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PAPER

Gelatin methacrylate as a promising hydrogel for 3D microscale organization and proliferation of dielectrophoretically patterned cells†

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Establishing the 3D microscale organization of cells has numerous practical applications, such as in determining cell fate (*e.g.*, proliferation, migration, differentiation, and apoptosis) and in making functional tissue constructs. One approach to spatially pattern cells is by dielectrophoresis (DEP). DEP has characteristics that are important for cell manipulation, such as high accuracy, speed, scalability, and the ability to handle both adherent and non-adherent cells. However, widespread application of this method is largely restricted because there is a limited number of suitable hydrogels for cell encapsulation. To date, polyethylene glycol-diacrylate (PEG-DA) and agarose have been used extensively for dielectric patterning of cells. In this study, we propose gelatin methacrylate (GelMA) as a promising hydrogel for use in cell dielectropatterning because of its biocompatibility and low viscosity. Compared to PEG hydrogels, GelMA hydrogels showed superior performance when making cell patterns for myoblast (C2C12) and endothelial (HUVEC) cells as well as in maintaining cell viability and growth. We also developed a simple and robust protocol for co-culture of these cells. Combined application of the GelMA hydrogels and the DEP technique is suitable for creating highly complex microscale tissues with important applications in fundamental cell biology and regenerative medicine in a rapid, accurate, and scalable manner.

Introduction

Microscale technologies have emerged as potentially useful tools in tissue engineering and biological applications. Such technologies render precise positioning for cells in order to define interactions of cells with their surrounding environment, mimicking the structure of native tissue constructs. They can be used in modulating different aspects of the cell behavior and in understanding the underlying biology.[1] The dielectrophoresis (DEP) method is one of various microscale technologies used in tissue engineering and in manipulating biological particles, such as DNA, proteins, and mammalian cells. DEP stands as a powerful microscale technology that could be used in combination with new biomaterials to embed cells within a given pattern. This allows precise positioning of cells in order to define cell–cell and cell–extracellular matrix (ECM) interactions. In this technique, particles are manipulated on the basis of the their interactions with an AC electric field, leading to a charge polarization in the particles and their surrounding medium.[2–4] It was first proposed by Pohl in 1951.[5] When an underlying particle is more polarizable than its surrounding medium, the net force directs the particle towards high electric field regions. This phenomenon denotes the positive DEP (p-DEP). The so-called negative DEP (n-DEP) occurs while the particle has less polarizability than its suspending medium in the presence of a non-uniform electric field and is characterized by the escape of the particle from high electric field regions.

A recent survey demonstrated that there are over 1000 publications utilizing the DEP technique in cellular applications encompassing 4 major categories, namely, cell characterization, cell separation, cell manipulation, and cell patterning, with over 700 papers published in the last decade.[6] The use of DEP forces for cell patterning has not been broadly followed since it was initially suggested by one of us (Professor Matsue) in 1997.[7] The

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reason is that the DEP forces are temporary and disappear when the electric field is switched off. Therefore, an immobilization step is needed after applying the DEP forces in order to keep the cells in a fixed position. Some immobilization strategies include the incorporation of cell-adhesive proteins,[8] trapping of the cells in microwells,[9] and the use of cross-linking agents. However, these methods often need a long exposure time to the electric field to generate good adhesion of the cells to the substrate; therefore, they are not well suited for mammalian cells. Recently, a promising approach to restraining dielectrophoretic patterned cells has been proposed; this particular method is based on the encapsulation of cells within hydrogel biomaterials.[11,12] We have also employed this approach to encapsulate colloidal particles patterned by n-DEP.[13] One of the main advantages of using hydrogels is that the cells encapsulated in hydrogels sense a 3D physical environment, which is a useful tool for generating cell-based assays (*e.g.*, cell-based biosensors[14]) and tissues that accurately model and predict *in vivo* behavior. Application of the DEP technique in the field of tissue engineering has recently gained more attention since it is simple to employ, scalable, capable of handling the cells in a high throughput manner, and provides the reconstruction of versatile and native cell patterns *in vitro*.[15] The DEP method, in conjunction with microfluidic-based 3D cell culture systems, has also been used to fabricate 3D tissue microstructures.[16]

Hydrogels are attractive soft materials for biological applications owing to their high water content and biocompatibility.[17] For many years, they have attracted a great deal of attention, and these materials have been designed, synthesized, and employed for numerous biological applications, for example as scaffolding in regenerative medicine,[18] drug delivery components,[19] and biosensors.[20] A novel use of hydrogels is immobilization of cells patterned by DEP forces.[11,12] A low-viscosity, low-conductivity hydrogel is required for efficient 3D cell patterning within hydrogels using DEP forces. However, many conventional natural or synthetic hydrogels such as collagen, alginate, and Matrigel™ are viscous, or hard to cross-link in a closed environment; therefore, they are not suitable for use with DEP electropatterning. To date, polyethylene glycol-diacrylate (PEG-DA) and agarose have been found to be the most suitable hydrogels for dielectric patterning of cells. However, these hydrogels do not support cell viability, spreading, migration, and function for a long culture time. One approach to tackle this problem is to make cell-containing microgels, which are then mixed with a bulk-phase agarose or PEG-DA precursor solution.[11] In this example, application of p-DEP forces first localized these microgels to regions of high electric field. The gels were then trapped in place by exposure of the bulk-phase polymer to light or a change in temperature. Compared to single-phase systems, this technology supported higher viability of liver progenitor cells.

In this study, we suggest the application of gelatin methacrylate (GelMA)[21] to fabricate 3D cell patterns using DEP. GelMA is a photopolymerizable semi-natural hydrogel comprised of modified gelatin with the methacrylic anhydride, and it is an attractive biomaterial for cell-based studies and tissue engineering applications. It can be synthesized by conjugating methacrylate groups to the amine-containing groups of natural gelatin. Gelatin is an inexpensive material made of denatured collagen and is comprised of natural cell-binding motifs such as RGD and matrix metalloproteinase (MMP)-sensitive degradation groups.[22] In our previous work,[23] we showed that, just by precise control of the microgeometry of micropatterned cell-laden 3D GelMA hydrogels as high-aspect-ratio rectangular constructs, it is feasible to attain high cellular alignment and elongation in the whole engineered tissue constructs along with long-term cell viability. The system we described has been successfully applied to some cell types (*i.e.*, human umbilical vein endothelial cells (HUVECs), rodent myoblasts, and rodent cardiac side population cells) since all of these cells require highly organized and elongated ECM–cell constructs to accurately mimic the corresponding complex organized microarchitectures *in vivo*. Due to the low viscosity and ion concentration of the GelMA hydrogel, we hypothesize that it could be a promising candidate for establishing 3D microorganization of cells, with long-term cell viability, by using the DEP method.

