METHODS ARTICLE

UV-Assisted 3D Bioprinting of Nanoreinforced Hybrid Cardiac Patch for Myocardial Tissue Engineering

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Biofabrication of cell supportive cardiac patches that can be directly implanted on myocardial infarct is a potential solution for myocardial infarction repair. Ideally, cardiac patches should be able to mimic myocardium extracellular matrix for rapid integration with the host tissue, raising the need to develop cardiac constructs with complex features. In particular, cardiac patches should be electrically conductive, mechanically robust and elastic, biologically active and prevascularized. In this study, we aim to biofabricate a nanoreinforced hybrid cardiac patch laden with human coronary artery endothelial cells (HCAECs) with improved electrical, mechanical, and biological behavior. A safe ultraviolet (UV) exposure time with insignificant effect on cell viability was identified for methacrylated collagen (MeCol) micropatterning. The effects of carboxyl functionalized carbon nanotubes (CNTs) on MeCol and alginate matrix morphology, mechanical properties, electrical behavior, and cellular response were investigated at different CNT mass ratios. A UV-integrated 3D-bioprinting technique was implemented to create hybrid hydrogel constructs consisting of CNT-incorporated alginate framework and cell-laden MeCol. The compressive modulus, impedance, and swelling degree of hybrid constructs were assessed over 20 days of incubation in culture medium at 37°C for different CNT mass ratios. The HCAEC viability, proliferation, and differentiation in the context of the bioprinted hybrid constructs were assessed over 10 days *in vitro*. The functionalized CNTs provided a highly interconnected nanofibrous meshwork that significantly improved viscoelastic behavior and electrical conductivity of photo-cross-linked MeCol. Alginate-coated CNTs provided a nanofilamentous network with fiber size of ~25–500 nm, improving not only electrical and mechanical properties but also HCAEC attachment and elongation compared to pristine alginate. The CNT-reinforced 3D-printed hybrid constructs presented significantly higher stiffness and electrical conductivity particularly in the physiologically relevant frequency range (~5 Hz). The CNT-reinforced hybrid implants maintained a significantly higher swelling degree over 20 days of culturing compared to CNT-free hybrid constructs. For a selected CNT mass ratio, HCAECs presented significant cellular proliferation, migration, and differentiation (lumen-like formation) over 10 days of incubation *in vitro*. Findings from this study deliver essential steps toward developing conductive, robust, and potentially prevascularized hybrid cardiac patches.

Keywords: cardiac tissue engineering, bioprinting, cardiac patch, nanotubes, hybrid scaffold, hydrogel, photo-cross-linking, 3D-printing

Introduction

MYOCARDIAL INFARCTION (MI) REMAINS the major cause of death associated with cardiovascular disease worldwide. Due to the very limited regenerative capacity of

myocardium, the heart is unable to repair itself after heart attack. Cardiac tissue engineering aims to stabilize, revascularize, and restore the ill-functioning myocardium through implantation of sufficiently large number of cells in MI territory through engineered cardiac-mimetic implants.

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Hydrogels increasingly play a pivotal role in engineering cardiac implants due to their ability to mimic specific aspects of the cardiac tissue microenvironment.^{1–3} Some of the widely used hydrogels in cardiac tissue engineering include alginate,⁴ gelatin,^{2,5} fibrin,⁶ collagen,² poly(ethylene glycol),¹ and poly(N-isopropylacrylamide) (PNIPAAm).⁷

Despite significant progress in engineering cardiac constructs, achieving hydrogel cardiac implants that are electrically conductive, mechanically robust, and biologically functional remains an area of intensive research. Hydrogel three-dimensional (3D) bioprinting has emerged as a very promising approach to fabricate complex tissue engineering constructs. For hydrogel bioprinting, the cell-containing hydrogel must be printed in an aqueous cross-linking fluid^{8–10} or aqueous gel (e.g., sacrificial gelatin medium) system, which significantly limits the choice of hydrogel material for bioprinting.¹¹

Currently among various hydrogel systems, alginate is a very popular printable hydrogel. It hardens by exposure to an ionic cross-linking agent such as calcium chloride solution following printing^{8,10} and can accommodate a high cell density¹² while maintaining high cell viability.¹⁰ Its drawbacks however include poor cellular adhesion,¹³ limited capacity for cellular proliferation and differentiation,¹¹ and poorly conductive pore walls that impede cardiac action potential propagation.¹⁰ Unlike alginate, collagen, which bears the majority of connective tissue proteins, promotes cellular adhesion, differentiation, and mobility. Specifically, type I collagen, a major constituent of cardiac extracellular matrix (ECM) has been considered as an excellent cell delivery platform for cardiac applications.^{14,15} However, layer-by-layer bioprinting of collagen is difficult due to its poor self-supporting property and low viscosity.

Compared to native myocardium features, some additional key characteristics of the alginate/collagen implants remain to be improved. Alginate lacks nanofibrous structural features that are one of the key components of native ECM for regulating cellular behavior, and unlike electrically conductive heart muscle tissue, electrical conductivity of alginate and collagen is poor, specifically at physiologically relevant frequencies (e.g., 1–5 Hz).¹⁶ Finally, mechanical properties of natural polymers such as collagen are weak, making them vulnerable to the stretching-contracting stresses associated with the beating myocardium.

Hydrogel nanoengineering,^{17,18} ionic- and photo-cross-linking,⁵ and micropatterning¹⁰ strategies have been used to modulate electrical, mechanical, and/or structural features of the hydrogel scaffolds. Methacrylated type-I collagen (MeCol) is a photo-labile derivative¹⁹ of collagen that can be obtained from adding methacrylate to the amine-containing side groups of the collagen (Fig. 1a). In the presence of a photoinitiator at a suitable pH, ultraviolet (UV) light induces collagen fiber cross-linking that improves hydrogel stiffness. The alginate implant mechanical and electrical behavior can be modulated by 3D printing¹⁰ and can also be improved by calcium ion-induced cross-linking between polysaccharide chains (Fig. 1). The incorporation of nanomaterials may also contribute to hydrogel improvement.

Surface functionalized carbon nanotubes (CNTs), which have no reported cytotoxicity²⁰ and previously used for drug delivery²¹ and medical imaging,²² have shown potential to improve hydrogel mechanical properties.¹⁷ Carboxyl functionalized CNTs can covalently bond with the alginate and reinforce the ionic cross-linked hydrogel structure (Fig. 1b).

