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Aqueous Two-Phase Emulsion Bioink-Enabled 3D Bioprinting of Porous Hydrogels

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Abstract

The three-dimensional (3D) bioprinting technology provides programmable and customizable platforms to engineer cell-laden constructs mimicking human tissues for a wide range of biomedical applications. However, the encapsulated cells are often restricted in spreading and proliferation by dense biomaterial networks from gelation of bioinks. Herein, we report a novel cell-benign approach to directly bioprint porous-structured hydrogel constructs by using an aqueous two-phase emulsion bioink. The bioink, which contains two immiscible aqueous phases of cell/gelatin methacryloyl (GelMA) mixture and poly(ethylene oxide) (PEO), is photocrosslinked to fabricate predesigned cell-laden hydrogel constructs by extrusion bioprinting or digital micromirror device-based stereolithographic bioprinting. Porous structure of the 3D-bioprinted hydrogel construct is formed by subsequently removing the PEO phase from the photocrosslinked GelMA hydrogel. Three different cells (human hepatocellular carcinoma cells, human umbilical endothelial cells, and NIH/3T3 mouse embryonic fibroblasts) within the 3D-bioprinted porous cell-laden hydrogel patterns showed enhanced cell viability, spreading, and proliferation compared to the standard (i.e. non-porous) hydrogel constructs. The new 3D bioprinting strategy is believed to provide a robust and versatile platform to engineer porous-structured tissue constructs and their models for a variety of applications in tissue engineering, regenerative medicine, and personalized therapeutics.

Keywords

Aqueous two-phase emulsion; 3D bioprinting; bioink; porous hydrogel; gelatin methacryloyl (GelMA); tissue engineering

Tissue engineering provides a viable strategy in restoring the structures and functions of damaged or diseased tissues.^[1] More recently, this technology has also been adapted to the generation of *in vitro* tissue models for applications in improved drug screening and personalized medicine.^[2] In an engineered tissue construct, the cells that form the biological basis, the growth/differentiation factors that induce proper cellular functions, and the

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biomaterial scaffold that mimics the extracellular matrix (ECM), are the basic elements typically necessary to achieve optimal tissue biofabrication.^[3, 4] In particular, the scaffold as a critical component in most scenarios, provide structural support for cell attachment, proliferation, and differentiation.

To mimic the ECM that cells reside in, a wide variety of biocompatible polymers have been used for scaffold fabrication. Among them, hydrogels, a class of highly hydrated polymer networks have been considered attractive, due to their cell friendly aqueous environments as well as suitable structural and mechanical properties that allow cell interactions and biochemical signaling.^[5] Numerous natural and synthetic hydrogel-forming materials, such as poly(ethylene glycol),^[6] collagen,^[7] gelatin,^[8] alginate,^[9] chitosan,^[10] and hyaluronic acid,^[11] have been exploited in the preparation of hydrogel scaffolds for tissue engineering applications. Ideally and in most of the scenarios, these hydrogels should be processed into three-dimensional (3D) crosslinked networks that simulate *in vivo* microenvironments to support spatial activities of the cells, instead of planar, non-structured forms.

The 3D bioprinting technologies have provided rapid and robust approaches to fabricate precisely custom-shaped scaffolds having the capacity to resemble the complex architecture of native tissues.^[3, 12] In general, 3D bioprinting employs cell-laden hydrogels as the bioinks with precision robotic or optical control to generate well-defined 3D tissue constructs through the use of different techniques, such as extrusion bioprinting,^[13] inkjet bioprinting.^[14] Despite the significant advancements in the 3D bioprinting technologies, challenges still remain. For example, dense biomaterial networks are usually necessary to maintain adequate mechanical strength and structural fidelity of the bioprinted constructs, thereby, oftentimes limiting the spreading, migration, and proliferation of encapsulated cells.^[15, 16] Thus, it is strongly desirable to bioprint hydrogel scaffolds ideally containing interconnected pores to allow for effective oxygen, nutrient, and waste diffusion as well as cell motility, facilitating the generation of functional tissues.