Materials and methods

Chemicals

Indium tin oxide (ITO) glass slides were provided by Sanyo Vacuum Industries Co., Ltd. (Tokyo, Japan). FITC-labeled polystyrene particles (diameter 6 μm) were provided by Polysciences, Inc. (Washington, PA, US). Hexamethyldisilazane was purchased from Tokyo Ohka Kogyo Co., Ltd. (Kanagawa, Japan). Positive g-line photoresist (*i.e.*, S1818) and developer (*i.e.*, MF CD-26) were obtained from Shipley Far East Ltd. (Tokyo, Japan). Methacrylic anhydride, 3-(trimethoxysilyl)propyl methacrylate, trichloro (1H, 2H, 2H-perfluorooctyl)silane, 3,3,4,4,5,5, 6,6,6-nonafluorohexyl trichlorosilane, gelatin (Type A, 300 bloom from porcine skin), and PEG-DA (MW: 4000) were provided by Sigma-Aldrich Chemical Co. (St. Louis, MO, US). 2-Hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (*i.e.*, Irgacure 2959) was purchased from Ciba Chemicals (Osaka, Japan). All other chemicals (reagent grade) were supplied by Wako Chemicals (Osaka, Japan) and used as they were received.

Gelatin methacrylate synthesis

GelMA hydrogel was synthesized as detailed in our previous work.[21] A high degree of methacrylation (~80%) was obtained by adding 8 mL of methacrylic anhydride to 10 g of gelatin in 100 mL of phosphate-buffered saline (PBS). The mixture was dialyzed in a 12–14 kDa cutoff membrane in the distilled water for one week at 40 °C and then lyophilized for one week. The GelMA prepolymer was stored at −20 °C until use.

Cell culture

An immortalized rodent myoblast (C2C12) cell line and HUVECs were provided by RIKEN Bioresource Center Cell Bank, Japan. HUVECs were cultured in the endothelial basal medium (EBM-2; Lonza) as supplemented with endothelial growth BulletKit (Lonza) and 1% penicillin/streptomycin (P/S) and were used or sub-cultured while they reached 70–80% confluence. The medium was changed once every 2 days. C2C12 cells were cultured in the Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS and 1% P/S and were used or passaged for future experiments when 70–80%

confluency was achieved. All cells were maintained in a standard cell culture incubator (Sanyo, Japan) with 5% CO_2 atmosphere at 37 °C.

Prepolymer preparation

GelMA prepolymer (10% w/v) was combined with sucrose buffer and 1% (w/v) photoinitiator (*i.e.*, Irgacure 2959), kept at 60 °C until fully dissolved, and then used for the experiments. Sucrose buffer was made from 0.25 M sucrose, 0.06 M D-glucose, 1×10^{-3} M $CaCl_2$, 2×10^{-3} M NaCl, 1×10^{-4} M KCl, 2×10^{-5} M $MgSO_4$, and 2×10^{-5} M NaH_2PO_4 at pH 7.2. Similarly, 40% (w/v) PEG-DA (MW: 4000) prepolymer was mixed in sucrose buffer with 2% (w/v) Igracure 2959 and kept at 40 °C until it fully dissolved.

Design and fabrication of the DEP device

A closed chamber was constructed to establish an AC electric field required for the dielectrophoretic cell patterning. Two dielectrophoretic patterning devices were constructed. For both devices, the effective electrode dimensions were 8×12 mm^2 as shown in the ESI, Fig. S1-A.† In the first device, the band electrode was 50-μm wide and had a 50-μm gap (from now on referred to as the 50-μm electrode–50-μm gap device) and in the second device, the band electrode was 50-μm wide with a 100-μm gap (from now on referred to as the 50-μm electrode–100-μm gap device). The schematic picture of the first design can be found in the ESI, Fig. S1-A.† The electrodes were patterned on the glass slide (thickness 1 mm; Matsunami Co., Japan) by the conventional photolithography procedure. Hexamethyldisilazane and S1818 were poured onto the glass slide and it was baked at 90 °C for 10 min. It was then irradiated by UV light through a mask aligner (MA-20; Mikasa Co. Ltd., Tokyo, Japan), and developed at MF CD-26. A Ti adhesive layer followed by a Pt film (thickness, 100 nm) were then seeded onto the glass slide. The electrode design was revealed by the lift-off technique. ITO glass was mounted on the top of this substrate to define the chamber for the DEP experiments. The chamber height was adjusted by a PET film spacer (thickness, 50 μm; Lintec Co., Japan) as shown in the ESI, Fig. S1-B.†

Numerical modeling

The electric field distribution within the fabricated device was computed using the finite element analysis as implemented in COMSOL Multiphysics 4.2, Stockholm, Sweden, for 4 different device designs (*i.e.*, 15-μm electrode-15-μm gap, 50-μm electrode-35-μm gap, 50-μm electrode–50-μm gap, and 50-μm electrode–100-μm gap devices). Potential difference and distance between the ITO electrode and the band electrode were set at 4 V and 50 μm, respectively. The conductivity of both GelMA and PEG-DA hydrogels in the sucrose buffer was determined to be 8 mS m^{-1}, as measured by the SG 3 conductimeter (Mettler Toledo, Zürich, Switzerland). The viscosity of the hydrogels was 0.111 cm^2 s^{-1} for 20% PEG-DA and 0.09 cm^2 s^{-1} for 5% GelMA, as reported in our previous work.[24]

DEP patterning of cell-laden hydrogels

AC voltage was exposed to the template interdigitated array (IDA) of Pt (Pt-IDA) electrodes using a waveform generator (No. 7075, Hioki EE Co., Japan). An oscilloscope (Wave surfer 424; LeCroy Co., Japan) was used to confirm the generated electric current. The n-DEP-induced behavior of the cells was created and recorded by using an optical microscope (IX71; Olympus Co., Japan) equipped with a digital CCD camera (C5985; Hamamatsu Photonics K.K., Shizuoka, Japan) and a Pentium IV computer.

To ensure the micropattern fidelity as depicted in Fig. 1-A, a PEG layer (thickness, ~1 μm) was created on the Pt-IDA prior to the DEP experiments. To do so, the PET film spacer (thickness, 1 μm) was inserted between the glass and Pt-IDA electrode. The chamber was then filled with PEG-DA prepolymer and UV light was irradiated from the underside of the device for 5 min, using the Pt electrode as the template. Because the Pt electrode is nontransparent, only the prepolymer solution in the gap regions of the device was polymerized upon exposure to the UV light. After removing the glass and rinsing the remaining unreacted prepolymer with PBS, the PEG-coated Pt-IDA electrode was ready for the DEP experiments. To perform the dielectrophoretic cell patterning experiments, the chamber was fabricated with the Pt-IDA electrode and the ITO electrode, which was treated with 3-(trimethoxysilyl)propyl methacrylate for 1 h in a vacuum. The 3-(trimethoxysilyl)propyl methacrylate was used to improve the polymerization of the GelMA hydrogel and improve the attachment of the hydrogel to the ITO surface. The PEG layer in the gap region was also helpful to remove the upper surface maintaining the cell pattern.