Since CNTs are highly electrically conductive at low CNT loading,²³ their presence in the hydrogel may improve the matrix electrical behavior.

In the light of the above description, we envision that UV-integrated bioprinting of CNT-incorporated hybrid implants consisting of alginate and MeCol may allow the biofabrication of hybrid implants with improved stiffness, conductivity, and cellular response. To assess our hypothesis, we conducted this study in several steps. First, using a UV-induced micropatterning technique, MeCol laden with human coronary artery endothelial cells (HCAECs) was micropatterned at various exposure times followed by cell viability assessment over 7 days to identify a safe UV exposure time for photo-cross-linking.

Next, the influence of CNT concentration on viscoelastic behavior, electrical conductivity, nanofibrous surface morphology, and biological performance of the UV cross-linked CNT-incorporated MeCol was assessed. Then, integrated with UV cross-linking, CNT-incorporated alginate and MeCol were 3D-printed into hybrid implants at various CNT concentrations followed by mechanical and electrical behavior assessments of the implants over 25 days of incubation at 37°C. The biological performance of the bioprinted hybrid hydrogel constructs laden with HCAECs was assessed *in vitro* over 10 days, while monitoring cell viability, proliferation, morphology, and migration within the implant.

Materials and Methods

Preparation of CNT-incorporated alginate and MeCol hydrogels

Multiwalled carboxyl acid functionalized CNTs with an average diameter of 30 nm and length of 5–20 μm (NanoLab, Inc., Waltham, MA) were incorporated into MeCol (Advanced BioMatrix, Inc., San Diego, CA) and sodium alginate (Sigma-Aldrich Co., Saint Louis, MO) at three levels of CNT mass ratios of 0, 5, and 10 μg CNT/mg MeCol and 0, 25, and 50 μg CNT/mg alginate, respectively. It should be noted that compared to single-walled CNTs, multiwalled CNTs have more structural complexity that causes a higher variability in their electrical and mechanical property.²⁴ In addition, single-walled CNTs have a better electrical conductivity than multiwalled CNTs; however, multiwalled CNTs offer advantages over single-walled CNTs with respect to the higher synthesis purity, less chance of defect during functionalization, higher tensile strength, and easier bulk synthesis, which make multiwalled CNTs the most commercially available CNTs for nanocomposite development.^{25,26} So, multiwalled CNTs were used in this study.

The CNTs were dispersed in biological grade sterile water and sonicated for 2 h at 4°C to obtain a homogenous CNT suspension at 10 mg/mL. Precalculated volumes of the CNT/water suspension were mixed with calcium-free DMEM that was used for dissolving a certain amount of sterile alginate to provide CNT mass ratios of 25 and 50 μg CNT/mg alginate at the final alginate concentration of 2.5% (w/v).¹⁰

The MeCol was dissolved in acetic acid (20 mM) at 4°C over 48 h. Precalculated volumes of the CNT/water suspension and sterile Irgacure (10% in Methanol), as the photoinitiator, were mixed with a selected volume of minimum essential medium-10X (MEM 10X) (Sigma-Aldrich) at 4°C. The MEM/CNT/Irgacure solution was then added to

FIG. 1. Schematic diagram of (a) MeCol formation and UV-induced cross-linking between collagen fibers and (b) calcium ion-induced cross-linking between alginate polysaccharide chains and covalent bonds between carboxylated CNTs and alginate. CNT, carbon nanotube. Color images available online at www.liebertpub.com/tec

MeCol followed by mixing with sterile sodium bicarbonate solution (2.34% w/v) and sodium hydroxide (0.5 M) to adjust the pH (7–7.4) of MeCol solution at the final concentration of 6 mg/mL. To avoid MeCol gel formation during mixing, the hydrogel preparation was performed at 4°C or on ice while protected from light. Experiments were conducted in triplicate.

HCAEC culturing and cell encapsulation in the hydrogels

The HCAECs (Cell Applications, Inc., San Diego, CA) were cultured with MesoEndo Growth Medium (Cell Applications, Inc.). Passages 5–6 with 90% confluence were

used for cell encapsulation in MeCol hydrogel. Cell pellets (at 4°C and 800 rpm centrifugation) were resuspended in a certain volume of calcium-free DMEM and counted using a hemocytometer. A selected volume of the cell suspension was mixed with precalculated volumes of MeCol (see Preparation of CNT-incorporated alginate and MeCol hydrogels section) and alginate at a final cell density of $0.8\text{--}1 \times 10^6$ cells/mL hydrogel for the hydrogel micropatterning, bioprinting, and cellular response assessments.

Micropatterning of MeCol laden with HCAECs

Managed by Objet Studio software™, a 3D-printing system (PolyJet Technology, Connex500, Vaughan, ON, Canada)

with a printing resolution of 16 μm was used to print a photo polymer (VeroWhite FullCure-830) into micromolds consisting of a base with the size of $8 \times 8 \times 2 \text{ mm}$ ($W \times L \times H$) and parallel strands and grooves with the height of 400 μm and thickness of 300 and 400 μm (Fig. 2a), respectively. The grooves were cleaned using a high pressure water jet cleaner at 100 bar (Power Blast International Ltd., Hampshire, United Kingdom), sodium hydroxide (1% w/v), ethanol, and ultra distilled water.

Glass coverslips were soaked in sodium hydroxide (10% w/v) for 3 h followed by washing with deionized water and drying at room temperature. The stack of coverslips was placed in a beaker containing 3-(Trimethoxysilyl) propyl methacrylate (TMSPMA) (Sigma-Aldrich Co.). The beaker was kept at 80°C overnight, allowing for TMSPMA to flow between the coverslips and coat them. Then, the coverslips were washed thrice with ethanol and dried at room temperature while being protected from light. The TMSPMA-coated coverslips and the micromolds were autoclaved before being used for UV-induced photo-cross-linking and micropatterning of cell-laden MeCol.

