodium pyruvate in α-MEM (Minimum Essential Medium) medium) for 7 days before seeding the EBs on the hydrogels. This time period is sufficient to induce the cardiac differentiation in the EBs [21].

2.9. Quantification of cell viability

A live/dead calcein AM/ethidium homodimer assay (Dojindo, Japan) was used to quantify the viability of stem cells cultured on the hydrogels according to the manufacturer's recommendations. At least ten EBs were counted in this experiment.

2.10. ES of EBs cultured on the hydrogels

The EBs cultured on the pure GelMA and GelMA gels containing the CNTs were electrically stimulated along with the CNT alignment at day 2 of culture using the interdigitated electrodes under the hydrogels. The ES was applied using a waveform generator (Hioki 7075, Hioki, Japan) under a specific program (frequency, 1 Hz; voltage, 3 V; duration, 10 ms) for two continuous days. Therefore, the total culture time of the EBs on the hydrogels was 4 days. The medium was replenished every day during the stimulation of the hydrogels to eliminate any side effect of the generated charge.

2.11. Extraction of RNA and complementary DNA (cDNA)

β-mercaptoethanol was used to extract RNA from the cells. The RNA was then purified (RNeasy© Micro Kit, Qiagen, Netherlands). RNA (1 μg) was reverse transcribed (QuantiTect Reverse Transcription, Qiagen, Netherlands). To synthesize cDNA, 12 μl of RNA (3 μg) was mixed with RNase-free water (14 μl) and gDNA wipeout buffer (4 μl). The mixture was then kept at 42 °C for 2 min and then cooled to 4 °C. Quantiscript Reverse Transcriptase and Reverse Transcriptase Primer Mix were added to the mixture, which was kept at 42 °C for 15 min and then at 95 °C for 3 min. The mixture was stored at 4 °C for the quantitative PCR (qPCR) experiment.

2.12. Real time PCR

The primers for Tnnt2, Nkx2-5, Actc1, and GAPDH were purchased from Nihon Gene Research Laboratories, Japan. Table 1 lists the complete primer names and their sequences. Real-time PCR was done with 2 μl of the primer set (5 μM), 2 μl of cDNA, and 14 μl of LightCycler FastStart DNA Master SYBR Green 1 (Roche, Germany) using a Roche LightCycler 1.5 (Roche, Germany). Denaturation step was performed at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 62 °C for 10 s, and 72 °C for 20 s. Gene expression was given as the normalized value to mouse GAPDH expression according to the Schmittgen and Livak's protocol [22]. Each individual PCR experiment was repeated at least four times with two replicates in each experiment and the reported gene expression values are the average of these eight independent experiments.

2.13. Immunostaining of differentiated EBs

The 4% paraformaldehyde was used to fix the EBs for 12 min and then permeabilization was performed using 0.3% Triton X-100 for 5 min. The cells were treated with 5% bovine serum albumin for 15 min. The cells were treated with a primary mouse monoclonal IgG antibody (ab-7784, Abcam®, Japan) diluted 500 times in DPBS to reveal cardiac Troponin T and the mixture was incubated at 4 °C for 24 h. The cells were then treated with a goat anti-mouse AlexaFluor® 488 antibody (Invitrogen, USA) diluted 500 times in DPBS and were kept at 37 °C for 1 h. 4,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., USA) was also used to show the cell nuclei. The EB immunostaining was imaged for four independent samples using a fluorescence microscope.

2.14. Beating analysis of differentiated EBs

The beating movies of the EBs were recorded under a microscope. ImageJ (ver. 1.42, National Institute of Health (NIH), USA) was used to quantify the beating area and the beating frequency of the EBs. At least 5 movies were considered for the beating analysis of EBs.

2.15. Statistical analysis

Statistically significant differences were determined using *t*-test and two-way ANOVA. A Tukey–Kramer post hoc analysis was used when the null hypothesis in ANOVA was rejected. The two-way ANOVA was used when there were two effective parameters (i.e., hydrogel type and electrical stimulation) in the analysis. Otherwise, the *t*-test was used. All the repeated data are presented as the average ± standard deviation, and differences with $p < 0.05$ were considered significant.

Table 1
Genes and their primer sequences.