Just prior to the DEP experiments, the cells were removed from the culture flask, counted, and suspended in sucrose buffer, and then mixed with the hydrogel prepolymer (1 : 1 ratio). Therefore, the final hydrogel concentration was 5% and 10% for the GelMA and PEG-DA hydrogels, respectively. The hydrogels were placed into a water bath at 37 °C before and during the dielectrophoretic cell patterning to maintain the liquid viscosity. The cell density was kept at 1×10^7 cells mL^{-1}. Twenty microliters of the cell-laden hydrogel (PEG-DA or GelMA) were pipetted between the ITO glass slide and the Pt electrode to fill the chamber volume ($8 \times 12 \times 0.05$ mm^3). A sinusoidal AC signal (10 kHz, 4 V) was applied to each microband array of the Pt-IDA template to align the cells within the periodical line patterns. An independent AC signal with the opposite phase with regard to the external ground was also applied to the ITO electrode. The cells were aligned within 3 min within the gap regions of the Pt-IDA electrode. Movie S1 in the ESI† shows the time evolution of cell patterning. The patterned cells within the GelMA hydrogel were then exposed to 365 nm UV light (3.5 mW cm^{-2}) (UVGL-25; UVP, LLC, Cambridge, UK) for 30 s. The UV exposure time for the PEG-DA was 4 min. Following polymerization, the patterned cells were gently removed from the Pt-IDA electrode and cultured on the ITO glass slide for up to 1 week in 35-mm Petri dishes (Milian) in a standard incubator by using the specific medium for each cell type. The medium was refreshed every 48 h. The procedure described here can be visualized in Fig. 1-B and C.

Assessment of cell viability

The calcein AM/ethidium homodimer live/dead assay (Invitrogen Co., Carlsbad, CA, US) was employed to quantify the cell viability

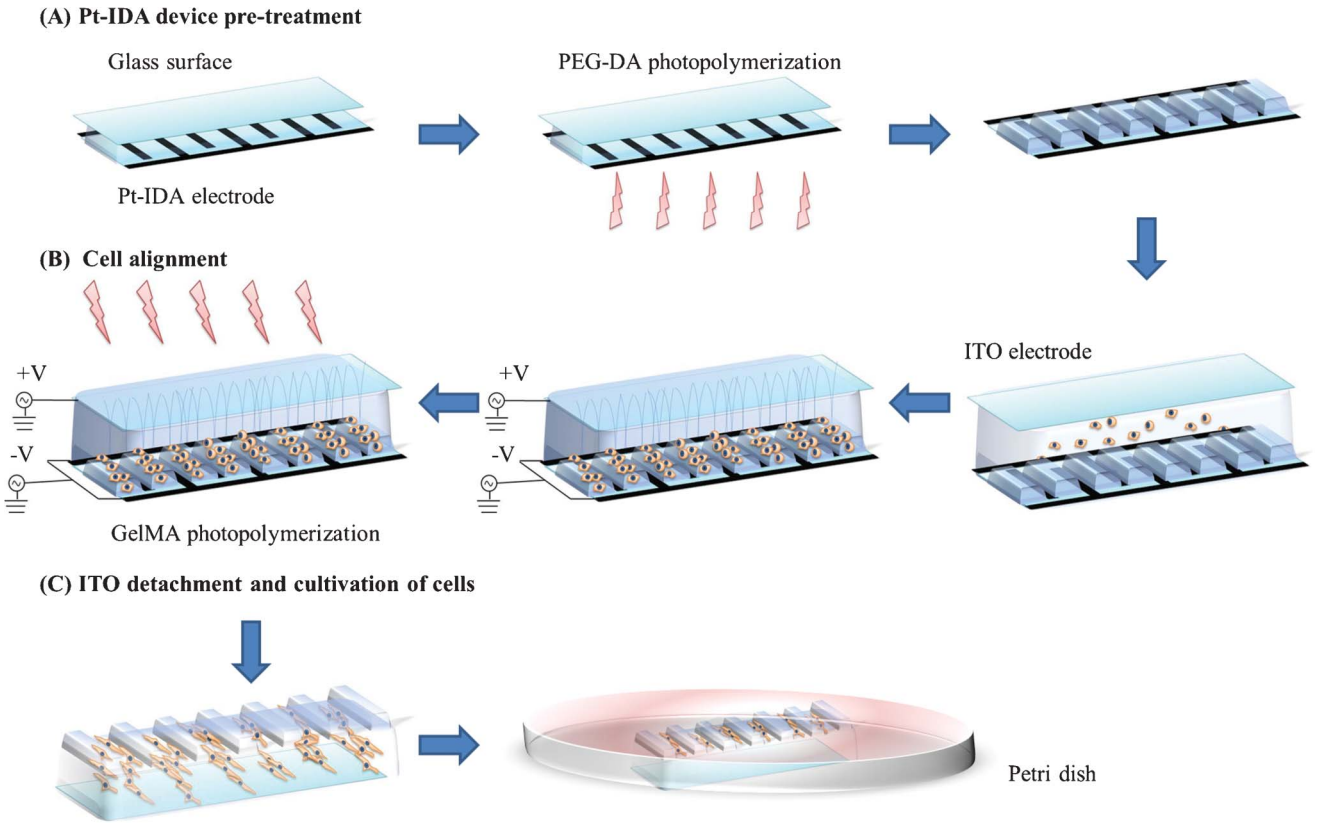

Fig. 1 Schematic representation of microscale cell alignment by dielectrophoresis (DEP) in gelatin methacrylate (GelMA) prepolymer. (A) A polyethylene glycol (PEG) layer was fabricated on the Pt-IDA electrode prior to the DEP experiments. The chamber was defined by a PET film with 1-μm thickness between the glass and the Pt-IDA electrode. The chamber was filled with PEG-diacrylate prepolymer and irradiated with UV light. Because the Pt electrode was nontransparent, it plays the role of the photomask, and therefore, only the prepolymer solution within the electrodes was polymerized. Unpolymerized prepolymer was washed out with PBS. (B) Cells in GelMA prepolymer solution were introduced in the 50-μm-high chamber and localized by n-DEP forces to the gap regions between the Pt-IDA electrodes on application of 4 V AC field. GelMA prepolymer was then exposed to UV light, embedding the cells in a stable microscale engineered organization. (C) Aligned cells within the GelMA polymer were removed from the Pt-IDA electrode and cultured.

encapsulated in the hydrogels according to the manufacturer instructions. Calcein AM is a cell-permanent dye that is changed to green fluorescent calcein in live cells through the action of intracellular esterases. Ethidium homodimer is a DNA-binding dyestuff that enters the damaged membrane of dead cells. Calcein AM/ethidium homodimer fluorescence colors were observed by using a fluorescence microscope (Carl Zeiss, Observer Z1). The NIH ImageJ software package (available free of charge at http://rsbweb.nih.gov/ij/) was used to quantify the fluorescence images of live/dead cells, and at least 5 images of 2 independent experiments were used for the quantification of cell viability.