As shown in Figure 2a, a volume of 20 μL of HCAEC-laden MeCol at 4°C was dispensed on the micromold grooves followed by spreading the droplet along the grooves using a TMSPMA-coated coverslip. With the coverslip on top, the micromold was incubated at 37°C for 6 min (Fig. 1b), before being exposed to UV light for photo-cross-linking (Fig. 2c). The vertical distance between the UV source (UV-365 rad-free; Schleicher & Schuell BioScience GmbH, Dassel, Germany) and the sample was adjusted at 8 cm with a spacer to obtain desired UV intensity for photo-cross-linking. By means of a UV radiometer (Digital UVX Radiometer; UVP, Upland, CA), the UV intensity power at

the sample position was measured as 6.1 mW/cm^2 , which was applied to all of photo-cross-linking experiments in this study. After photo-cross-linking, the micromold was gently detached from the cross-linked MeCol, leaving the micropatterned cell-laden MeCol strands on the coverslip (Fig. 2d), which was subsequently transferred to the culture medium.

UV-integrated bioprinting of the hybrid implants

Using a pneumatic 3D-bioplotter system (EnvisionTEC GmbH, Gladbeck, Germany), alginate (with or without CNTs) was printed into calcium chloride solution (50 mM) in culture plate wells coated with 0.1% (w/v) polyethylenimine (PEI) (Sigma-Aldrich) (Fig. 2e).^{8,10} The sample stage and dispensing head temperatures were maintained at 37°C and 22°C, respectively, during printing. The bioprinting was performed with a nozzle-moving speed of 8–10 mm/s and pneumatic pressure of 0.1 bar using a plastic cone needle (EFD Nordson, Westlake, OH) with an internal diameter of 250 μm . The overall printing dimension was $11 \times 11 \times 2 \text{ mm}$ ($W \times L \times H$). Based on our previous study,¹⁰ an intrastrand distance (ISD) of 1500 μm with strand alignment angle of 15/165° and strand thickness (ST) of $\sim 230 \mu\text{m}$ provided a higher implant porosity than other examined printing patterns. We applied this printing pattern to hydrogel printing in the present study.

Following alginate printing in the first layer, calcium chloride was removed from the culture plate well by a pipettor, allowing for the second dispensing head to print cell-laden MeCol (at 15°C) (Fig. 2f). Since cell-laden MeCol is not immediately self-supportive after printing, it is very difficult to direct-print MeCol using dispensing-based printing techniques. In contrast, alginate strand hardening is reasonably

FIG. 2. Fabrication steps for MeCol micropatterning and hybrid implant bioprinting: (a) the dimensions of grooves and strands of the 3D-printed micromold for loading cell-laden MeCol before cross-linking, (b) placing TMSPMA-coated coverslip on the top of the hydrogel-loaded micromold to allow cell-laden MeCol to fill the grooves followed by incubation at 37°C, (c) UV exposure for MeCol photo-cross-linking, (d) micromold detachment to obtain the micropatterned MeCol hydrogel laden with HCAECs, (e) printing CNT-alginate into cross-linking fluid in PEI-coated culture plate well, (f) draining CaCl_2 and bioprinting of cell-laden MeCol into the spaces between alginate strands at sample stage of 37°C, (g) applying UV light for MeCol photo-cross-linking, and (h) adding CaCl_2 to the culture plate well and alginate printing into the second layer of the hydrogel construct. HCAEC, human coronary artery endothelial cell; MeCol, methacrylated collagen; PEI, polyethylenimine. Color images available online at www.liebertpub.com/tec

fast upon exposure to calcium chloride after printing. As such, we used alginate as the implant framework to contain cell-laden MeCol within spaces between alginate strands in each printed layer.

For MeCol printing, the coordination origin was shifted by 750 μm to allow for dispensing MeCol in spaces between alginate strands in the same layer. A cone plastic needle with an internal diameter of 200 μm at a nozzle-moving speed of 25–32 mm/s was used for MeCol bioprinting. The variability of the speed (25–32 mm/s) could be related to some degrees of collagen fibrillogenesis in the neutral pH that increases the MeCol viscosity in the syringe with time. Allowing for collagen cross-linking at 37°C, the printed layer was given 6 min at the sample stage temperature of 37°C following exposure to UV light (45 s) to the sample for MeCol photo-cross-linking (Fig. 2g). Then, calcium chloride at 37°C was gently added to the well, and the first dispensing head was allowed to print alginate in the second layer (Fig. 2h). Alginate and cell-laden MeCol were printed into three layers with the height of $\sim 700 \mu\text{m}$ before replacing calcium chloride with the culture medium and incubating the samples at 37°C under 5% CO_2 . The culture medium was refreshed every 2 days.

Cell viability and morphology assessments

Hoechst 33342 (Sigma, ON, Canada) and Calcein-AM (Sigma) at concentrations of 2.5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$, respectively, were used for staining total and live cells, respectively. Based on fluorescence microscope images, the cell viability was assessed by ImageJ software by dividing the number of live cells by total number of cells from 10 randomly selected areas of the cell-laden hydrogel strands. For immunocytochemistry, the cultured implants were fixed in 4% paraformaldehyde for 30 min at room temperature. After fixation, the cell-laden hydrogels were washed with PBS and then treated with 0.1% Triton X-100 for 20 min. Bovine serum albumin (BSA) (1%) was used as the blocker agent for 20 min before the samples were treated with DAPI (1:10,000) and Alexa Fluor 488 phalloidin (1:40) for 60 min. Cell viability and immunocytochemistry assessments were performed over 7 days of the implant culturing.

Characterization of electrical and mechanical properties

Viscoelastic behavior of CNT-incorporated MeCol hydrogel was assessed at three CNT mass ratios in the absence and presence of UV exposure using a rheometer (AR-G2, TA Instruments Ltd, West Sussex, NB). A volume of 200 μL of MeCol hydrogel (see Preparation of CNT-incorporated alginate and MeCol hydrogels section), which was a sufficient volume for a 500 μm gap between a 20 mm top parallel hatch plate and a lower plate of the rheometer, was loaded onto the sample stage that was set at 37°C.

For impedance measurements, MeCol (75 μL) was loaded between two gold-coated glass slides connected to a LCR meter (Good Will Instrument Co., Ltd., New Taipei City, Taiwan) with an A.C. bias sweeping between 5 Hz and 1 MHz.¹⁰ The measurements of impedance, storage modulus, and loss modulus were performed for three CNT mass ratios of 0, 5, and 10 $\mu\text{g CNT/mg MeCol}$ in the presence and absence of photo-cross-linking.