Gene symbol	Gene name	Left primer (5′ → 3′)	Right primer (3′ → 5′)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	tgtccgtcgtggatctgac	cctgcttcaccaccttcttg
Tnnt2	Troponin T type 2	tggagggtacatccagaagc	tcctctctgccaggatcttc
Nkx2-5	NK2 homeobox 5	gacgtagcctggtgtctcg	gtgtggaatccgtcgaaagt
Actc1	Actin alpha, cardiac muscle 1	ggtcatcaccattggcaac	atgccagcagattccatacc

3. Results and discussion

Prior to the DEP procedure, carboxyl groups were chemically bonded to the CNTs to make their stable dispersion in aqueous medium. The high quality of the synthesized CNTs was demonstrated using Raman spectroscopy and microscopy images in our previous work [18]. The CNTs were then dispersed in a 10% (w/v) GelMA prepolymer solution. For the DEP experiment, an IDA-ITO was made on a glass slide using lithography and chemical etching techniques (Fig. 1A). A SU-8 microstamp (well diameter, 700 μm) was fabricated and mounted on the IDA-ITO to make a chamber for the DEP experiment (Fig. 1B). The GelMA prepolymer-CNT dispersion was then introduced into the chamber and aligned along the direction of the applied sinusoidal electric field (voltage, 20 V; frequency, 1 MHz) (Fig. 1B). The electric field induced a dipole moment within the CNT structures and forced them to align. This process is rapid and takes 10 s to complete (Movie S1 and Fig. 1C). Photopolymerization of the GelMA prepolymer fixed the aligned CNTs in the GelMA gels. Microwells were created in the gels by removing the top SU-8 microstamp. Scanning electron microscopy (SEM) (JSM-6500F, JEOL, Japan) images of the dielectrophoretically aligned CNTs (Fig. 1D) illustrated the highly aligned CNTs between the ITO electrode bands. Note that the SEM pictures were taken in the absence of GelMA hydrogel. We previously demonstrated the horizontal alignment of CNTs in GelMA hydrogels using the DEP technique [18]. In the previous study, the IDA electrodes were prepared using platinum (Pt). As Pt is not transparent, here, ITO was used to prepare the IDA electrodes for the DEP experiment. ITO is optically transparent, and it has high electrical conductivity. In addition, ITO is non-toxic and is a stable electrode material under both humid and warm environments [23].

An AFM technique was used to determine the stiffness of pure GelMA and GelMA gels containing 0.5 mg/mL CNTs (Fig. 2A and B). The AFM tip approached the aligned CNTs in a per-