Quantification of cellular alignment and elongation

Cells within hydrogels were fixed with 3–4% paraformaldehyde for 12 min, following a wash with PBS. 0.3% Triton X-100 was used to make the permeable cells for 5 min at room temperature and then placed in 5% bovine serum albumin (BSA) dissolved in PBS for 15 min. The samples were stained with phalloidin (AlexaFluor® 594, Invitrogen, US) and 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc., Burlingame, CA, US) as recommended by the manufacturers, in order to visualize filamentous F-actin and cell nuclei, respectively. The stained samples were observed with the fluorescence microscope. The nuclear shape index (or circularity) and alignment of DAPI-stained cell nuclei were quantified to evaluate the average cell elongation and alignment. The nuclear alignment angle was defined as the orientation of the major elliptic axis of individual nuclei to the vertical pattern line and it was measured using the ImageJ software package. All measured nuclear alignment angles were then normalized to the preferred nuclear orientation to define the mean orientation of all nuclei per samples. The alignment angles were subsequently categorized in 10-degree increments with respect to the preferred nuclear orientation and all cells within less than 10 degrees counted as the aligned cells. In addition, the nuclear shape index (circularity = $4 \times \pi \times$ area/perimeter^2) of each individual cell nucleus, showing the nuclear elongation, was quantified using the ImageJ software package. A shape index of 1 represents a non-elongated cell. At least 200 cell nuclei were considered to determine the cell alignment and circularity parameters and all calculated values were checked manually to ensure the results were accurate.

Co-culture of C2C12 and HUVEC cells

The protocol for dielectrophoretic cell patterning was followed as explained above, with some modifications. After patterning the C2C12 cells using the DEP technique, the GelMA

prepolymer was polymerized with UV light underneath the device for 30 s by using the Pt-IDA electrode as the template. After removing the Pt-IDA electrode and rinsing the non cross-linked prepolymer with PBS, the new cell type suspended in the culture medium was added onto the device and incubated for 15 min. The device was then gently washed out with PBS to trap the HUVEC cells just within the C2C12 cell patterns. The procedure described here can be visualized in the ESI in Fig. S4.† The delineated process was also performed for FITC-labeled polystyrene particles and C2C12 cells.

Statistical analysis

Statistically significant differences were revealed by the independent Student *t* test for 2 groups of data by using the MINITAB 16.0 statistical software package (Minitab Inc., State College, PA, US). All data are represented by average ± standard deviation, and *p*-values less than 0.05 were deemed to be statistically significant.

Results

Modeling and optimizing the DEP experiments

Maintaining cell viability is an important criterion when applying dielectrophoretic force. When cells are subjected to a dielectrophoretic force, cell damage occurs for two main reasons: (i) there may be excessive charging of the cell membrane exposed to the electric field;[25] (ii) the cells are suspended in a non-physiological medium. To avoid the first problem, n-DEP, which was employed in this investigation, is preferable to p-DEP for cell patterning because the cells are trapped away from high electric field regions and are less likely to experience high temperatures and large potentials at the cell membrane. To minimize the dielectrophoretic force on the cells, the cells' velocity during their exposure to the non-uniform electric field was recorded. This approach has been used to quantify the dielectrophoretic force on particles and cells. Accordingly, the speed and direction of cell movement in the electric field at different frequencies were measured as shown in the ESI, Fig. S2.† Here, the aim was to minimize the applied voltage and to adjust the frequency to obtain the maximum cell velocity value. 4, 8 and 12 V were tested for the experiments. A decrease in the cell viability (around 40%) was observed for 8V, and with 12 V the cell viability decreased dramatically (data not shown). As a result, 4 V was recognized to be strong enough to manipulate cells inside the chamber. The maximum repulsion speed when applying this voltage was obtained at a frequency of 10 kHz. The second problem results from the fact that an effective DEP cell manipulation needs a low conductive buffer that is non-optimal for cell viability and function. In order to find the optimum DEP buffer, several buffers with different conductivities were tested, while keeping the cells in each of them for 1 h. The results obtained are reported in the ESI, Table S1.† From this experiment, sucrose buffer was chosen for further experiments since it showed low conductivity and a high percentage of live cells.

Design of the electrode device is a key factor in the development of DEP systems. Mathematical modeling of DEP phenomena helps to optimize the electrode design prior to the DEP experiments, leading to considerable savings in the time and cost of experiments. As mentioned previously, finite element analysis was used to simulate the induced electric field for different Pt-IDA electrode designs. The results are demonstrated in the ESI, Fig. S3.† The area of low electric field within the electrode lines where the cells tend to be seeded depends on the distance between neighboring electrodes. The results clearly show that the minimal distance between 2 neighboring electrodes to maintain cells in those areas is 50 μm. Thus, in further experiments, only 2 of 4 underlying designs (*i.e.*, 50-μm electrode–50-μm gap and 50-μm electrode–100-μm gap devices) were employed.

Dielectrophoretic cell patterning

As recommended by Albrecht *et al.*,[12] surface modification of electrodes in the DEP experiments using trichloro(1H, 2H, 2H-perfluorooctyl)silane prevents the adhesion of PEG to the electrode surface. We followed this procedure to make a non-adhesive surface for the GelMA hydrogel. However, the obtained pattern fidelity was low. Even a more hydrophobic silane (*i.e.*, 3,3,4,4,5,5,6,6,6-nonafluorohexyl trichlorosilane) was not found to be effective. The reason may be the free amino groups in the GelMA hydrogel, which cause adhesion between the silanes and the GelMA hydrogel itself. In order to tackle this problem, the Pt-IDA electrode surface was treated prior to the DEP experiments with a thin layer of PEG (See Fig. 1-A) to facilitate easy detachment of the GelMA polymer from the electrode surface. Fig. 2 shows the microscopic images of the aligned C2C12 cells at different culture times. The C2C12 cells were patterned within the 5% GelMA hydrogel with the aid of the DEP method, and this helped preserve the cell patterns with high fidelity. Since cells can easily proliferate in the GelMA hydrogel, after a few days of culture a highly patterned tissue was obtained. The same trend was observed in the case of HUVEC cells within GelMA hydrogel, except that the proliferation rate for the HUVEC cells in the GelMA hydrogel was less than that for the C2C12 cells in GelMA, as shown in Fig. 3 and 4. In contrast to the patterned cells in the GelMA hydrogel, C2C12 and HUVEC cells embedded in 20% PEG could not grow and keep their shape during the culture period, as shown in the ESI in Fig. S5.†

Viability of the dielectrophoretic patterned cells within GelMA and PEG hydrogels

The viability of the dielectrophoretic patterned cells was investigated by a live/dead assay. The experiment was performed using a 50-μm electrode–50-μm gap device (Fig. 3) and a 50-μm electrode–100-μm gap device (Fig. 4) for different culture times. The viability of the cells was higher for the 50-μm electrode–100-μm gap device. These results show the importance of the distance within electrodes and cells to improve the cell viability with n-DEP patterning experiments. The C2C12 and HUVEC cells encapsulated in GelMA hydrogel can readily proliferate and the number of live cells increases with respect to the culture time. It was observed that PEG did not support the cell viability of either C2C12 or HUVEC cells for long period. The viability of C2C12 and HUVEC cells in the PEG hydrogels decreased as a function of culture time and, after 5 days of culture, it reached less than