Representing the stiffness and electrical conductivity, compressive elastic modulus and impedance of the 3D-printed hybrid constructs were measured over 20 days of incubation at 37°C. For compressive modulus measurements, the constructs were 3D printed into 10 layers with the final construct height of $\sim 2 \text{ mm}$. Before each measurement, the implant top and side views were imaged using a stereomicroscope, and the surface area and thickness of the implant were measured by ImageJ software (U.S. National Institutes of Health, Bethesda, MD). The texture analysis material testing system (Texture Technology Corp., Hamilton, MA) with a load cell of 250 N and a crosshead speed of $10 \mu\text{m s}^{-1}$ for a 1 mm displacement (compression) was used for measuring force and axial deformation of the implants through a uniaxial unconfined compression test. Applying the surface area and thickness of each implant, the stress-strain behavior of each sample was analyzed.

For impedance measurement, the superficial fluid (culture medium) was removed from the cultured 3D-printed construct using Kimwipes tissue. For each sample, two gold-coated probes of the LCR meter (Good Will Instrument Co., Ltd) were attached to both sides of the implant, while the clearance between two probes was set at 5 mm using a glass spacer. With A.C. bias sweeping between 12 Hz and 1 MHz, the impedance was measured for each 3D-printed implant. The measurements for elastic modulus and impedance of the implants were performed for CNT mass ratios of 0, 25, and 50 $\mu\text{g CNT/mg alginate}$ at four time intervals over 20 days of incubation.

For measuring swelling degree, the implants were weighed after 3D printing before being immersed in the culture medium and their “weight change” was monitored at different time intervals over 20 days of incubation in the culture medium.²⁷

Surface morphology assessment

Scanning electron microscopy (SEM) was used to examine the surface morphology of the hydrogels. After snap-freezing over liquid nitrogen, the CNT-incorporated MeCol and alginate samples were freeze-dried at -80°C and 0.01 mbar for 24 h. The freeze-dried samples were placed on carbon tape attached to SEM stubs. The samples were coated with a 5-nm layer of chromium (Q150T; Quorum Technologies, United Kingdom) and then examined using a field-emission scanning electron microscope (SU8010; Hitachi High-Technologies, Japan) at 10 kV.

Statistical analyses

Comparisons between two or more groups were performed with paired Student's *t*-test and one-way analysis of variance, respectively. The statistical analyses were performed using SPSS software (IBM SPSS Statistics, Ver. 21.0. Armonk, NY: IBM Corp.) at a significance level (*p*-value) of <0.05 for all measurements.

Results

HCAEC viability assessment of photo-cross-linked cell-laden MeCol with respect to UV exposure time and CNT concentration

Figure 3a and b depicts the stereomicroscope and fluorescence images of HCAEC-laden MeCol strands after 2 h

FIG. 3. *In vitro* cell distribution, viability, and morphology assessments of HCAECs in UV-induced micropatterned cell-laden MeCol sheets at the cell density of 8×10^5 cells/mL: (a) Bright field microscopy image and (b) Calcein-stained cells distributed in the hydrogel filaments (yellow arrows) and spaces (red arrows) of the UV-induced micropatterned MeCol hydrogel (6 mg/mL) at 45 s UV exposure after 2 h following microfabrication, and live cell distribution and density of the cell-laden MeCol strands micropatterned at (c) 45 s, (d) 120 s, and (e) 270 s after 3 days following MeCol sheet microfabrication; and live-dead cell assay (Calcein-AM Hoechst staining) and cell morphology (Phalloidin F-actin and DAPI) assessments corresponding to UV exposure times of (f) 45 s, (g) 120 s, and (h) 270 s after 3 days. Color images available online at www.liebertpub.com/tec

following MeCol micropatterning at varying UV exposure times at 37°C and pH 7.4. The yellow and red arrows on the images represent ST and ISD measured as $402 \pm 30 \mu\text{m}$ (yellow arrows) and $329 \pm 21 \mu\text{m}$ (red arrows), respectively, which were reasonably consistent with the dimensions of the

3D-printed micromolding stamp channels and grooves. Figure 3b depicts the live cell distribution within the micropatterned sheet of MeCol. As seen, cells are well distributed along the patterned strands. While the intrastrand space is expected to be cell free after micropatterning, a

small randomly distributed cell population can be identified in the spaces between the MeCol strands (red arrow) (Fig. 3b).

Figure 3c–e depicts the fluorescence microscope image of Calcein-AM stained cells (live cells) in the micropatterned MeCol sheet for three UV exposure times of 45, 120, and 270 s, respectively, after 3 days of incubation. The live cell population decreases with UV exposure time, and clearly, the highest cell damage (smaller live cell density) is associated with the UV exposure of 270 s (Fig. 3e). The superimposed images of Calcein-AM and Hoechst stained cells (live–dead cell assay) have been shown in Figure 3f–h. As shown, the dead cell density, represented by blue dots alone, increases with UV exposure time (Fig. 3f–h). The magnified images shown in Figure 3f–h depict the phalloidin F-actin and DAPI stained cells, indicating cellular morphology changes with UV exposure time. While HCAECs preserve their ability to stretch in the 3D MeCol hydrogel at UV exposure of 45 and 120 s, they largely stay round at UV exposure of 270 s after 3 days of incubation (Fig. 3f–h).

Figure 4 illustrates the quantitative assessment of cell viability in CNT-incorporated MeCol with respect to UV exposure time and CNT mass ratio over 7 days of culturing. Compared to the control samples (no UV, no CNT), UV exposure time of 45 s causes no significant effect on cell viability over 7 days. For UV exposure of 120 s, no significant cell damage is observed within 12 h, but cell viability decreases significantly in the following 7 days (Fig. 4a). The highest cell damage impact of UV is observed at the exposure time of 270 s (Fig. 4a). The CNT mass ratio ranging between 0 and 10 μg CNT/mg MeCol had no significant effect on cell viability over 7 days for the HCAEC-laden MeCol photo-cross-linked with UV at 45 s exposure time (Fig. 4b). Notably, the cell viability largely remains above 90% over 7 days of incubation for all levels of CNT mass ratios (Fig. 4b).

