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Biomanufacturing of heterogeneous hydrogel structures with patterned electrically conductive regions

Ali Nadernezhad[a,b] , Navid Khani[a,b] , Bahattin Koc[a,b,]*

[a]*Faculty of Engineering and Natural Sciences, Sabanci University, Istanbul, Turkey*
[b]*3D Bioprinting Laboratory, Sabanci University Nanotechnology Research and Application Center, Istanbul, Turkey*

* Corresponding author. Tel.: +90-216-483-9557; fax: +90-216-483-9550. *E-mail address:* bahattinkoc@sabanciuniv.edu

Abstract

In this paper, we present the biomanufacturing of patterned electrically conductive and cell- embedded hydrogel structures, in which selectively patterned regions of hydrogels including carbon nanotubes (CNTs) and NIH 3T3 cells were demonstrated. We employed a recently developed method of multimaterial additive manufacturing of hydrogels, by using an aspiration-on-demand protocol. The printed structures were characterized by electrical resistivity measurements and microscopy techniques. The results showed that by selectively patterning the electrical conductivity, each region can be considered as a resistor in series circuit. Moreover, due to the novel approach in assembly of cell-patterned segments in low viscosity, the cell viability in printed structures was high after printing. The prospects of combining the functionalities of two fabricated structures were further discussed and highlighted.

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Keywords: Multimaterial bioprinting; Hydrogels; Patterned electrical properties

1. Introduction

Bio-additive manufacturing of structures from multiple bioinks, each inducing its own functionalities in the fabricated structures, was the subject of several studies in the past few years. However, there were challenges in further development of the field until the recent time; such as preserving the structural integrity of printed objects and the geometrical macro-precision of multimaterial regions; and also the difficulties in development of hardware platforms. Recent studies [1-4] tried to address those needs by development of hardware platforms able to process a wide range of materials, and opened up the window for further research in fabrication of multifunctional structures by using biomanufacturing technologies.

As an active field of research in biomanufacturing, a great deal of interest was spent on multimaterial 3D printing of hydrogel structures [5, 6] due to their promising properties in the field of tissue engineering. Hydrogels are extremely swollen polymer networks capable of uptaking high water content, and can be used as biomaterials serving different functions like as the scaffolding matrix, cell carrier, carrier for targeted drug delivery, or being incorporated as a part of a bio-sensing device [7-10]. Hydrogels undergo a significant change in their physical properties usually triggered by some external stimuli like change in thermal or chemical properties of the environment, or the presence of light. The extent of changes in physical properties depend on each specific hydrogel system and the process is usually referred to as gelation. Hydrogels with gelation conditions similar to human body's environment are of particular interest, since the process can be triggered upon implantation, or the properties of the hydrogel will not degrade rapidly in the native or simulated body's environment.

Hydrogels can be used as carriers for nanomaterials and nanostructures to enable different functionalities within the mimicked tissue, including electrical conductivity. Several studies were dedicated to modification of different hydrogel nanocomposites with electrically conductive materials like

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metallic nanoparticles, graphene and graphene oxide, and carbon nanotube (CNT) [10]. Inclusion of a conductive nanomaterial within the hydrogel allows electrical stimulation of cells, which has been shown to have effects on cell proliferation and differentiation [11]. Electrical stimulation is particularly effective in nerve and cardiac tissue engineering [12, 13], due to the electric nature of these tissues. Moreover, it is reported that electrical stimulation of stem cells results in change in expression of differentiation markers [14]. Incorporation of CNTs inside hydrogel matrix increases the electrical conductivity of the nanocomposite, and hence its ability to conduct the electrical stimuli signals, and makes it ready to be used in different platforms like tissue models or implantable scaffolds. A number of studies have investigated the contribution of hydrogel-CNT nanocomposites in stimulation of cells and altering their behavior [10]. However, the aim of the most of efforts was not to develop a pattern of electrically conductive regions within the fabricated structure, rather it was only to demonstrate the effects induced by presence or absence of electrically conductive region on the overall biological response of the system. In addition to the external stimulation of cellular constructs *in vitro* or *in vivo*, other factors should be considered to achieve the multifunctional structure of the mimicked tissue. Native tissue comprises a heterogeneous structure with distinct regions of different cell types, different micro/macro-structures, different cellular densities, or different geometrical features like porosity. One approach in mimicking the natural structure of tissue by the aid of biomanufacturing is to fabricate objects with patterned cell densities/types. In this way, each region of the fabricated structure would have its own bio-functionality. Computational and experimental studies have been conducted to prove this concept [15].