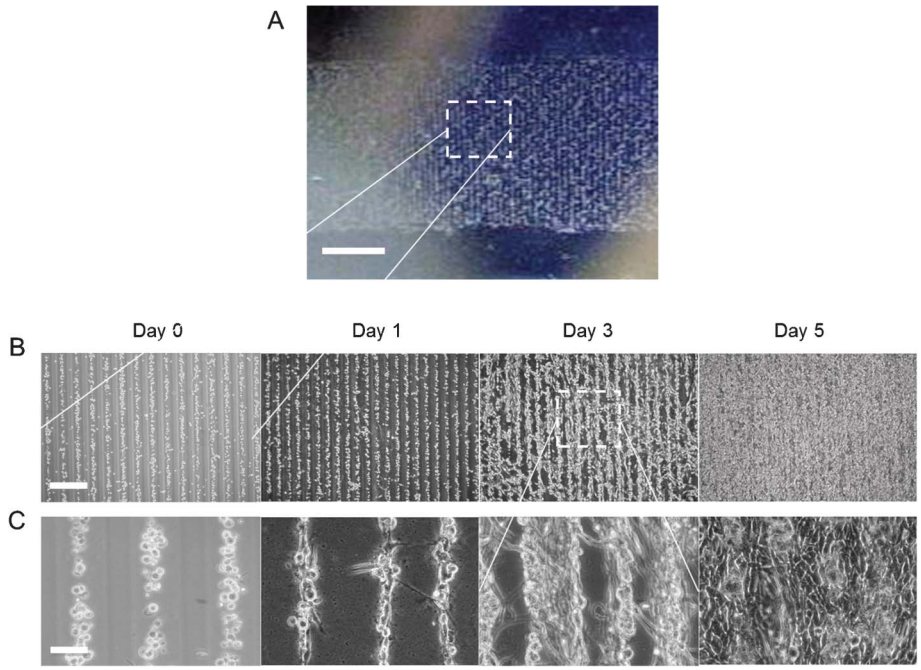

Fig. 2 Picture of aligned C2C12 muscle cells within 5% gelatin methacrylate (GelMA) hydrogel as obtained by the dielectrophoresis (DEP) technique using the 50 μm electrode–50 μm gap device (A). Phase contrast images of the aligned C2C12 muscle cells within 5% GelMA hydrogel at different culture times (B and C). Scale bars show 0.25 cm, 400 μm, and 50 μm in A, B, and C, respectively.

10% for both C2C12 and HUVEC cells. It is intriguing to note that there is a statistically significant discrepancy ($p < 0.001$) between the cell viability of the cells in GelMA and PEG hydrogels even after 1 day of culture.

Evaluation of cell alignment and elongation within GelMA and PEG hydrogels

Fabrication of a highly organized cell structure, which recapitulates the function and structure of the corresponding tissue *in vivo*, is important for making many functional tissues. Biomaterials and methods to construct highly aligned cells and, consequently, tissues are a subject of great interest among researchers who are working in the field of regenerative medicine. Therefore, we investigated the alignment and elongation of C2C12 and HUVEC cells in GelMA and PEG hydrogels. Fig. 5 shows the fluorescence images of filamentous F-actin and cell nuclei of the patterned cells. As can be observed, the cells in the GelMA hydrogel grew along the specified pattern direction and remained aligned during the culture period.

Quantitatively determined nuclear alignment and elongation of C2C12 and HUVEC cells in GelMA and PEG hydrogels is shown in the ESI in Fig. S6 and S7.† As mentioned previously, the degree of alignment is defined as the normalized number of cells within less than 10 degrees deviation with respect to the desired nuclear orientation. The degree of alignment for both C2C12 and HUVEC cells in GelMA and PEG hydrogels was around 40%. Here, both GelMA and PEG are shown to be suitable candidates for preserving cell alignment using the DEP technique, and there is no statistical difference between the degrees of alignment for both hydrogels. Notice that the degree of alignment for unpatterned cells within the hydrogels was about 10% (data not shown).

Another important factor to judge cell growth is to track cell circularity. A decrease in the circularity of the cells with respect to the time implies tendency of the cells to elongate and proliferate. The results, as indicated in ESI Fig. S7 and S8†, clearly demonstrate that the cells were able to proliferate within the GelMA hydrogels; however, the underlying PEG hydrogels were not able to provide an appropriate environment for the cells to proliferate. There is no statistical difference between the cell types with regard to cell circularity after 1 day of culture. However, in the case of C2C12 cells, after 3 days of cell culture, a significant discrimination between the cell circularity in GelMA and PEG hydrogels was observed ($p < 0.05$ for the 50-μm electrode–50-μm gap device and $p < 0.01$ for 50-μm electrode–100-μm gap device). This discrepancy increases after 5 days of culture and reaches $p < 0.001$ for both the 50-μm electrode–50-μm gap and the 50-μm electrode–100-μm gap devices. The proliferation rate for HUVEC cells was less than that for C2C12 cells. Therefore, the difference between the cell circularity in GelMA and PEG hydrogels for this cell type was detectable only after 5 days of culture. The corresponding *p*-values for the 50-μm electrode–50-μm gap and the 50-μm electrode–100-μm gap devices were measured to be less than 0.01 and 0.05, respectively.

Co-culture of C2C12 and HUVEC cells

C2C12 cells were aligned and trapped following the same protocol described above; however, to facilitate the trapping of the second cell type (HUVEC cells), the polymerization procedure was performed from the underside of the device, using the Pt-IDA electrode as the photomask. Therefore, there were grooves between the C2C12 pattern lines where the HUVEC cells could settle. As can be seen in Fig. 6, C2C12 cells were aligned inside the GelMA hydrogel and some HUVEC cells were trapped between them. An