Viscoelastic and electrical behavior of photo-cross-linked CNT-incorporated MeCol

Figure 5 illustrates the effect of CNT mass ratio on the impedance and viscoelastic properties of the photo-cross-linked MeCol compared to the control sample. The impedance of CNT-free MeCol is generally higher than CNT-incorporated photo-cross-linked MeCol for all frequencies (Fig. 5a). Although MeCol presents relatively low impedance at high frequencies regardless of CNT mass ratio, at a low

frequency range (e.g., ~ 5 Hz), which is considered to be more physiologically relevant, the hydrogel impedance significantly (p -value < 0.05) decreases with CNT mass ratio (Fig. 5b).

Compared to the control sample, the photo-cross-linked MeCol storage modulus (E') is significantly increased with CNT mass ratio while the loss modulus is only slightly improved (Fig. 5c). For hydrogel elasticity, the phase angle significantly decreases with CNT mass ratio for the photo-cross-linked MeCol compared to the control sample, indicating the impact of CNT and photo-cross-linking on the hydrogel stiffness (Fig. 5d).

UV exposure and CNT concentration effects on MeCol surface morphology

Figure 6a–e illustrates the nanofiber size distribution of CNT-incorporated MeCol matrix photo-cross-linked at 37°C and neutral pH (~ 7.4), compared to the control sample. The fraction of small nanofibers ranging 50–100 nm significantly increases with CNT mass ratio (Fig. 6a–e). An increase in the CNT mass ratio from 5 μg CNT/mg MeCol (Fig. 6b, c) to 10 μg CNT/mg MeCol (Fig. 6d, e) results in an elevation in the fraction of 100-nm fibers regardless of UV exposure. In particular, the nanofibers ranging 50–200 nm predominantly define the nanofibrous structure of the MeCol matrix at the CNT concentration of 10 μg CNT/mg MeCol (Fig. 6e). Figure 6f–h depicts SEM images of MeCol with different CNT mass ratios. In the absence of CNTs, MeCol matrix presents numerous collagen sheets with some fibers (Fig. 6f), but the incorporation of CNTs, along with photo-cross-linking, leads to highly interconnected nanofibers, rather than sheets (Fig. 6g, h).

Cellular response to CNT-induced nanofibrous morphology of alginate matrix

Figure 7a depicts the surface morphology of the CNT-incorporated alginate compared to that of pristine alginate (Fig. 7b). The alginate-coated CNTs represented by the nanofilaments in the alginate matrix can be observed in Figure 7a. In particular, the magnified image clearly shows the nanowiring CNTs (red arrows) with a diameter of ~ 25 –50 nm in alginate. This nanowired morphology (red arrows) may be considered as a mimicking structure for the purkinje fiber network on the surface of the ventricular muscle fibers. In contrast, the pristine alginate (Fig. 7b) demonstrates a nonporous fiber-free surface morphology.

FIG. 4. Quantitative assessment of cell viability in micropatterned cell-laden MeCol hydrogel with respect to (a) four levels of UV-exposure time and (b) CNT mass ratio over 12 days in the culture medium at 37°C and 5% CO_2 . Color images available online at www.liebertpub.com/tec

FIG. 5. Effect of CNT mass ratio on viscoelastic and electrical properties of photo-cross-linked MeCol hydrogel: variations of (a) frequency-dependent impedance, (b) impedance at a physiologically relevant frequency (e.g., 5 Hz), (c) storage modulus and loss modulus, and (d) $\tan(\delta)$ with respect to CNT mass ratio at 37°C and UV exposure time of 45 s compared to the control sample (no CNT, no UV exposure). Color images available online at www.liebertpub.com/tec

Figure 7c depicts phalloidin- and DAPI-stained HCAECs in CNT-incorporated alginate at 25 μg CNT/mg alginate after 7 days of culturing. The superimposed bright field and fluorescence microscope images, representing alginate-coated CNT network and stained HCAECs, are shown in Figure 7d. Figure 7e and f depicts HCAEC morphology based on phalloidin- and DAPI staining for CNT-incorporated alginate and pristine alginate, respectively. The HCAECs are well attached and elongated in the CNT-incorporated alginate matrix (Fig. 7c–e), while the cells seem to be largely round and unattached in the pristine alginate (Fig. 7f).

Mechanical, electrical, and swelling behavior of 3D-printed hybrid hydrogel construct

Figure 8a depicts the stereomicroscope image of the 3D-printed CNT-incorporated alginate framework with a thickness of $\sim 265 \pm 37 \mu\text{m}$ of the hybrid implant in the calcium chloride solution (50 mM) immediately after printing. Figure 8b and c shows the stereomicroscope and phase contrast images of the representative architectural design of the hybrid implant in the culture medium after 2 and 4 days of incubation, respectively. The photo-cross-linked cell-laden MeCol can be distinguished in the images,

filling the intrastrand spaces between CNT-incorporated alginate strands (Fig. 8b, c). Notably, an increase in the average alginate strand thickness ($696 \pm 59 \mu\text{m}$) was noticed after 2 and 4 days of incubation. This significant increase in the alginate strand thickness can be attributed to the hydrogel swelling that occurred during the incubation time in the culture medium.

Figure 8d illustrates the degree of swelling of the implants at three levels of CNT mass ratios of 0, 25, and 50 μg CNT/mg Alginate over 20 days of incubation in the culture medium. The implants reached their highest swelling degree after 4 days of incubation; however, the CNT-incorporated alginate implants experienced as much as 50% higher swelling after 4 days (Fig. 8d) compared to CNT-free alginate implants. Notably, the pristine alginate swelling degree declined after day 4, most probably due to the alginate degradation, while the CNT-incorporated alginate implants retained their high swelling degree of $\sim 200\%$ (Fig. 8d) over 20 days. It indicates that the incorporation of carboxyl functionalized CNTs in alginate can improve not only the water retention but also the structural integrity of the alginate over the incubation time.