Indeed, previous studies have shown that porous hydrogels or void-forming hydrogels possess many advantages in 3D cell cultures over their non-porous counterparts.^[17] Leachable solid particles as sacrificial templates have been used to create porous hydrogel constructs. However, successful and faithful generation of hydrogel constructs with large pore sizes using the 3D bioprinting techniques is limited, since particles with larger sizes tend to easily clog the nozzles in particular in the case of extrusion bioprinting. To this end, integration of templating with 3D printing provides a promising strategy to produce porous scaffolds by simultaneous printing two immiscible phases. The benefit of this technology is that it could control the porosity and interconnectivity of the printed scaffold by tuning composition, viscosity, and concentration of the emulsion inks. The reported porous structures were previously formed by using UV-curable inks based on water-in-oil or oil-in-water emulsions.^[18] However, these conventional emulsion inks possessed the organic phases that served as porogens, which would exert different levels of cytotoxicity limiting their use as bioinks, i.e. when cell encapsulation during the bioprinting processes is preferred.^[19]

A fully aqueous environment ensures convenience mass exchange and good cell survival.^[20] A templating method using Pluronic micelles was shown to produce porous hydrogel scaffolds through extrusion bioprinting.^[21] However, generation of scaffolds with large pore sizes that could enhance nutrient exchange and increase cell growth, spreading, and proliferation remains a challenge, due to the size limitation (maximum size: ~8 μm) of the sacrificial micelles that could be formed. Aqueous two-phase systems containing two different water-soluble polymers with low interfacial energy have been used to partition biomolecules in water, including nucleic acids, proteins, and cells.^[22] The benefits of having such a system as the bioinks lie in i) the enhanced convenience in tuning the stability as the phase separation can be controlled at a wide range of temperatures, phase volume and mass fraction ratios, and osmotic pressure,^[23] ii) the maintained aqueous environment and high biocompatibility to the biomolecules of the two phase-forming polymers with high molecular weights at low concentrations,^[20] and iii) a readily tunable pore size of the bioink in a wide range (up to several tens of micrometers). We therefore hypothesized that the aforementioned drawbacks of the conventional emulsion bioinks could possibly be overcome with an aqueous two-phase (i.e. water-in-water) emulsion method wherein aqueous solutions of two incompatible hydrophilic biomaterials are mixed to form an emulsion.^[24] Such an emulsion, which consists of droplets of one immiscible aqueous phase dispersed within the another continuous aqueous phase, is formed due to the thermodynamic incompatibility between aqueous solutions of certain water-soluble polymers without the need of surfactants, particles, or organic solvents.^[25]

The present study, therefore, aimed to formulate a novel bioink formulation based on aqueous two-phase emulsion of two biocompatible solutions, consisting of gelatin methacryloyl (GelMA) and poly(ethylene oxide) (PEO), leading to the formation of highly interconnected and hierarchical pores in the bioprinted structures upon subsequent photocrosslinking and leaching (Figure 1 and S1). Combination of different concentrations of these two biopolymers was assessed to find the optimal conditions to balance the rheological properties, printability, pore size, and structural integrity of bioprinted constructs, as well as cellular behaviors. Importantly, the feasibility for the use of the cell-laden GelMA-PEO emulsion as the bioink was demonstrated in two distinct bioprinting methods, extrusion bioprinting and digital micromirror device (DMD)-based stereolithographic bioprinting. The 3D-bioprinted porous hydrogel constructs encapsulating various cell types showed high biocompatibility as well as excellent cell viability, proliferation, and metabolic activity as compared to the controls bioprinted with pure GelMA.

GelMA is a biocompatible gelatin-based polymer that have been widely used to fabricate cell-laden 3D tissue analogs due to their suitable biological properties and tunable physical characteristics.^[26, 27] Similarly, PEO as a synthetic polyether (also known as poly(ethylene glycol) (PEG) at small molecular weights), is prevalently used as hydrogel scaffolds for biomedical applications owing to its biocompatibility, inertness, and readily available molecular modifications.^[28] Studies have shown that the addition of PEG increases the strength of hydrogen bonding between gelatin chains and results in phase separation of the gelatin/PEG aqueous solutions at both above and below the gelation temperature of gelatin.^[29, 30]