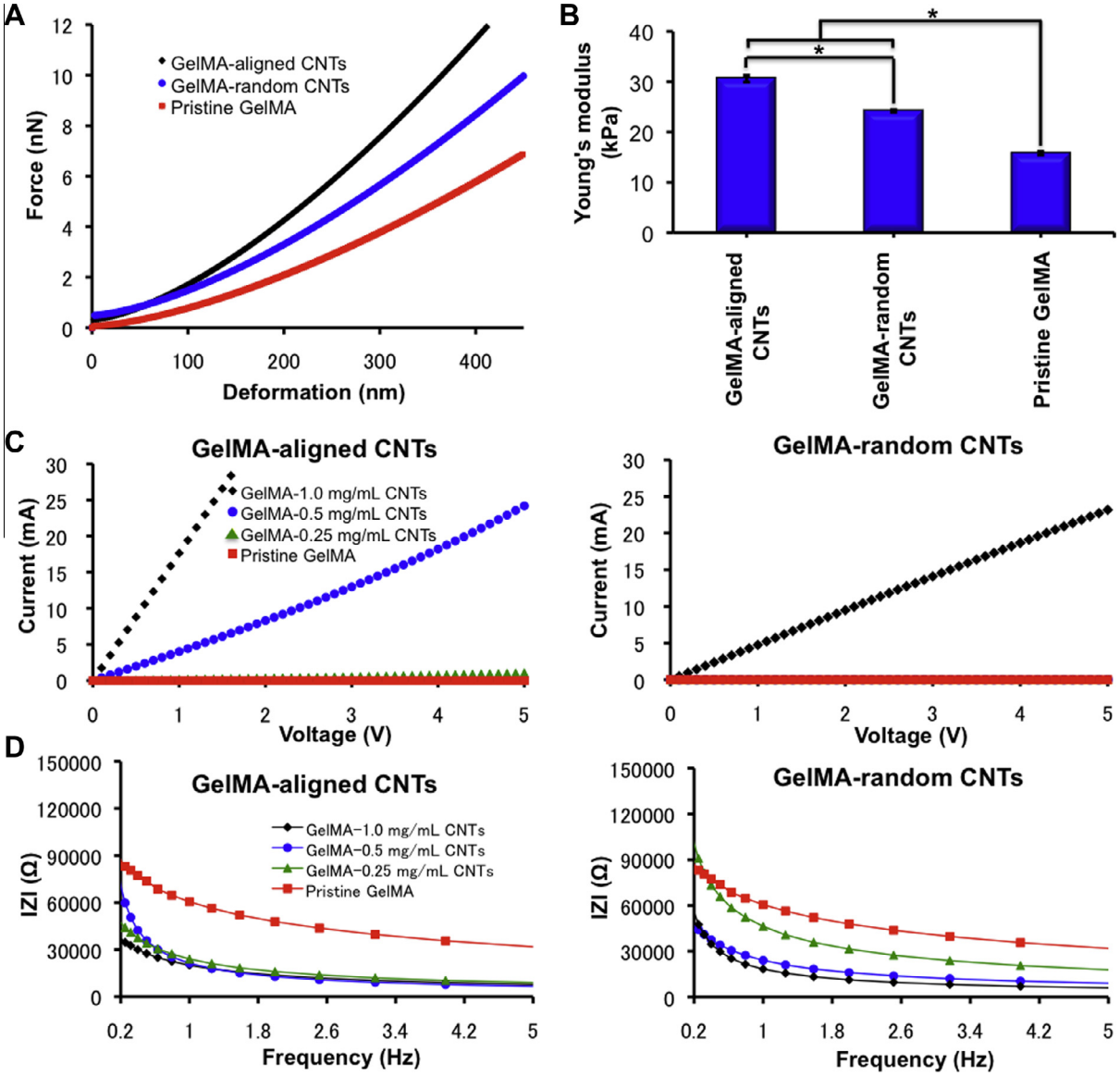

Fig. 2. Mechanical properties and electrical conductance of pure GelMA and hybrid GelMA-CNT hydrogels. (A) Force–deformation curves of pure 5% (w/v) GelMA and GelMA gels containing the 0.5 mg/mL random and aligned CNTs. AFM was employed to obtain the curves. The AFM tip approached the aligned CNTs in a perpendicular direction. (B) Stiffness of the underlying hydrogels. The slope of force–deformation curves resulted in the stiffness values taken into consideration the Hertzian model. (C) Current–voltage measurements of pure GelMA and hybrid GelMA-CNT gels. The curves were obtained using IDA-ITO electrodes under the hydrogels. In the right panel, the blue and green lines were hidden on the back of red line due to data overlapping. (D) Absolute impedance as a function of frequency for the pure GelMA and hybrid GelMA-CNT gels. The impedance values were obtained using the IDA-ITO electrodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pendicular direction. The pristine GelMA had a Young's modulus of 15.8 ± 0.4 kPa. The CNTs significantly increased the stiffness of pure GelMA hydrogels due to their high mechanical properties. The Young's modulus values were 24.2 ± 0.2 and 30.6 ± 0.7 kPa for the GelMA hydrogels containing the random and aligned CNTs, respectively. This slight difference between the latter values was due to additional reinforcement provided by the aligned and bundled CNTs. Numerous chemical and physical signals from the stem cell niche affect stem cell differentiation. Although numerous chemical factors that direct stem cell differentiation have been identified (e.g. small chemicals that modulate the Wnt signaling pathway [24]), mechanical signals and their molecular mechanisms of action need to be further studied. In a recent investigation, Hazeltine et al. assessed the effect of stiffness of polyacrylamide hydrogel substrates on the differentiation of human embryonic stem cells towards cardiomyocytes [25]. They found that the hydrogels with an intermediate stiffness (50 kPa) were the most efficient substrates for the cardiac differentiation of stem cells. This effect was also reported for mouse embryonic stem cells [26]. Small quantities of CNTs, especially aligned CNTs, in GelMA hydrogels may be effective and powerful tools for changing the mechanical features of hydrogels without significantly influencing their biological characteristics and function for potential stem cell culture and differentiation studies.