Recently, a method for multi-material 3D printing of hydrogels structures was proposed based on aspiration-on-demand of bioinks [2], to fabricate heterogeneous objects with several multifunctionality. In this paper, we employed the previously developed method of biomanufacturing of hydrogel structures to fabricate structures in two categories, one composed of patterned electrically conductive regions, and the other had patterned cell densities. The prospects of the concept of combining these two multifunctional structures will be further discussed, while the possible applications and the potentials of the proposed combination will be highlighted.

2. Materials and methods

Agarose hydrogels were used as the primary bioink. Variation in electrical conductivity of bioinks was done by inclusion of CNTs in agarose hydrogels. NIH 3T3 cells embedded in agarose hydrogel matrix (cell laden) were used to provide different cell densities over the fabricated structure.

2.1. Preparation and characterization of electrically conductive hydrogels

Agarose-CNT nanocomposite hydrogels with three different concentrations of CNTs were prepared by dissolving 2 wt% agarose powder (Sigma) in water using the boiling water bath method. Agarose powder was added to proper amounts of aqueous dispersion of 0.1 wt% CNTs with 0.1 wt% Polyvinilpyrrolidone (OSCiAl) kept in a boiling water bath while the mixture was stirred vigorously for 30 minutes. The mixtures were sonicated at 80 °C by using a probe sonicator (Q700, QSonica) for 30 minutes with 50% amplitude and pulse on/off intervals of 5 seconds. The resulting nanocomposite hydrogels were kept at 60 °C until further use. Three batches of hydrogels with 0.01, 0.03, and 0.05 wt% CNTs were used. Electrical conductivity of hydrogels with different CNT concentrations was measured by Agilent U1273A handheld digital multimeter connected to Cascade PM5 Port Probe Station. Gallium-Indium eutectic (Sigma) was used for the connection between probes and hydrogel filaments.

2.2. Preparation and imaging of NIH 3T3 cell laden

NIH 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (Gibco) and passaged two times a week by incubation in humidified atmosphere with 5% CO_2 at 37 °C. Cell laden bio-ink were prepared by using cell cultures washed with 1X PBS and trypsinized for 5 min followed by centrifuging at 1100 rpm for 5 min. After re-suspension of cell pellets in growth medium and counting with hemocytometer after staining with trypan blue (Sigma), the proper number of cells was added to autoclaved mixture of low melting temperature agarose (Biozym Sieve 3:1) and 1X phosphate buffer saline (PBS, Hyclone by Thermo Scientific). Cell laden bioink was kept at 38 °C with 1 × 106 cells/ml density. The patterned segments in printed constructs were visualized and quantified by the following cell staining protocols. Cell viability in cell suspension was evaluated by using the trypan blue exclusion test and cells were visually examined and scored. Cell viability in cell laden in patterned constructs was assessed by applying a live/dead fluorescence assay after printing. Printed constructs were first stained by incubating in 2 μM calcein-AM (Invitrogen, green fluorescence) and 0.5 μM propidium iodide (Invitrogen, red fluorescence) for 20 min at 37 °C, followed by washing by 1X PBS for three times. Tiled z stacks were then captured by Carl-Zeiss LSM 710 inverted confocal microscope and 3D reconstructed images were analyzed by ImageJ software.