Figure 9 illustrates the variation of compressive elastic modulus and impedance of CNT-reinforced hybrid implants with respect to CNT mass ratio and incubation time

FIG. 6. Quantitative and surface morphology assessments of fibrous structure of MeCol hydrogel with respect to UV exposure and CNT concentration: nanofiber size distribution of MeCol hydrogel matrix cross-linked at (a) no UV exposure without CNT incorporation (control), (b) no UV exposure and 5 μg CNT/mg MeCol, (c) 45-s UV exposure and 5 μg CNT/mg MeCol, (d) no UV exposure and 10 μg CNT/mg MeCol, (e) 45-s UV exposure and 10 μg CNT/mg MeCol, and SEM images of MeCol matrix cross-linked with (f) no UV and no CNT, (g) 45-s UV and 5 μg CNT/mg MeCol, and (h) 45-s UV and 10 μg CNT/mg MeCol. Color images available online at www.liebertpub.com/tec

in culture medium. The incorporation of CNT in the alginate generally increases the stiffness of the hybrid construct (Fig. 9a). Notably, the implant stiffness increases over 3 days for all levels of CNT ratios, but declines after 3 days and 10 days for CNT ratios of 0, 0.025, and 0.05 mg CNT/mg alginate, respectively (Fig. 9a). It can be noticed that the 3D-printed implant with CNT ratio of 25 μg CNT/mg alginate maintains a higher compressive modulus over 20 days of incubation compared to CNT ratios of 0 and 50 μg CNT/mg alginate (Fig. 9a).

The CNT incorporation into the alginate of the 3D-printed implants generally reduces the construct impedance over 20 days of incubation in the culture medium (Fig. 9b). For all levels of CNT mass ratios of alginate, the impedance of the 3D-printed construct is first decreased within the first 3 days of incubation but it is elevated over the following 17 days of incubation (Fig. 9b). An increase in the alginate CNT mass ratio from 25 to 50 μg CNT/mg alginate slightly

improves implant electrical conductivity (lower impedance) at 5 Hz (Fig. 9b).

In vitro biological assessment of bioprinted CNT-reinforced hybrid implant

Figure 10 shows the fluorescence microscopy images representing HCAEC proliferation, migration, and differentiation in the context of the CNT-incorporated bioprinted hybrid patch (at 5 μg CNT/mg MeCol and 25 μg CNT/mg alginate) at three time points of implant culturing. Figure 10a depicts the distribution of HCAECs in the implant stained with Calcein-AM after 2 days in the culture medium. Cells are largely situated in the MeCol hydrogel that fills spaces between CNT-alginate strands (Fig. 10a). A significantly higher cell population density due to the cell proliferation can be observed in MeCol segment of the hybrid patch after 7 days (Fig. 10b) compared to day 2 (Fig. 10a).

FIG. 7. Surface morphology and cellular response of HCAECs to the CNT-incorporated alginate hydrogel: (a) SEM images of alginate-coated CNT in the hydrogel matrix at CNT concentration of 0.025 mg/mg alginate, (b) CNT-free alginate cross-linked at 50 mM calcium chloride solution after printing, (c) fluorescence image, (d) bright field and fluorescence composite images of HCAECs stretched along the alginate-coated CNT filaments in the alginate matrix, (e) cell morphology assessed using phalloidin F-actin and DAPI staining for CNT-incorporated alginate at 0.05 mg CNT/mg alginate, and (f) CNT-free alginate laden with HCAECs. Color images available online at www.liebertpub.com/tec

FIG. 8. Top view of the 3D-bioprinted hybrid implant made of CNT-incorporated alginate and photo-cross-linked cell-laden MeCol at 5 μ g CNT/mg MeCol and the implant swelling pattern in the culture medium: stereomicroscope images of the 3D-printed hybrid construct (a) after printing (day 0) in calcium chloride and (b) after 2 days in the culture medium and (c) the phase contrast bright field image of the implant after 4 days and (d) the variation of implant swelling degree over 20 days of incubation in the culture medium for three CNT mass ratios of alginate. Color images available online at www.liebertpub.com/tec

FIG. 9. Variations of the (a) compressive elastic modulus and (b) impedance (5 Hz) of the hybrid 3D-printed construct (ISD of 1500 μm , SAA of 15/165°, and ST of 250 μm) with respect to incubation time and CNT mass ratio. Color images available online at www.liebertpub.com/tec

After 7 days, HCAECs migrated from one MeCol area (rhombus shape) to another, distinguishable from the strips (white arrows) over CNT-incorporated alginate strands (Fig. 10b). Notably, HCAECs have been not only proliferating and migrating but also beginning to create some lumen-like configurations (yellow circles) after 7 days (Fig. 10b). Figure 10c depicts the Hoechst-stained implant, representing the distribution of total cell density (live and dead) in a selected area of the hybrid implant after 10 days (Fig. 10c). The red arrows indicate the non-cell-migrated areas of the CNT-incorporated alginate strands of the implant, and the yellow circles indicate the areas where the lumen formation is apparently taking place (Fig. 10c). The HCAECs have obviously migrated into a larger area of the alginate strands, which were cell free initially (Fig. 10c).

Figure 10d clearly shows the live cell distribution across MeCol segments and alginate strands of the hybrid implant. The HCAEC elongation (yellow arrows) can be noticed at the interfacial areas between MeCol and alginate strands (Fig. 10d). Figure 10e–g depicts phalloidin- and DAPI-stained HCAECs forming lumen (yellow circles) in the hybrid construct (Fig. 10d). The HCAECs with elongated and interconnected actins exhibit lumen formation morphology in MeCol segments of the hybrid implant after 10 days (Fig. 10d–g).

Discussion

MI represents the leading cause of mortality with globally significant socioeconomic burden.²⁸ Due to the negligible regenerative capacity of mammalian adult cardiac cells, the progressive loss of cardiomyocytes due to MI eventually leads to heart failure.²⁹ Intra-myocardium hydrogel injection or pericardium hydrogel patch implantation³⁰ is considered as promising cardiac tissue engineering approach for MI repair. Cardiac-mimetic constructs with structural support and contractile activity offer great potential for cardiac regenerative therapy; however, the need for development of cardiac patches with more complex myocardial features is still needed. The 3D bioprinting represents a promising strategy that allows for creating complex cardiac implants laden with various types of cells.³¹

Ideally, a 3D-bioprinted cardiac patch must be biocompatible, biodegradable, bioprintable, mechanically robust and elastic, electrically conductive, and promote cellular growth, proliferation, and differentiation. Hybrid scaffolding, in which two or more hydrogels with synergistic roles are printed into a single 3D construct, is an alternative to improve essential characteristics of a cardiac patch. Nanomaterials can be incorporated into the hydrogels to obtain superior properties and desired functionality.^{32–34} In this study, we demonstrated that nanoreinforced alginate and HCAEC-laden MeCol can be bioprinted into a hybrid cardiac implant using a UV-integrated dispensing-based 3D-printing technique. We also demonstrated that the incorporation of functionalized CNTs into alginate and MeCol significantly enhanced the hydrogel electrical and mechanical properties over 20 days of incubation. The CNT-incorporated bioprinted hybrid patch presented a remarkable HCAEC growth, proliferation, and migration within 7 days in culture medium *in vitro*.