The pure GelMA gel and GelMA gels containing dielectrophoretically aligned and random CNTs at different CNT concentrations (0.25, 0.5, and 1 mg/mL) were prepared. The electrical conductance between the IDA-ITO electrodes under the gels was quantified to ensure accurate and reproducible conductivity measurements of the gels. Fig. 2C shows the results of the DC electrical conductivity measurements. The electrical conductivity was significantly enhanced upon the addition of CNTs to the gels. Moreover, the GelMA hydrogels containing the aligned CNTs had a higher conductivity compared with the GelMA-random CNT hydrogels. For instance, the measured currents at an applied voltage of 5 V for the GelMA gels containing the 0.5 mg/mL aligned and random CNTs were 24.2×10^{-7} mA and 1.36×10^{-7} mA, respectively. The higher electrical conductivity of the GelMA gels containing the aligned CNTs in contrast with the GelMA-random CNT hydrogels results from the aligned CNT network within these hydrogels in the direction of applied electric field, which leads to the facile propagation of electrical current. We observed a concentration-dependent in the electrical conductivity of both GelMA-aligned CNT and GelMA-random CNT gels. However, the conductivity values for GelMA-random CNT gels did not show a significant difference except in 1 mg/mL CNT concentration in which a sudden increase in the conductivity was occurred. In the case of GelMA-aligned CNT gels, there was higher control on the position and alignment of CNTs in the gel compared with GelMA-random CNT gels. Therefore, the concentration-dependent conductivity of GelMA-aligned CNT hydrogels was more obvious. The measured impedance values of the underlying hydrogels showed a similar trend to the DC conductivity values (Fig. 2D). For example, the measured impedances at a frequency of 0.2 Hz for the pure GelMA and GelMA gels containing the 1.0 mg/mL aligned and random CNTs were 3.7×10^4, 5.6×10^4, and $8.7 \times 10^4\ \Omega$, respectively. Again, we anticipated a concentration-dependent in the electrical conductivity of both GelMA-aligned CNT and GelMA-random CNT gels. However, here, 0.25 mg/mL CNT concentration was sufficient to minimize the impedance for GelMA-aligned CNT gels. Therefore, further increase in the CNT concentration did not have a significant decrease on the impedance of GelMA-aligned CNT gels, while we still could observe a concentration-dependent in the electrical conductivity for GelMA-random CNT gels. The GelMA-aligned CNT gels, with the highest electrical conductance compared with the pure GelMA and GelMA-randomly dispersed CNT gels, provided the most efficient platform for the ES of cellular structures. ES can be easily performed using the IDA-ITO electrodes deposited below the hydrogels. Interestingly, electrical conductivity of CNTs is stable under cell culture conditions [27], ensuring robust and enduring ES for cellular or tissue constructs using fabricated GelMA-aligned CNT hydrogels.

Mouse EBs generated using the hanging drop technique were seeded in the microwells created on the pure GelMA and GelMA gels containing the 0.5 mg/mL aligned and random CNTs before receiving ES (Fig. 3A). The ES parameters included a frequency of 1 Hz, a voltage of 3 V, and a duration 10 ms for two continuous days. EBs are 3D packed aggregates of stem cells that mimic early stages of embryonic development [28]. Therefore, EBs have been the subject of intense research in fundamental embryology, stem cell biology, and clinical applications [29]. We initially analyzed the viability of EBs on the hydrogels with and without the ES using a live/dead assay (Fig. 3). All the EBs exhibited high cellular viability, indicating that the CNTs and the applied ES were safe for the EBs. Many parameters affect the potential toxicity of CNTs, such as CNT diameter, length, functionalization, and purity. These parameters have been reviewed elsewhere [30]. Here, the CNTs were dispersed in GelMA hydrogels, which significantly reduces their potential cytotoxicity [31]. Interestingly, it has been shown that water-soluble and carboxylated CNTs with diameter 20–30 nm and length 0.5–2 μm, which we studied here can be removed from blood circulation via renal excretion [32]. All together, we confirmed the biocompatibility of GelMA-CNT hydrogels and the applied ES to the mouse EBs.