Based on this prior knowledge, we generated our emulsion bioink by mixing GelMA and PEO aqueous solutions both in phosphate-buffered saline (PBS), to induce the formation of discontinuous PEO droplets dispersed in the continuous GelMA phase, under physiological conditions. We chose PEO with a molecular weight of 300,000 Da since it has been reported that the phase separation is affected by the molecular weight of PEO, and low molecular weights of PEO do not generally induce phase separation.^[29] Figure S2 shows photographs of the GelMA-PEO emulsion after mixing the GelMA and PEO aqueous solutions at a 1:1 volume ratio by pipetting for 5 s. To distinguish the two phases of the bioink, PEO was labeled with fluorescein isothiocyanate (FITC) (greenish yellow), while GelMA was conjugated with rhodamine B (red). No clear difference was observed during the first 20 min. Phase separation started to occur after 30 min and became complete after 100 min. Therefore, our GelMA-PEO emulsion is anticipated to be stable to produce microporous hydrogels using bioprinting during the 30-min time window and this duration could possibly be prolonged by lowering the temperature.^[30] The relative good stability of the emulsion bioink ensures adequate time for bioprinting.

To ensure the appropriate mechanical property of the porous hydrogels, 10% (wt% for all expressions unless otherwise indicated) GelMA pre-gel solution was selected as the base for all our bioink formulations, and different concentrations of PEO solutions were introduced into this base solution and vigorously mixed. The stability of the PEO phase was dependent on the concentration of PEO used to prepare the emulsions. When the PEO phase was high in concentration of 1.6 % (w/v) (undissolved PEO could be observed at higher concentrations thus not used, Figure S3) in the mixture, the boundaries of the two phases were clearly observed, indicating the formation of microscale phase separation; when the PEO phase was at low in concentration (0–0.5%), phase separations poorly formed (Figure 2a). Therefore, the 1.6% of PEO was chosen to prepare the GelMA-PEO emulsions.

Similarly, we also investigated the size of PEO droplets, dependent on the volume ratio of the 1.6% PEO solution and the 10% (w/v) GelMA solution. To obtain the size distribution of the PEO droplets in the GelMA-PEO emulsion, GelMA was conjugated with rhodamine B and photocrosslinked by using lithium phenyl-2,4,6-trimethyl benzoyl phosphinate (LAP) as the photoinitiator. When exposed to UV light for 40 s (0.5 W cm^{-2}), the continuous phase of GelMA was solidified to form a hydrogel containing the emulsion droplets of PEO. The hydrogel was imaged using a fluorescent microscope (Figure 2b and S4). Rhodamine B conjugation did not seem to affect the formation of the emulsion. The quantitative analysis of size distributions of the PEO droplets dispersed in the GelMA continuous phase was derived from ImageJ analysis. By measuring 100 individual PEO droplets directly from optical microscopic images, a narrow distribution of $22.7 \pm 5.5 \text{ }\mu\text{m}$ could be obtained at the volume ratio of 4:1. The distribution became broader and the average size of the PEO droplets increased to $52.7 \pm 17.7 \text{ }\mu\text{m}$ as the volume ratio of GelMA to PEO was increased to 1:1 (Figure 2c). To observe the porosity of the formed hydrogels, PEO droplets were removed by immersing in PBS for 24 h, leaving voids within the crosslinked GelMA hydrogel. The interconnected porous structure of the GelMA hydrogel was investigated by a diffusion test using a blue dextran solution (molecular weight: 2,000,000 Da), where it was observed that the blue dextran could diffuse through the fabricated porous GelMA hydrogel but not the standard GelMA hydrogel (Figure S5). The interconnected porous structure was

further characterized by 3D reconstruction of the z-stack using the confocal fluorescence microscopy (Figure 2d). The rhodamine B-conjugated GelMA hydrogel was shown in red, while the dark areas represented the interconnected pores. The dependency of the pore size of the hydrogel on volume ratio of GelMA and PEO, and the interconnected micropores were further characterized by using scanning electron microscopy (SEM, Figure 2e). Higher uniformity and smaller pore sizes ($24.8 \pm 10.3 \mu\text{m}$) were measured in the GelMA-PEO emulsion at the volume ratio of 4:1 as compared with the emulsion at the volume ratio of 1:1 having an average pore size of $58.7 \pm 24.9 \mu\text{m}$, matching those observed from optical micrographs.