2.3. Biomanufacturing by SU^{3D} bioprinter

The constructs with patterned electrical conductivity and encapsulated cell densities were fabricated by using a method published in [2]. In summary, the inks were loaded in glass vials and kept at proper temperature during printing from which, a robotic dispensing nozzle first aspirated the sequential segments of inks in each filament of the patterned structure in the low viscous state, followed by in-situ gelation of inks within a glass capillary tube and their extrusion. Two different structures were fabricated, one composed of a patterned regions of agarose-CNT nanocomposite hydrogels with different CNT

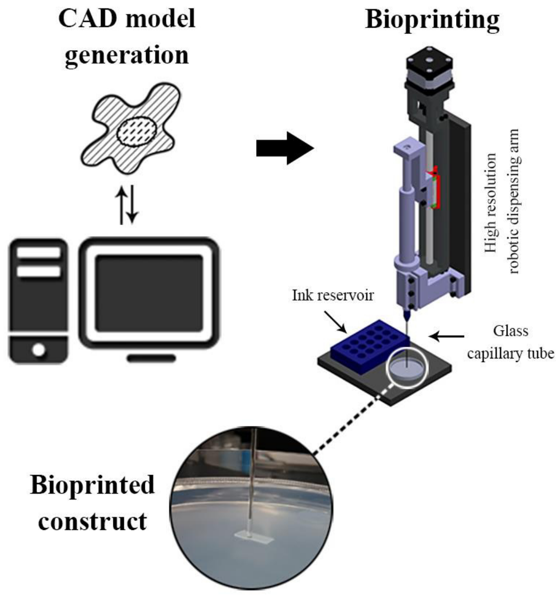

Fig. 1. The process of biomanufacturing of a typical heterogeneous structure by using aspiration-on-demand protocol to assemble bioink segments prior gelation

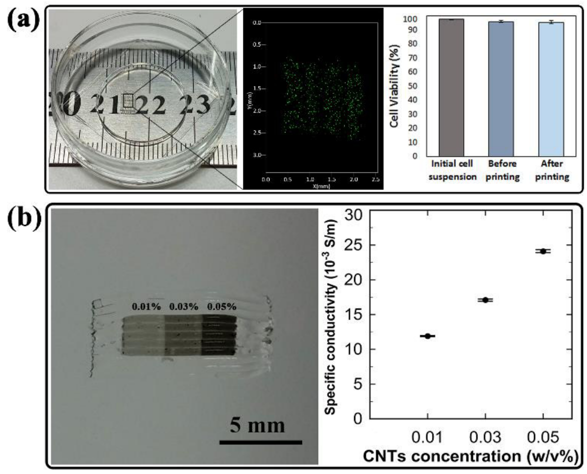

Fig. 2. (a) patterned cell densities across bioprinted hydrogel structure with high cell viability; (b) patterned electrical conductivity in printed object by variation of CNT concentration along each deposited filament.

concentrations, and the other with a rectangular cell laden region within a cell-free hydrogel matrix. The electrically conductive structure was composed of three adjacent squares of agarose-CNT nanocomposite hydrogels with 0.01, 0.03, and 0.05 wt% CNT, respectively.

3. Results and discussion

The overall process of biomanufacturing for printing of heterogeneous structures is shown in Fig. 1. In a typical session, the computer aided design (CAD) model is used to produce tool-path for the robotic dispensing head, and the constitutive material segments of each filament are assembled in the glass capillary tube prior gelation. The details of this process can be found in [2]. The separation of bioink's reservoir from the dispensing head makes all the bioinks available for the aspiration throughout the printing and aspiration-on-demand of bioinks is conducted based on tool path.