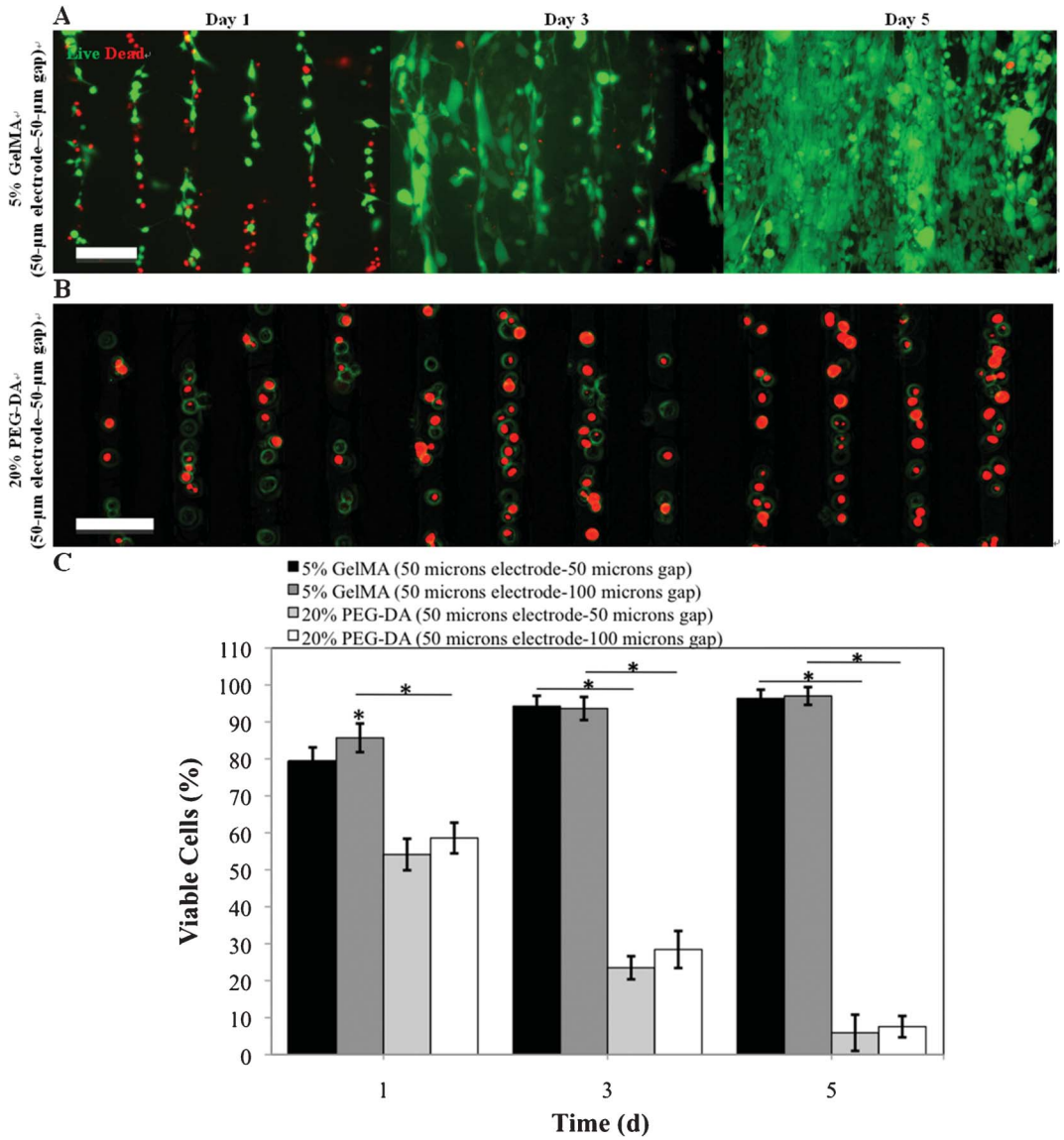

Fig. 3 Results of the live/dead assay for the patterned C2C12 cells encapsulated in 5% gelatin methacrylate (GelMA) (A) and 20% polyethylene glycol (PEG) (B) using a 50-μm electrode–50-μm gap device for different culture times. Since the cells can proliferate within the GelMA hydrogel, an increase in cell viability as a function of culture time was observed for the GelMA hydrogel. However, the cells within the PEG are not able to grow and migrate; the cell viability was therefore decreased. Scale bar shows 100 μm (*$p < 0.001$).

interesting point is that the dielectrophoretic cell patterning was more precise than the conventional approach to seed the HUVEC cells inside the constructed micropattern. The robustness of the delineated process is also shown in the case of FITC-labeled polystyrene beads and C2C12 cells as a co-culture model.

Discussion

The DEP technique is as an efficient tool to direct cells to a state in which specific cell functions can be promoted. For instance, Ho *et al.* designed a DEP device that could biomimetically pattern hepatic and endothelial cells to mimic the function of liver cells.[26] However, versatile application of DEP in tissue engineering has been limited by a lack of suitable materials and methods to maintain the dielectrophoretic patterned cells after removing the electric field.

Dielectrophoretic patterned particles can be immobilized on substrates by covalent bonding through cross-linkers such as disuccinimidylsuberate[10] and succinimidyl 4-(*p*-maleimidophenyl)-butyrate.[13] In the former case, it was found that an aggregation of particles and a random reaction between the activated substrate and particles reduced the precision of the pattern.[10] Additionally, both cross-linkers required a considerable amount of time (in the order of a few hours) to be effective. Therefore, such techniques are not suitable for immobilizing the dielectrophoretic patterned cells after switching off the AC current. Another approach for immobilizing dielectrophoretic patterned cells is to allow the cells to aggregate while the electric current is on. Such cell aggregates remain stable after removal of the electric field.[27] In addition to requiring a considerable amount of time (10–15 min) for aggregation, this stabilization method significantly limits the control over the size and shape of