First, we showed that cell-laden MeCol could be micro-patterned with a thickness of $\sim 400\ \mu\text{m}$ using UV light (365 nm, 6.1 mW/cm²) as a result of photo-cross-linking. We identified a safe UV exposure time ($\sim 45\ \text{s}$) for micro-patterning that led to an insignificant UV effect on cell viability for the development of hybrid implants. Bertassoni *et al.* reported similar UV exposure time ($<60\ \text{s}$) as a safe time window for hydrogel photo-cross-linking.³⁵ Furthermore, CNT incorporation into MeCol significantly improved the hydrogel electrical conductivity. In particular, at the physiologically relevant frequencies of $\sim 5\ \text{Hz}$, the impedance of CNT-incorporated MeCol hydrogel was significantly (p -value < 0.05) reduced compared to that of the pristine MeCol. The higher electrical conductivity of CNT-incorporated MeCol can be attributed to the highly interconnected nanofibrous network associated with the incorporation of highly conductive CNTs in the hydrogel matrix,¹⁷ which was morphologically assessed using SEM imaging.

The formation of the nanofibrous interconnected network could be attributed to the cross-linking of MeCol-coated CNTs through the acrylic groups. SEM images revealed that CNT-incorporated MeCol had similar porous structure as the pristine MeCol, but with highly interconnected nanofibers, mostly ranging between 50 and 200 nm. Notably, more than 57% of the fibers had a thickness of $\leq 100\ \text{nm}$ at 10 μg CNT/mg MeCol while this amount was 10% and 42% at 0 and 5 μg CNT/mg MeCol, respectively. Along with the highly

FIG. 10. Fluorescence microscopy imaging of live cells stained using Calcein-AM and/or Hoechst after (a) 3 days, (b) 7 days, and (c, d) 10 days, and (e–g) immunohistochemistry analysis of HCAEC morphology based on phalloidin F-actin and DAPI staining indicating lumen formation in the CNT-incorporated MeCol of the hybrid implant biofabricated at UV exposure of 45 s and CNT concentration of 5 mg CNT/mL MeCol and 0.025 mg CNT/mg alginate. Color images available online at www.liebertpub.com/tec

interconnected fibers, the smaller fiber thickness and the higher CNT content could be other factors contributing to the higher electrical conductivity of the MeCol matrix.³⁶

The storage modulus (E') that represents the hydrogel ability to store deformation energy in an elastic manner was significantly improved (p -value < 0.01) by photo-cross-linking and CNT incorporation in the matrix. This improvement is directly related to the extent of cross-linking of the matrix, where the highly interconnected fiber networks that are resulted from CNT incorporation and photo-cross-linking exert a significant impact on the hydrogel strength.³⁷ Furthermore, evaluated from $\tan(\delta)$ values, the phase angle (δ) significantly reduced from 19.6° for the control sample (no UV, no CNT) to 12.4° for CNT-free photo-cross-linked MeCol and 11.2° and 9.3° for CNT-incorporated photo-cross-linked MeCol at CNT mass ratios of 5 and 10 $\mu\text{g}/\text{mg}$, respectively. Based on the fact that phase angles (δ) of 0° and 90° represent purely elastic and fluidic materials, obviously the phase angles of 11.2° and 9.3° indicate significant improvement in the elastic behavior of CNT-incorporated photo-cross-linked MeCol hydrogel.

Similar to MeCol, CNTs improved the structural features of alginate hydrogel. Unlike, the pristine alginate surface morphology, the incorporation of carboxyl functionalized CNTs into alginate provided a nanowired meshwork with

nanofilaments ranging between 25 and 500 nm. In addition to the covalent bonding carboxyl functionalized CNTs and alginate polysaccharide chains (Fig. 1), some studies have suggested that hydrogel printing^{38,39} and UV irradiation,⁴⁰ which were implemented in this study for hybrid patch bioprinting, may play an important role in CNT alignments and interaction with the alginate matrix. From a biological point of view, cellular response assessment indicated that the incorporation of CNTs into the alginate matrix significantly improved cellular attachment and elongation with respect to the CNT-induced filamentous structure of alginate. In contrast, HCAECs largely stayed round and unattached in the pristine alginate. Similar observation has been reported for HeLa cell adhesion and proliferation, which were significantly improved on CNT-incorporated alginate compared to alginate alone.⁴¹

Immediately after printing and upon exposure to calcium chloride solution, the alginate strands harden due to the ionic cross-linking. Alginate strands showed a good printing resolution of $265 \pm 37 \mu\text{m}$ with respect to the size of the printing nozzle of $250 \mu\text{m}$. However, the strand thickness increased by $\sim 200\%$ over 2 days of incubation in the culture medium due to the alginate swelling. Generally, the CNT-incorporated alginate presented a higher swelling degree compared to alginate alone. The higher swelling degree

could be attributed to free carboxylic groups of non-cross-linked CNTs that enhance water absorption in the matrix compared to the pristine alginate.⁴¹ The CNT-incorporated alginate presented a more stable hydrogel matrix with respect to water retention over 20 days of incubation compared to pristine alginate, indicating a distinct role of CNTs to reinforce hydrogel cross-linking in addition to the ionic cross-linking.

The stereomicroscope and phase contrast images of the hybrid implant after 2 and 4 days of incubation showed a good structural integrity of the photo-cross-linked cell-laden MeCol within the alginate framework. Despite implant swelling, no significant disintegration between alginate strands and MeCol was observed. This might be attributed to a possible interaction between alginate polysaccharide chains and non-cross-linked CNTs in MeCol that may take place at the interface between alginate and MeCol strands of the hybrid implant.