Figure 2 shows the printed objects with patterned electrical conductivity and NIH 3T3 cell densities. the patterned electrical conductivity across the printed sample in Fig. 2a is obtained by changing the CNTs concentration in different regions. The specific electrical conductivity of regions with 0.01, 0.03, and 0.05 wt% CNTs were measured as 11.9, 17.1, and 24.1 × 10^{-3} S/m, with standard deviation of 0.19, 0.41, and 0.9 × 10^{-3} S/m, respectively. The density of embedded cells within the agarose hydrogel was 1 million cells/mL (the square region within the printed object in Fig. 2b). Cell viability measurements before and after printing indicated that cell remain highly viable during the process of printing (95.9% and 95.3%, respectively).

Our previous study [2] showed that the nanocomposite hydrogels including electrically conductive materials like CNTs can turn the printed structure into an array of segments with tunable electrical properties, and each segment can be treated as an independent resistor. This provides the possibility of tuning the overall and regional electrical conductivity across the object, in which the net electrical flux in each geometrical region of the construct can be controlled upon applying a defined potential.

The concept of combining two printed structures, one with patterned electrically conductive regions and the other with patterned cell densities within the structure, is shown in Fig. 3. Combination of two structures will result in formation of regions in which the cells can be dispersed over regions which has designed electrical conductivity, by controlling the concentration of the conductive material within the hydrogel matrix. The multifunctionality of the hypothetical structure has several advantages compared to the current methods of bulk-enhancement of the electrical conductivity of culture environment. The primary advantage is the possibility of region-based enhancement of electrical signaling potential between the cells. This can be beneficial in development of tissue constructs with electrical nature, which need to be selectively simulated in specific regions of culture, during the incubation period *in vitro*. A possible application of such structures is in development of *in vitro* models for neuroelectrodes to study the tissue-material interactions in nervous system [16]. On the other hand, the ability to selectively pattern the cell embedded regions during biomanufacturing of hydrogel structures enables the possibility of triggering cellular responses for a specific cell type in a construct hosting multiple cell types. In this way, a combination of electrically conductive environment with a selectively patterned cell laden, mimicking the heterogeneous structure of tissue, will result in selective electrical stimulation of the responsive cell type, while the other cells remain almost unaffected. An important application could be selective promotion of differentiation of stem cells in heterogeneous cell-laden structure of bone tissue constructs [14].

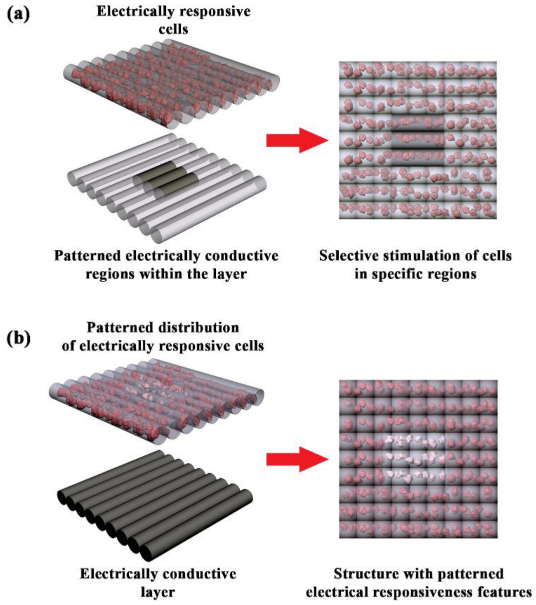

Fig. 3. (a) combination of two successive layers with patterned electrically conductive regions to selectively stimulate electrically responsive cells; (b) combination of two successive layers with patterned distribution of electrically responsive cell over an electrically conductive layer.

4. Conclusions

In this study, biomanufacturing of heterogeneous hydrogel structures with different multifunctionalities was demonstrated. The printed constructs were selectively patterned with electrically conductive regions or different cell densities with high cell viability after printing. The results show the potential of the combination of these two structures with their unique structural features, in production of multifunctional objects with properties specifically tuned for the targeted applications. The advantages of the hypothesized structure were further discussed and the prospective application in the field of tissue engineering were suggested.

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