Mechanical properties of the formed hydrogels were further evaluated with different formulations of GelMA-PEO emulsions (Figure 2f, S7, and Tables S1, S2). To ensure proper mechanics, 10% GelMA was chosen for emulsion bioink preparation. It has been found that when the volume ratio of GelMA and PEO was maintained at 1:1, the Young's modulus of the GelMA-PEO hydrogel increased from $0.9 \pm 0.3 \text{ kPa}$ to $1.4 \pm 0.1 \text{ kPa}$, as the PEO concentration increased from 0.5% to 1.6%; when the PEO concentration was kept constant at 1.6%, as the volume ratio of GelMA and PEO increased from 1:1 to 4:1, the Young's modulus increased from $2.3 \pm 0.4 \text{ kPa}$ to $9.8 \pm 1.6 \text{ kPa}$. It was evidenced that Young's moduli of the porous GelMA hydrogel with different formulations were higher than the 5% pure GelMA (Figure 2g).

The rheological properties of a hydrogel as the bioink are critical to understanding its printability, in particular for extrusion bioprinting. GelMA is a temperature-sensitive material, which is more viscous at lower temperatures than at higher temperatures because of the reversible physical crosslinking.^[27] Thus, the rheology of the GelMA bioinks can be readily regulated by adjusting the temperature. To examine the effect of PEO on the rheological properties of the GelMA-PEO emulsion bioinks, we examined the viscosity of different formulations of the GelMA-PEO emulsion bioinks as a function of temperature (Figure 2f). The shear rate was kept constant at 50 s^{-1} and the viscosity of the defined bioinks was measured from 36°C to 6°C at a cooling rate of 5°C min^{-1} . All the GelMA-PEO emulsion bioinks tested had an obvious decrease in viscosity when the temperature increased from 6°C to 25°C . The increased volume ratio of GelMA to PEO from 1:1 to 4:1 also resulted in the increment of viscosity when the temperature was lowered. As such, while the phase ratio significantly affected the viscosity of the emulsion in different formulations of the bioinks, the temperature was the key factor determining the emulsion viscosity for a defined formulation. Thus, similar to the pure GelMA bioink,^[31] the emulsion bioink was anticipated to allow for direct extrusion bioprinting at the 15°C . Combining the abovementioned results of different formulations in GelMA-PEO emulsion, to achieve printable GelMA hydrogels with interconnected pores, the GelMA-PEO aqueous two-phase emulsion containing 10% GelMA solution and 1.6% PEO solution at a volume ratio of 1:1 was used for subsequent studies.

To further demonstrate the advantages of the porous structure of the GelMA hydrogel scaffolds, the cell-laden GelMA-PEO emulsions were photocrosslinked in polydimethylsiloxane (PDMS) molds, where subsequently removed to achieve the porous GelMA structures. Several mammalian cell types including human hepatocellular carcinoma

cells (HepG2), human umbilical endothelial cells (HUVECs), and NIH/3T3 mouse embryonic fibroblasts (NIH/3T3 fibroblasts), were chosen to encapsulate in the cell-laden porous hydrogel constructs. The cells were cultivated for 7 days and their viability, spreading, and proliferation within the porous GelMA hydrogel were characterized by Live/Dead assay and Prestoblu[®] test. Cells encapsulated within standard GelMA hydrogels (5% to match the concentration of GelMA in the emulsion bioink) were used as the control group. The viability of HepG2 cells in the porous hydrogels was 11% lower than that in the standard hydrogels possibly due to the low cell adaptability to the PEO phase in the emulsions at Day 1; however, the viability increased to 7% higher than that in standard GelMA hydrogel at Day 7 with the leaching of PEO and the formation of pores (Figure 3a and Table S3). In comparison, HUVECs and NIH/3T3 fibroblasts exhibited similar viability within porous hydrogels as compared with standard hydrogels encapsulated cells (Figure S8), which demonstrated the high biocompatibility of the emulsions and the porous GelMA hydrogels. HUVECs proliferation and spreading were enhanced by 3 fold and 4 fold in the porous GelMA hydrogels as compared with standard hydrogels on Day 3 and Day 7 respectively, as evidenced by Prestoblu[®] assay (Figure 3b and Table S4). Cell spreading in the porous GelMA hydrogel was further evaluated by measuring cell volumes in 3D reconstruction.^[16] When encapsulated in the porous hydrogel constructs, the NIH/3T3 fibroblasts could well-spread, resulting in a 3-fold larger volume than that of the same cells in the counterparts on Day 7 (Figure 3c). Additionally, a great number of spreading cells was observed in the porous GelMA scaffolds in comparison to those in the standard GelMA scaffolds in both of HUVECs and NIH/3T3 fibroblasts on Day 7 (Figures 3d and S9). Therefore, our developed emulsion bioink formulation and subsequently formed porous hydrogels were able to promote the growth and spreading of encapsulated cells as compared to their non-porous counterparts.