Furthermore, the presence of CNT in the alginate significantly improves the stiffness of the hybrid implant over 20 days of incubation compared to the implants with pristine alginate. Interestingly, the CNT mass ratio of 25 μg CNT/mg alginate offered a generally higher elastic modulus over 20 days of incubation compared to the higher CNT mass ratio of 50 μg CNT/mg alginate. This might be attributed to the higher chance of CNT aggregation at the higher CNT mass ratios in the hydrogel. When aggregated, CNTs tend to form isolated microclusters, rather than being homogeneously distributed in the hydrogel matrix. Consequently, the interaction surface area between CNTs and alginate is significantly reduced. As a result, the isolated CNT microclusters introduce vulnerable stress centers that can initiate microfractures upon compression.

At the CNT mass ratio of 25 μg CNT/mg alginate, the swollen 3D-printed hybrid patch offered an elastic modulus of 43–54 kPa over 20 days of incubation, which is similar to rat right ventricular myocardium elastic modulus (20 ± 4 – 54 ± 8 kPa).⁴² Furthermore, this level of elastic modulus of the CNT-reinforced hybrid implant is significantly higher compared with reported values for gold nanoparticle-incorporated alginate implant (3.5 kPa).⁴³ Compared to the pristine alginate, the CNT-incorporated implants offered higher conductivity (lower impedance) at the physiologically relevant frequency (~ 5 Hz). Considering that electrically conductive cardiac implants can significantly contribute to electrical signal propagation among seeded cardiomyocytes,^{43,44} the proposed hybrid cardiac patch can be a promising implant, from electrical and mechanical perspective, for myocardial tissue engineering.

It should be noted that while CNTs show the ability to improve electrical conductivity in the bioprinted patch *in vitro*, the mechanism of signal propagation in CNTs is quite different from that in cardiomyocytes. The mechanism of current flow in cardiomyocytes is based on the cell membrane depolarization, which takes place due to ion flow (Na^+ , K^+ , Ca^{2+}) through various channels in the cell membrane.⁴⁵ In contrast, the conductivity of CNT-reinforced polymeric composites is governed by “Tunneling” or electron “hopping”, which occurs along CNT interconnects and provides a continuous electron path in CNT network.^{46,47}

Although a recent study has demonstrated that individual small single-walled CNTs (1.5 ± 0.4 nm on diameter and

20 μm in length) have the ability to transfer ions to mimic the protein ion channels in cellular membranes,⁴⁸ the study provides no direct electrophysiological evidence to show that those CNTs could contribute to the action potential propagation between electroresponsive cells (e.g., cardiomyocytes) and further study would be needed to address this point. In addition, most commercially available CNTs are multiwalled with an average diameter larger than 1.5 μm and participate as a CNT network, rather than individual CNTs, in the matrix of tissue engineered constructs. Thus, the predominant mechanism of signal transfer in CNTs in the cardiac patch is based on the electron transportation. Therefore, although the electrical behavior of the patch in this study holds true for *in vitro* evaluation, it may not be able to mimic the electrophysiological condition *in vivo* in the heart muscle.

Despite the difference between the mechanisms of signal transfer of cardiomyocytes and conductive materials (e.g., CNTs), studies aim to enhance electrical signaling through conductive materials, similar to those effects observed in exogenous electrical pacing.⁴⁹ This is based on the hypothesis that the electric field associated with the passing current through the conductive material (e.g., CNTs) could cause depolarization of the membrane of cardiomyocytes, which are cultured on the construct *in vitro*. The redistribution of cytoplasmic materials, the change in cell membrane potential, the electrophoretic accumulation of surface molecules responsible for cell-substratum adhesion,⁵⁰ and the improvement in vital gap junction protein connexin-43 (Cx43) organization and density⁵¹ are among possible mechanisms through which the electric field of the current in the conductive patch may affect cardiomyocytes.

Although these theories may explain the effect of the electric field of the current in a conductive material on the cells, the mechanism of signal transfer at the interface between CNTs and cardiomyocytes is still unclear. Furthermore, the recent findings on ion permeability of small single-walled CNTs⁴⁸ could potentially reveal a new avenue toward building conductive biomimetic cardiac patches; however, further electrophysiological investigations are required to confirm the mechanism of signal transfer through those CNTs between cardiomyocytes.

In addition to the electrical and mechanical behavior, the hybrid implant presented a very promising biological performance *in vitro* with respect to HCAEC proliferation and migration and lumen-like formation in the construct. The photo-cross-linked CNT-incorporated MeCol segments of the hybrid construct promoted HCAEC attachment and elongation within 3–4 days. Unlike the pristine alginate that presented no physical cue for cell attachment or migration, HCAECs demonstrated their tendency to migrate from one MeCol to another over the CNT-incorporated alginate strands by day 7 of culturing. This suggests that CNT-induced filamentous structures, as shown in SEM images, can promote cell attachment and migration, facilitating cellular integrity across the entire hybrid patch by day 10.

In addition, the lumen-like formation of HCAECs represents the ability of cells to attach, elongate, and connect with neighboring cells in the 3D-printed implant. This kind of cellular response to the 3D hydrogel microenvironment is vital to recreate native tissue morphology in engineered tissues. In consideration of the electrical, mechanical,

morphological, and cellular behavior assessments, overall, the proposed nanoreinforced 3D-bioprinted hybrid implants show promise for cardiac tissue engineering applications.

Conclusion

Implementing UV-integrated 3D-printing technique, we bioprinted CNT-reinforced alginate and photo-labile MeCol into hybrid cardiac constructs that offered significantly higher electrical conductivity and compressive modulus with a distinct *in vitro* biological performance. A UV exposure time of ~45 s was identified with insignificant effect on cell viability, while inducing sufficient photo-cross-linking for micropatterning MeCol laden with HCAECs. Incorporation of carboxyl functionalized CNTs into MeCol improved the MeCol nanofibrous matrix with highly interconnected nanofibers (50–200 nm). Likewise, alginate-coated CNTs provided a nanofilamentous structure to alginate matrix that remarkably improved cell elongation and attachment compared to pristine alginate.

The CNT-reinforced MeCol and alginate presented significantly higher storage modulus and compressive elastic modulus, respectively, leading to stiffer and more conductive hybrid 3D-printed patches. Notably, the CNT-reinforced 3D-bioprinted hybrid implants promoted HCAEC proliferation, migration, and differentiation represented by lumen-like formation in the implant within 7–10 days *in vitro*. The proposed hybrid hydrogel construct offers great potential for myocardium regeneration and vascularization, which will be assessed in MI rat models in our future study.

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Disclosure Statement

No competing financial interests exist.

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