We further examined the performance of pure GelMA and GelMA gels containing the 0.5 mg/mL aligned and random CNTs in supporting the cardiac differentiation of mouse EBs with and without the ES. The EBs were immunostained to assess Troponin T (a cardiac protein) expression (Fig. 4A). Higher expression of this protein suggests greater cardiac differentiation of EBs [33]. The EBs cultured on the GelMA-aligned CNTs differentiated more towards cardiomyocytes in contrast with the EBs on the pure GelMA and GelMA containing the random CNTs. This effect was more pronounced when the ES was applied. The differentiation results were further confirmed by analyzing the expression of cardiac genes (i.e. Tnnt2, Nkx2-5, and Actc1) (Fig. 4B). Tnnt2 gene has been recognized as a late-stage marker of the cardiogenesis of stem cells [34]. Nkx2.5 appears in the early stages of cardiogenesis and regulates the expression of several cardiac genes [35]. Actc1 is widely expressed in muscle tissues, and it has a major impact on the contractility of cardiomyocytes [36]. The Tnnt2 expression levels were 1.71 ± 1.20, 0.86 ± 0.25, and 2.51 ± 0.19 for the non-stimulated EBs cultured on the pure GelMA and GelMA gels containing the random and aligned CNTs, respectively. ES up-regulated Tnnt2 gene expression to 3.24 ± 0.50, 2.43 ± 0.95, and 10.17 ± 3.39 in EBs on pure GelMA and GelMA gels containing the random and aligned CNTs, respectively. Nkx2-5 and Actc1 gene expression also demonstrated similar trends. The profound impact of ES on the differentiation of stem cells to the cardiomyocytes in the GelMA hydrogels containing the aligned CNTs in contrast with the pure GelMA and GelMA-randomly dispersed CNT hydrogels is largely because of the higher electrical conductance of the GelMA containing the aligned CNT gels in the direction of the applied ES. Moreover, it appeared that the combined effects of the electrical conductivity and Young's modulus of the gels had a predominant effect on the differentiation of stimulated or non-stimulated EBs cultured on the underlying gels. Cells sense and respond to their mechanical environment through the mechanotransduction pathway. The mechanical properties of the ECM, especially the ECM stiffness, lead to conformational changes that affect the binding affinity of cellular integrins that regulate cell behaviors, function, differentiation, and fate [37]. Therefore, the measured differences