To demonstrate the 3D fabrication of the porous structures, extruding bioprinting was first utilized to deposit the cell-laden GelMA-PEO emulsion bioink. The emulsion bioink was prepared in the same way as described before, followed by cooling at 4 °C for 20 min and then extruding at 15 °C with simultaneous crosslinking of GelMA *via in situ* UV exposure (0.5 W cm^{-2} , 15 s) (Movie S1). Additional UV exposure (15 s) was performed to strengthen the crosslink density of the hydrogel. Several pre-designed patterns were bioprinted using the emulsion bioink and compared with that of the pure GelMA bioink (5%) (Figure 4a–c). The bioprinted structures using the emulsion bioink showed the structural integrity while possessing interconnected pores (Figure 4b–ii, iii, iv). On the contrary, the pattern bioprinted with the standard GelMA hydrogel was homogeneous and showed no macroscale porosity as anticipated (Figure 4b–i). We were also able to bioprint multi-layered 3D structures using the GelMA-PEO emulsion bioink, demonstrating its excellent printability (Figure 4c–iii, iv). As with the pure GelMA bioink,^[13, 32] the GelMA-PEO emulsion bioink could also be readily bioprinted. Standard cell-laden GelMA patterns, used as the control, was achieved by 3D bioprinting of the pure GelMA/cell mixture (Figure 4c–i, ii). Similar to the scaffolds formed by the molding technique, the proliferation of encapsulated cells in the bioprinted 3D porous GelMA constructs were significantly higher as compared to the counterparts bioprinted with standard GelMA bioink (Figure 4d and S10). The presence of interconnected pores within the bioprinted porous GelMA constructs also allowed for improved spreading

of the cells. Moreover, the structural integrity of the hydrogel constructs was maintained, even after cell spreading (Figure 4d).

Similarly, we performed bioprinting of the GelMA-PEO emulsion bioink using a DMD bioprinting method to demonstrate the versatility of our developed emulsion bioink. DMD bioprinting is based on layer-by-layer crosslinking of the bioink in a reservoir, which avoids the shear stress the cells experience associated with the extrusion process and is generally faster, making it preferable in certain applications.^[33] We demonstrated DMD bioprinting of cell-laden constructs using a predefined serpentine pattern, while the uncrosslinked bioink together with the PEO droplets was washed away with PBS immediately after bioprinting (Figure 5a). As with the extrusion bioprinting, all encapsulated cell types showed enhanced spreading and proliferation within the DMD-bioprinted porous GelMA structures (Figure 5b) compared to corresponding bioprinted standard GelMA patterns (Figure 5c and S11).

In conclusion, we have described an aqueous two-phase emulsion method to prepare biocompatible and bioprintable bioinks using a blend of GelMA and PEO solutions. The concentrations of the two water-soluble polymers were optimized for phase separation and subsequent emulsion formation, where discontinuous PEO droplets were dispersed in the continuous GelMA phase. Droplet size, mechanical and rheological properties, and printability of the GelMA-PEO emulsion bioinks were carefully characterized, to obtain optimal combination to achieve proper rheological properties and good printability of the bioink as well as larger pore sizes and desired structural integrity of the bioprinted constructs. The feasibility of the GelMA-PEO emulsion for use as the bioink was demonstrated in both extrusion and DMD-based bioprinting methods. The 3D-bioprinted porous GelMA constructs, encapsulating various cell types, showed high biocompatibility as well as excellent viability, proliferation, and metabolic activity of the cells as compared to those bioprinted with conventional pure GelMA as the bioink. The mechanical properties of the bioprinted porous hydrogel constructs may be enhanced by incorporating carbon nanomaterials, laponite clay-based nanocomposite, and micro/nanofibers.^[34] It is anticipated that our 3D bioprinting strategy utilizing the aqueous two-phase emulsion bioink could be widely extended to engineering various tissue constructs with further optimizations in a tissue type-specific manner.^[35]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematics showing 3D bioprinting of (a) a porous hydrogel structure using the two-phase aqueous emulsion bioink and (b) a conventional hydrogel structure.