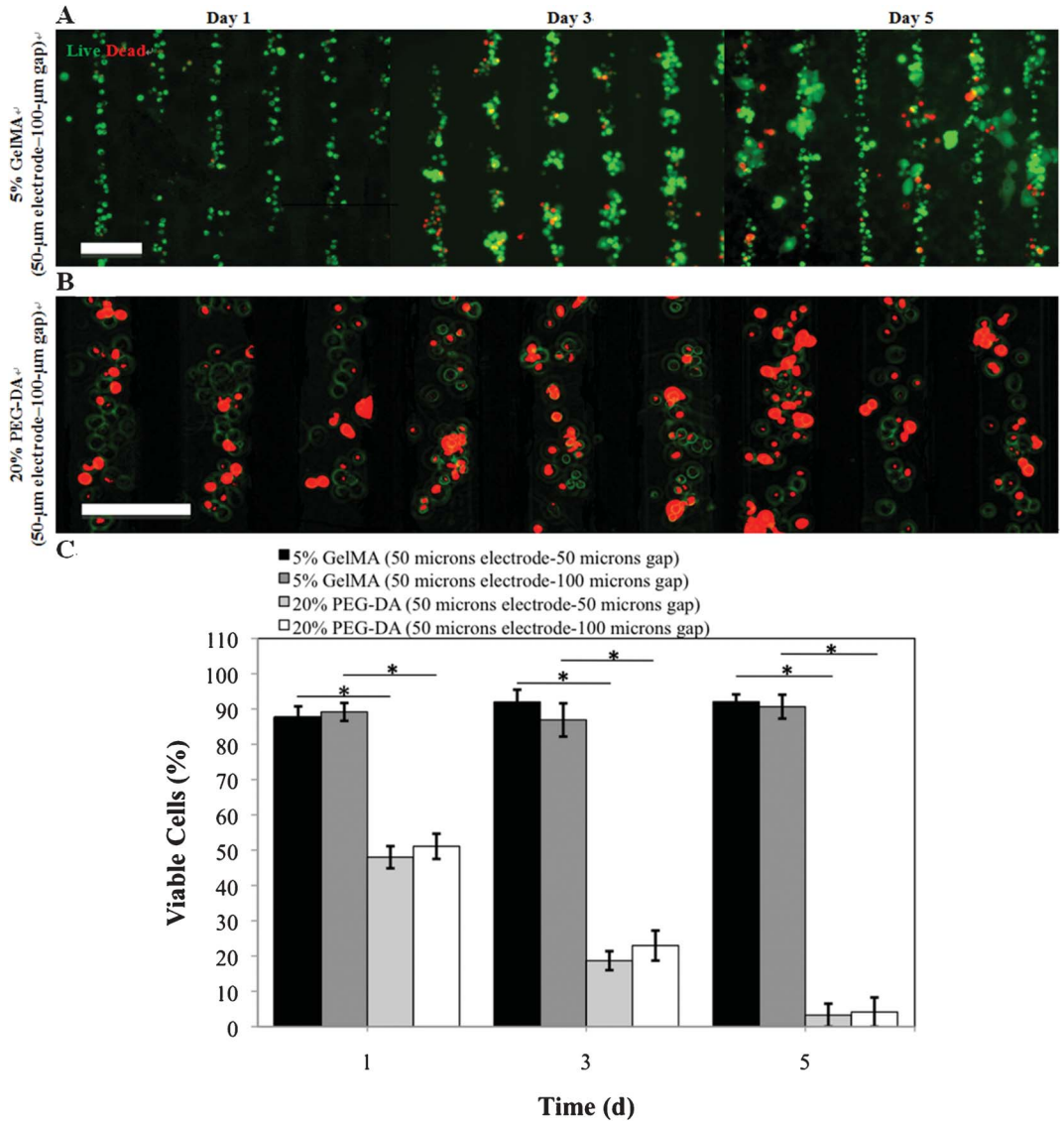

Fig. 4 Results of live/dead assay for the HUVEC cells encapsulated in 5% gelatin methacrylate (GelMA) (A) and 20% polyethylene glycol (PEG) (B) after using 50-μm electrode–100-μm gap device at different culture times. Since the cells can proliferate within the GelMA hydrogel, an increase in the cell viability with respect to culture time was observed for the GelMA hydrogel. However, the cells within the PEG are not able to grow and migrate and the cell viability was therefore decreased. Scale bar shows 150 μm (*$p < 0.001$).

the resultant cell arrays. In a recent attempt by the same research group,[28] this approach was improved to produce highly predictable cell patterns. As this method was not always successful, Markx *et al.*[29] suggested that the cell aggregates need fibrin to proliferate and prevent the aggregate loss. It is worth mentioning that cell viability is fairly high after the short manipulation time with DEP, provided that extreme conditions (*i.e.*, very low frequencies and/or high voltages) are avoided.[11] Lastly, breakthrough work has been done by Albrecht *et al.*, who used PEG-DA and agarose[12] to immobilize dielectrophoretic patterned cells. Owing to the low viscosity and ionic concentration of these hydrogels, they are well-suited candidates for use in the DEP apparatus. Other hydrogel materials can, however, be qualified after some modifications. For instance, alginate hydrogel was modified with irradiation treatment to decrease the sol molecular weight, and therefore the viscosity of the precursor solution.[30] However, it is difficult to decrease the polymer conductivity because of inherent material characteristics that are usually required for polymerization, particularly charged groups. One of the major challenges when using PEG-DA and agarose hydrogels is that they do not support cell viability and function over a long period. As indicated previously, one approach to tackle this problem is to make cell-containing microgels that are mixed with a bulk-phase agarose or PEG-DA precursor solution.[11] In that work, the application of p-DEP forces first localized these microgels to regions of high electric field. The gels were then trapped in place by exposing the bulk-phase polymer to light or a change in temperature. Compared to single-phase systems, this technology supports higher viability of liver progenitor cells. Unlike other complicated procedures used to construct and implement such multiphase systems, the proposed approach does not provide a

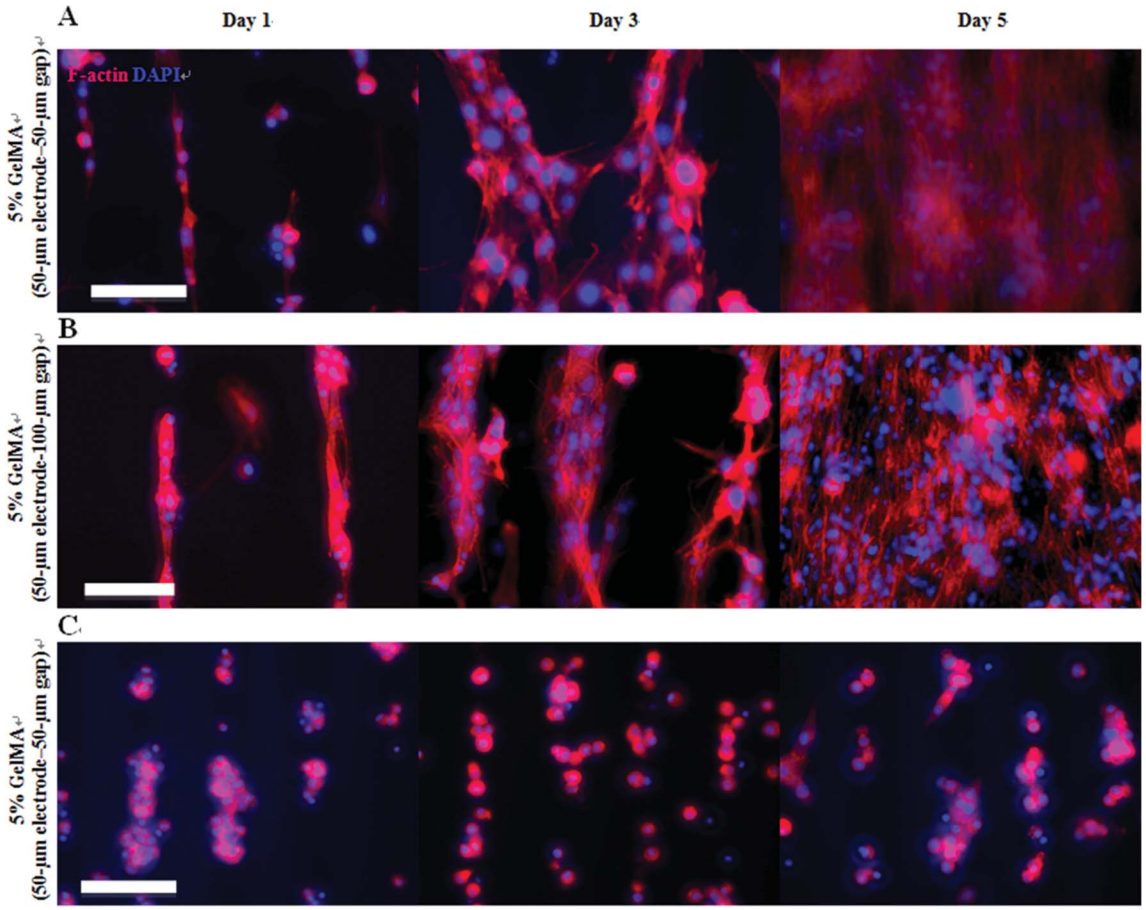

Fig. 5 Fluorescence images of DAPI/F-actin staining of the aligned C2C12 (A and B) and HUVEC (C and D) cells encapsulated in 5% gelatin methacrylate (GelMA) using the 50-μm electrode–50-μm gap (A and C) and 50-μm electrode–100-μm gap (B and D) devices. The cells remained aligned for 5 days of culture while growing up to make a tissue construct. Scale bars show 100 μm in A and C and 75 μm in B and D.