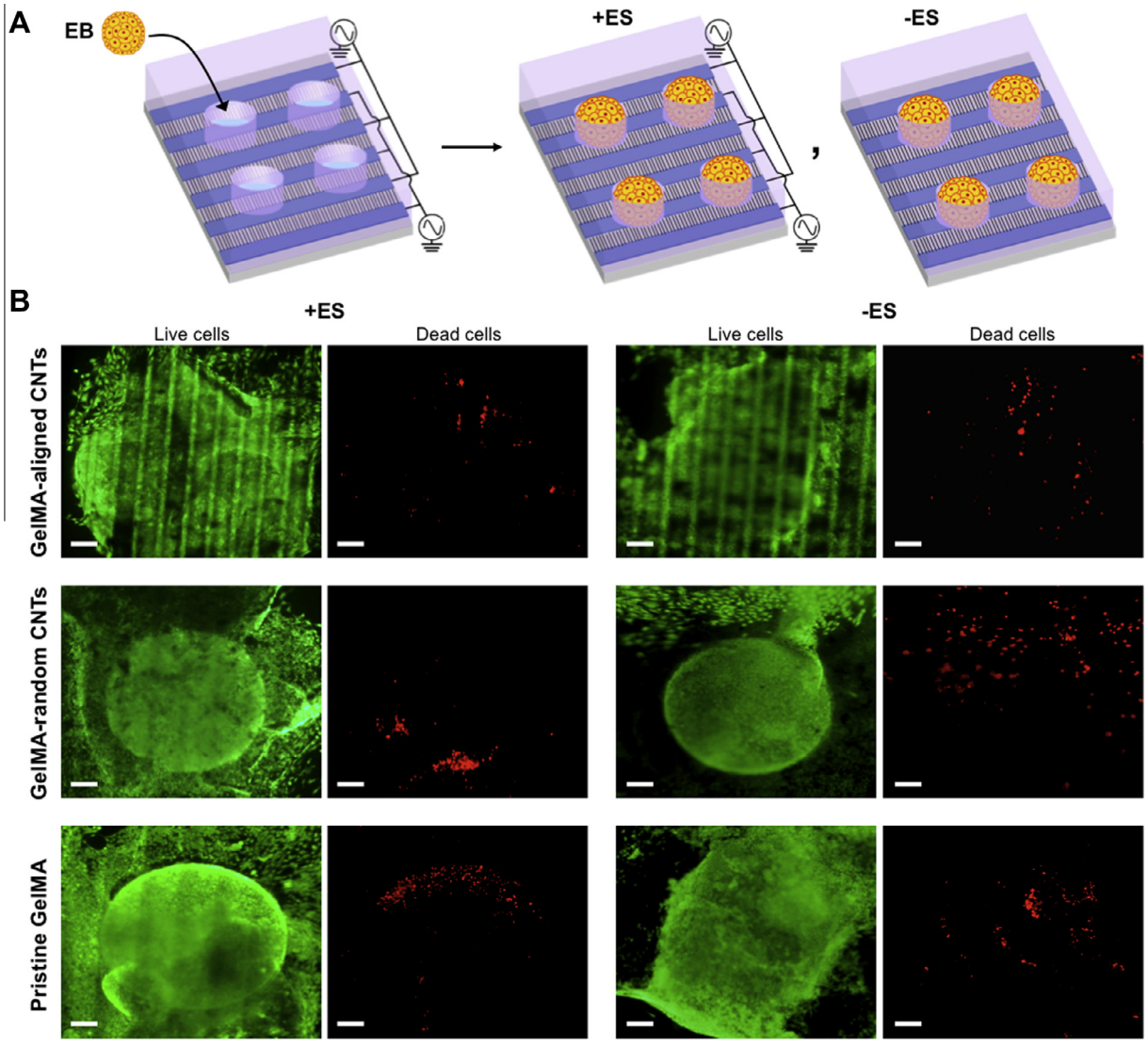

Fig. 3. Evaluation of stem cell viability on pure GelMA and GelMA gels containing the 0.5 mg/mL random and aligned CNTs with and without ES. (A) Schematic of the stem cell culture on the hydrogels and ES. The ES was applied after 2 days of the EB seeding on the gels. (B) Live and dead pictures of EBs cultured on pure GelMA and GelMA-CNT hydrogels with and without the ES (+ES and −ES, respectively). Live and dead cells were shown as green and red colors, respectively. Scale bars, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the stiffness of the underlying hydrogels may affect the cardiac differentiation of stimulated or non-stimulated EBs cultured on these hydrogels.

The beating activity of EBs cultured on the pure GelMA and GelMA gels containing the 0.5 mg/mL aligned and random CNTs with and without applying the ES was recorded and analyzed (Movies S2–S7 and Fig. 5). The ratio of the EB beating area to the whole EB area and the beating frequency of the EBs on various gels were measured. In agreement with the protein and gene analyses, the EBs seeded on the GelMA hydrogels containing the aligned CNTs had greater beating activity in contrast with the EBs on the pure GelMA and GelMA-random CNT gels. The ES further increased the beating activity of the EBs, and the greatest effect was observed for the EBs that were seeded on the GelM hydrogels containing the aligned CNTs. For example, the beating frequency was significantly enhanced (nearly 2-fold) in EBs on the GelMA gels containing the aligned CNTs in response to the ES, whereas a significant increase was not observed for the other EBs. Although the beating frequency of the cardiac muscle cells seeded on these gels was lower than that of native murine cardiac tissue (~10 beats/s) [38], the GelMA-aligned CNT hydrogels had a higher yield than the other hydrogels in generating contractile and functional cardiomyocytes. The employed differentiation medium specifically directed cardiac differentiation in the EBs. Other people have also used this medium to induce cardiac differentiation in embryonic stem cells [39,40]. It is interesting to know which other lineages the EBs tend to differentiate on the underlying hydrogels, which was not the aim of this study. However, it is difficult to judge about this issue based on the difference in electrical conductivity and Young's modulus of the hydrogels. Taken together, the GelMA-dielectrophoretically aligned CNT hydrogels were better at inducing the cardiac differentiation of EBs compared with the pure GelMA and GelMA-random CNT hydrogels, particularly upon the application of ES. It seems that mechanical properties of hydrogels have a minor effect on the cardiac differentiation of the EBs compared with the ES because the gels did not exhibit a wide range of Young's modulus values and all values were slightly lower than the Young's modulus of the mouse heart ECM (~32 kPa) [41]. Significant effect of ES on cardiomyogenic differentiation of stem cells has been reported previously [42]. Functional cardiac tissue requires electrical