Figure 2.

Characterization of the GelMA-PEO emulsions. (a) Effect of PEO concentration on emulsion droplet size, at GelMA:PEO phase volume ratio of 1:1, where the optical images show the morphologies of the emulsions as the PEO concentrations were varied from 0% to 1.6%. (b) Effect of volume ratio of GelMA (10%) and PEO (1.6%) on emulsion droplet size. Fluorescence micrographs of rhodamine B stained porous GelMA with varied GelMA:PEO volume ratios from 1:1 to 4:1. (c) Pore size distribution of porous GelMA at various GelMA:PEO phase volume ratios. Inset shows average pore size as a function of GelMA:PEO volume ratio. In each case 100 random pores were analyzed. (d) 3D reconstruction confocal fluorescence image of the hydrogel with interconnected pores; inset shows the top view. (e) SEM micrographs showing the porous GelMA hydrogels with the GelMA:PEO volume ratios of 1:1 (left) and 4:1 (right). (f) Young's modulus of the porous GelMA hydrogels as functions of (i) PEO concentration and (ii) volume ratio. (g) Viscosity of GelMA-PEO emulsions as a function of temperature. Pure GelMA hydrogel at the concentration of 5% was used as the control group. (* $p < 0.05$, ** $p < 0.01$, $n = 3$).

Figure 3.

Characterizations of cells encapsulated in the porous GelMA constructs (cylinder, 10 mm in diameter and 2 mm in thickness). Standard GelMA hydrogels (5%) were used as the control. (a) Fluorescence micrographs showing viability of encapsulated HepG2 cells on Day 1, Day 3, and Day 7, where live cells were stained in green and dead cells in red (i), and quantifications of cell viability (ii). (b) Quantifications of proliferation of HUVECs on Day 1, Day 3, and Day 7 using the Prestoblu[®] assay. (c) Quantifications of NIH 3T3 fibroblast volumes within the GelMA constructs. Graph shows the comparison between cell volumes in standard GelMA and porous GelMA constructs on Day 7. (d) Cell spreading within the GelMA constructs. Confocal fluorescence micrographs show morphologies of NIH/3T3 fibroblasts within (i and iii) standard GelMA constructs and (ii and iv) porous GelMA constructs. The cells were stained for nuclei (blue) and F-actin (green). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$).

Figure 4. Extrusion bioprinting of the aqueous two-phase emulsion bioink. (a) Designed patterns. (b) Optical micrographs of corresponding printed standard GelMA hydrogel patterns (i and v) and porous GelMA hydrogel pattern (ii-iv, vi-viii) at low magnification (i-iv) and high magnification (v-viii). (c) Photographs of 3D printed multi-layered standard (i-ii) and porous GelMA hydrogel patterns (iii-iv). The bioinks in (i, ii) were stained with rhodamine B prior to bioprinting to aid visualization. (d) Fluorescence micrographs showing bioprinted HUVECs and NIH/3T3 fibroblasts in standard GelMA hydrogel patterns (i-iv) and porous (v-viii) GelMA hydrogel patterns on Day 7 of culture.

Figure 5. DMD bioprinting of the two-phase emulsion bioink. (a) Principle of the DMD bioprinting. (b) Bioprinted standard GelMA hydrogel pattern (i) and porous (ii) GelMA hydrogel pattern. (c) Fluorescence micrographs showing cell spreading within the standard (i-iii) and porous (iv-vi) GelMA hydrogel patterns on Day 7 of culture. The cells were stained for F-actin (green) and nuclei (blue).