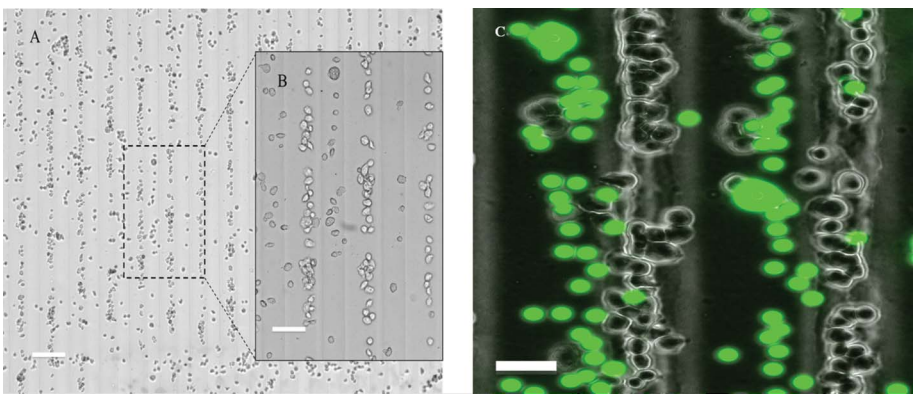

Fig. 6 Phase contrast image of aligned C2C12 cells in 5% gelatin methacrylate (GelMA) hydrogel using the dielectrophoresis (DEP) technique and trapped HUVEC cells in the gap regions between the hydrogel (A). High magnification of this picture in which bright cells are C2C12 cells and dark cells are HUVEC cells (B). FITC-labeled polystyrene beads (green) and C2C12 cells as a co-culture model (C). Scale bars show 400 μm, 100 μm, and 50 μm in A, B, and C respectively.

homogeneous cell-laden microenviroment. In this study, we proposed the application of GelMA hydrogel as a promising biomaterial for use with the DEP technique for patterning cells in a rapid and scalable way, and for maintaining cell function over a long period. Unlike the p-DEP method used by Albrecht *et al.*,[11,12] here, we used n-DEP in which cells are directed to regions with low electric fields, so that less field-induced stress is exerted on the cells.

It was demonstrated that cell proliferation is feasible on the ITO glass slide. Growing the cells on the ITO glass slide ensures both excellent conductivity and optical transparency, unlike other electrode materials such as gold or platinum, which are not

optically transparent and therefore are not optimal materials for cell cultivation. Note that ITO appears to be non-toxic and stable at warm and humid conditions related to the cell culture environment. Moreover, the electrical conductivity of the ITO glass slide makes it particularly appealing for the pulsed electrical stimulation of a variety of cells, such as neural stem cells,[31] cardiac cells,[32] and muscle cells,[33] since the location and time of the stimulation can be precisely controlled. We note that electrical stimulation is an invaluable tool to improve the proliferation, alignment, and differentiation of such cell types. ITO glass slides required chemical or physical pretreatment in order to provide specific cues for each cell type. For instance, Tandon *et al.*[32] employed a laser-based method to prepare micropatterned ITO glass slides for primary cardiomyocytes and human adipose-derived stem cells. They claimed that their proposed process is much simpler and faster than conventional photolithography approaches to creating micropatterned ITO glass slides. However, our method provides a microscale cell pattern on the ITO glass slide without any further lengthy pretreatment procedure for the ITO glass slide. In addition, such cell patterning makes it possible to decouple the effects of physical or chemical cues for cell patterning and other parameters, such as applying electrical stimulation, on cell fate.

Cellular micropatterns have in principle been generated through surface modification or micropatterning methods, such as soft lithographic approaches[34] or electrochemical lithography methods.[35] They usually require a cell-adhesive region such as fibronectin-coated sections, leaving other areas intact for the cell adhesion. These passive methods depend upon cell adhesion processes, which are divergent for each cell type and occur over relatively long timescales, and thus, cannot be generalized to non-adherent cell types. On the contrary, the dielectrophoretic cell patterning technique is independent of cell adhesion and therefore decreases time for the cell patterning and extends its capabilities in dealing with non-adherent cell types or cells that vary their behavior when they meet a rigid surface as compared to a stiff surface.[36] In addition, dielectrophoretic cell patterning is deemed to be a promising technique to construct co-culture cell arrays,[37] which are well-suited for the fabrication of tissue constructs with multiple cell types and for the study of cell–cell interactions at a microscale resolution at which cell–cell contact and communication can be established. Our research group has already developed some techniques to manipulate different cell types on a single chip.[38] However, the lifetime of such co-culture systems is limited because of a lack of an immobilizing post-procedure for the dielectrophoretic patterned cells. In this study, we established a procedure to make a co-culture system of C2C12 and HUVEC cells that is simpler and provides a 3D construct with an immutable stability.

Because of their thickness and extensive swelling, spatial patterning of hydrogels in 3D is not trivial.[39] The need for spatial patterning of cells has been a growing topic of interest, in particular for making well-defined niches for 3D cell culture, understanding the important role of biomechanical and bio-chemical cues in the cell function, and finally in regenerating more realistic tissue structures.[40] The DEP technique, along with newly developed hydrogels such as GelMA, which have adjustable chemical and physical properties, can provide such an opportunity and thereby enable the construction of highly complicated 3D culture constructs in a short time and with high pattern fidelity.

Conclusions

A novel biomaterial (GelMA hydrogel) was employed to establish 3D microscale organization of cells with the aid of DEP. Particular features of the DEP technique include high accuracy and speed, scalability, and the ability to manipulate both adherent and non-adherent cells. To date, PEG-DA and agarose have been the most suitable hydrogels used in dielectropatterning of cells. However, they do not support cell viability and function for a prolonged culture time. In this investigation, we suggest the application of GelMA as a promising hydrogel for cell electropatterning, as a result of its low viscosity and ionic concentration. It was found that the GelMA hydrogel has a superior performance in generating myoblast (C2C12) and endothelial (HUVEC) cell patterns with high fidelity as well as in maintaining cell viability and growth as compared with PEG. A simple and robust protocol was developed to make a co-culture system for these cells. Combined application of the GelMA hydrogel and DEP technique is appropriate for precisely fabricating complex microscale tissues in a rapid and scalable manner for both fundamental biological studies and tissue engineering applications.

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S.A. and J.R. conceived the idea and designed the research. S.A., J.R., H.K., H.S., A.K., and T.M. analyzed the results. S.A. and J.R. contributed equally to the work and wrote the paper. G. C-U. synthesized the GelMA hydrogel. S.A. and J.R. performed all other experiments. H.K., H.S., A.K., and T.M. supervised the research. All authors read the manuscript, commented on it, and approved its content. This work was supported by World Premier International Research Center Initiative (WPI), MEXT, Japan.

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