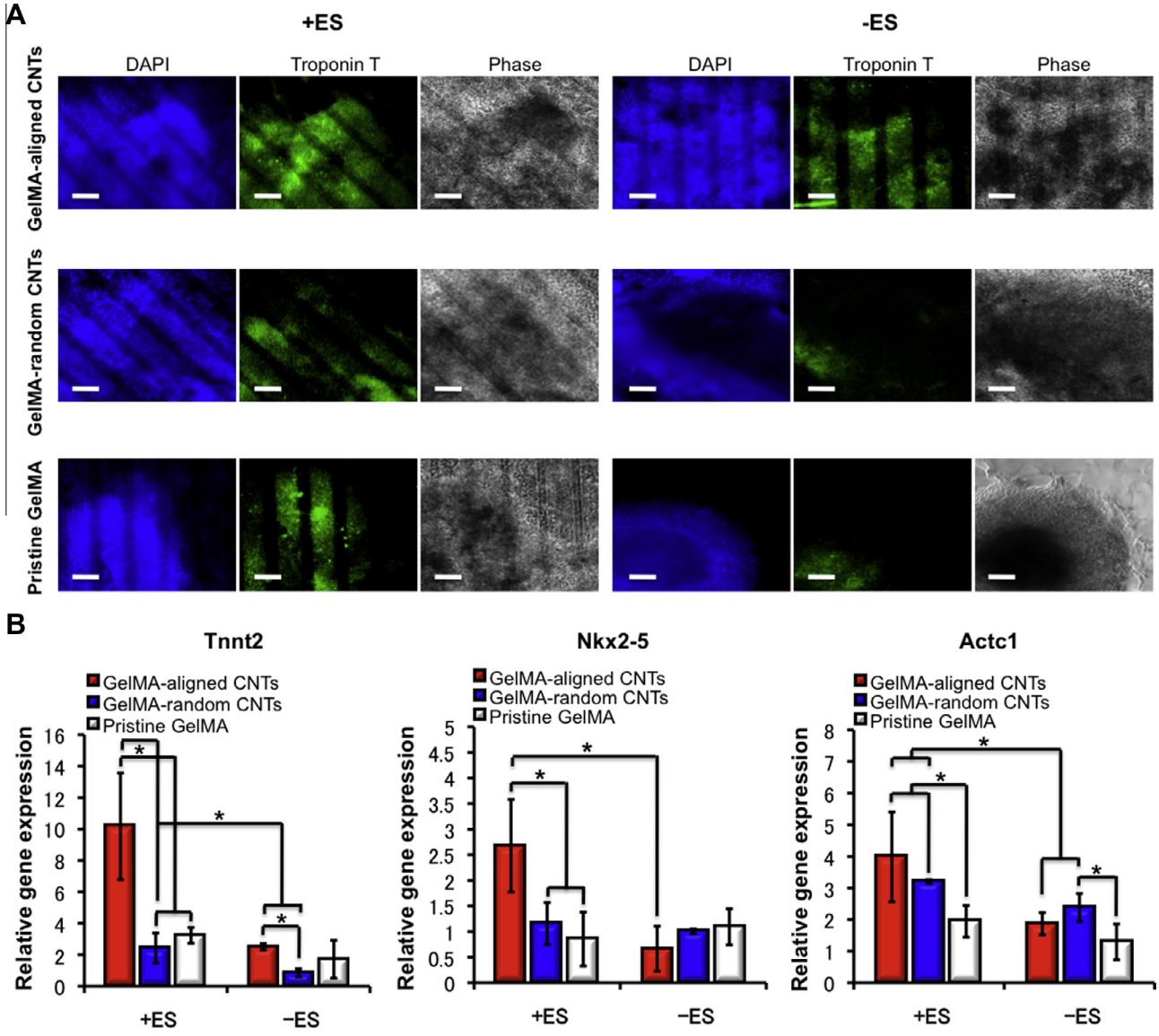

Fig. 4. Analysis of the cardiac differentiation of the EBs on pure GelMA and GelMA gels containing the 0.5 mg/mL random and aligned CNTs with and without ES. (A) Immunostaining of nuclei (DAPI) and troponin T and corresponding phase contrast images of EBs on pure GelMA and GelMA-CNT hydrogels with and without the ES (+ES and −ES, respectively). High expression of troponin T indicates high cardiac differentiation of the EBs. (B) Expression analysis of cardiac genes (i.e. Tnnt2, Nkx2-5, and Actc1) in the differentiated EBs cultured on the pure GelMA and GelMA-CNT hydrogels with and without the ES. Again, high expression of these genes indicates high cardiac differentiation of the EBs. The gene expression values were given as the normalized values to mouse GAPDH expression. Scale bars, 150 μm.

signaling and pathways for efficient cell–cell communication and cooperative contractility [43]. The GelMA-aligned CNT hydrogels may provide such signaling pathways and therefore may develop and maintain the physiological activity and function of cardiomyocytes due to the high and interconnected electrical conductivity of the gels.

In cell-based therapeutics for damaged or diseased cardiac tissue, cardiomyocytes cannot directly be used because they cannot efficiently integrate with the native cardiac tissue, resulting in arrhythmias [44]. In addition, cardiac cell therapy using stem cells requires a pre-differentiation step to avoid the formation of teratomas. The commonly used protocols for cardiac differentiation of stem cells use soluble factors that are often costly and have a low yield [45]. Therefore, more efficient materials or tools are necessary to improve stem cell differentiation toward cardiac cells. Such technologies and materials are of great importance not only for stem cell therapeutic applications but also for fabricating functional cardiac tissues for regenerative medicine, drug discovery and development, disease modeling, and toxicological studies [46]. For instance, Nunes et al. proposed a robust and high-throughput platform (called as biowire) for the maturation of cardiomyocytes [47]. Here, we generated GelMA-dielectrophoretically aligned CNT hydrogels that directed and controlled stem cell differentiation in an effective and reliable manner. In contrast to Matrigel™ or feeder cells as traditional materials to culture stem cells, the developed gel is inexpensive, easy to fabricate, and has defined and tunable physicochemical properties. In addition, the integration of this scaffold with IDA-ITO is an asset for the delivery of spatially controlled electrical signals to stem cells to enhance their cardiac differentiation and function.

4. Conclusions

DEP approach was employed to generate GelMA-aligned CNT hydrogels in a rapid and facile manner. The GelMA hydrogels containing the aligned CNTs exhibited superior performance in sup-

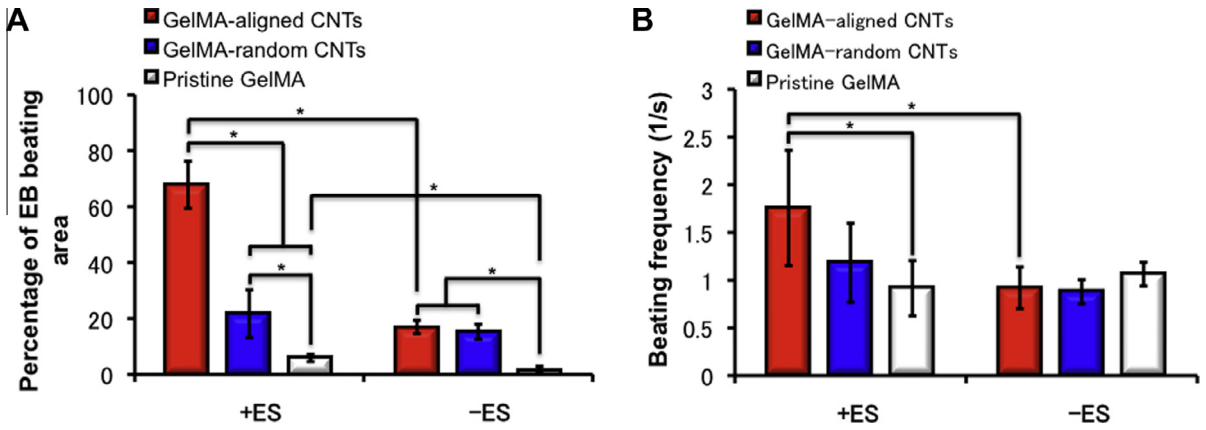

Fig. 5. Beating activity of EBs on pure GelMA and GelMA gels containing the 0.5 mg/mL random and aligned CNTs with and without ES. (A) Percentage of the EB beating area for the EBs on the pure GelMA and GelMA-CNT hydrogels with and without the ES (+ES and −ES, respectively). (B) The beating frequency of the differentiated EBs cultured on the pure GelMA and GelMA-CNT hydrogels with and without the ES. These parameters were calculated from spontaneous beating of the EBs.

porting the cardiac differentiation of EBs in contrast with the pristine GelMA and GelMA-random CNT gels, as confirmed by the protein and gene expression analyses and the beating activity of the EBs. The obtained cardiomyocytes may be useful in broad applications within regenerative medicine and cell therapy.

Disclosures

The authors declare no conflict of interest.

Acknowledgments

S.A. conceived the idea, designed the experiments, and analyzed the results. M.E. functionalized the CNTs and performed the I–V measurements. X.L. helped with the AFM measurements under the supervision of K.N. S.Y. and S.A. performed all other experiments. S.A. wrote the paper. H.S., T.M., and A.K. analyzed the results and supervised the project. All authors read the manuscript, commented on it, and approved its content. This work was supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.11.047.

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