Visible Light-based Stereolithography Bioprinting of Cell-adhesive Gelatin Hydrogels

Zongjie Wang, *Student Member, IEEE*, Zhenlin Tian, Xian Jin, *Member, IEEE*, Jonathan F. Holzman, *Member, IEEE*, Frederic Menard, and Keekyoung Kim, *Member, IEEE*

***Abstract*—Stereolithography-based bioprinting offers advantages in resolution and rapid printing time, and thus has received major attention in recent years. However, traditional stereolithography-based bioprinting utilizes a ultraviolet light which may cause mutagenesis and carcinogenesis of cells. In this paper, we present a new visible light crosslinkable bioink that is based on cell-adhesive gelatin. The bioink consists of Eosin Y (EY) based photoinitiator and gelatin methacrylate (GelMA) pre-polymer solution. We examined the feasibility of using visible light from a commercial beam projector to pattern the EY-GelMA bioink. We measured the absorbance of bioink to characterize its sensitivity to visible light and performed bioprinting to test its ability to promote cell adhesion. It is found that the EY-GelMA bioink has an absorption peak at roughly 522 nm and that it can be successfully crosslinked by visible light from the commercial projector. We performed the bioprinting experiments and visualized the cell morphology using nuclei/F-actin staining. Experimental results show that most of the cells attached to the EY-GelMA bioink after five days' culturing. Ultimately, the EY-GelMA bioink can support both visible light crosslinking and cell adhesion, offering great potential in bioprinting and tissue engineering.**

I. Introduction

Organ shortage crisis has become problematic in recent years. According to the U.S. Department of Health and Human Service [1], in 2015, there were roughly 120,000 people waiting for organ transplants, but less than 20% of these people could receive organs. Organ donation has not been significantly increased in the last ten years and is not likely to meet the deficit of donor organs. Therefore, there are high demands of artificial organs and tissues as a solution to the organ shortage [2]. To allow cells to proliferate and mature to form tissues, an appropriate cell microenvironment is required. It has been well established that cell behavior is more native to a culture in a 3-D microenvironment, rather than a 2-D microenvironment [3], [4]. Thus, biomaterial scaffolds are needed to localize cells in the 3-D microenvironments. Bioprinting can be used to create such scaffolds.

Bioprinting is an additive manufacturing process of biocompatible materials and/or living cells. It has become one of the most promising solutions for fabricating artificial organs [5]. In the bioprinting process, a combination of cells and biomaterials, referred to as bioinks, are dispensed on the micrometer scale to form artificial tissues. Such bioprinting can offer high-resolution, scalability, and cost-effectiveness. As a result, the development and application of bioprinting has greatly increased since 2010. In recent years, bioprinting has successfully generated vascularized tissues [6], cartilage [7], and bone [8] and has been applied to the formation of a cell microarray and a drug screening platform [9], [10].

Bioprinting techniques can be divided into four categories: inkjet [11], extrusion[12], laser-based [13], and stereolithography (SLA) bioprinting [14]. The SLA bioprinting uses a light reflected from a digital micromirror device (DMD) to selectively cure the bioinks layer-by-layer. Its advantages over the other three techniques include its high resolution (< 100 μm) and high cell viability (> 85%) [14]. However, the light source of the conventional SLA bioprinting is in the ultraviolet (UV) spectrum, which may cause mutagenesis and skin carcinogenesis [15]. Therefore, UV light is not appropriate for printing artificial organs.

We previously reported a visible light crosslinkable bioink being a mixture of poly(ethylene glycol) diacrylate (PEGDA) and gelatin methacrylate (GelMA) [16]. Although the bioink offered controllable mechanical properties and high cell viability after printing, it did not allow cells to attach and spread in the hydrogel. Such cell attachment is critical to the formation of tissues from individually printed cells [17]. With this in mind, in this work, we present a new bioink solution that provides both visible light crosslinking and cell adhesion. The bioink solution is based on Eosin Y (EY) photoinitiation and GelMA hydrogel. It has an absorption peak at approximately 522 nm and can be crosslinked by visible light generated from a commercial beam projector. The EY-GelMA bioink has improved biological properties, over those of existing visible light crosslinkable hydrogels.

II. Materials and Methods

A. Hydrogel Preparation

GelMA hydrogel was synthesized in accordance with a previously reported process [18]. Briefly, 5 g of gelatin was dissolved in 50 mL of dimethyl sulfoxide (VWR International, Radnor, PA, USA) at 50ºC with stirring. Then 0.3 g of 4-dimethylaminopyridine and 2 mL of glycidyl methacrylate were added. The mixture was stirred for two days at 50ºC as shown in Figure 1A. The mixture was then dialyzed against reverse osmosis (RO) water at room temperature for five days. We changed the water twice a day. After dialysis, the sample

*This research is supported by the Natural Sciences and Engineering Research Council of Canada Discovery Grant and the Canada Foundation for Innovation.

Zongjie Wang, Xian Jin, Jonathan F. Holzman, and Keekyoung Kim are with the School of Engineering, University of British Columbia, Kelowna, BC, V1V 1V7, Canada (email: keekyoung.kim @ubc.ca)

Zhenlin Tian and Frederic Menard are with the Irving K. Barber School of Arts and Sciences, University of British Columbia, Kelowna, BC, V1V 1V7, Canada.

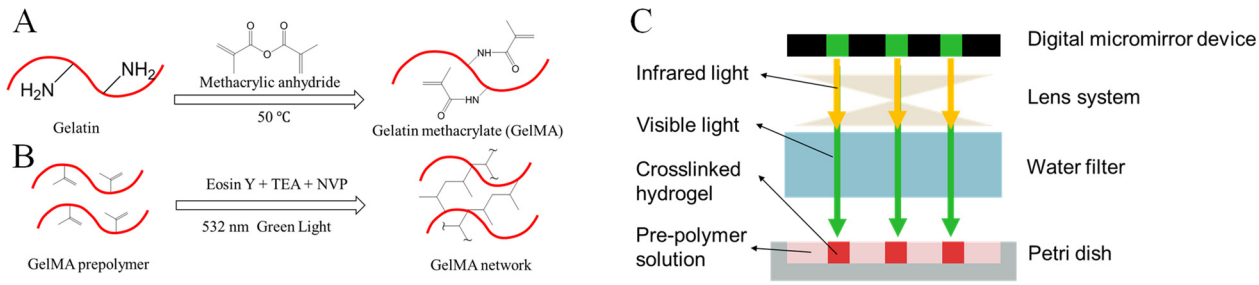

Figure 1. Synthesis and bioprinting of EY-GelMA hydrogel. (A) The methacrylation group is introduced to the gelatin via reaction with methacrylic anhydride to form GelMA. (B) GelMA pre-polymers can be crosslinked to build a hydrogel network in the presence of EY-based photoinitiator and green visible light. (C) The patterned light reflected from the digital micromirror device array selectively irradiates the pre-polymers to pattern desired 3-D structures.

was freeze-dried via lyophilization. The EY-GelMA bioink was prepared by dissolving 15% w/v freeze-dried GelMA, 0.02 mM Eosin Y disodium salt, 0.2% w/v triethanolamine (TEA), and 74 nM 1-vinyl-2 pyrrolidinone (NVP) in phosphate-buffered saline (PBS) as shown in Figure 1B. All chemicals were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA), unless otherwise specified.

B. Working Principles

We previously reported a SLA bioprinting system [16]. A beam projector (Acer, Taipei, Taiwan) was used as the device for generating light patterns. The projector was placed 4 cm away from the reflection mirror. The distance between the mirror and printing plate was 6 cm. A 4 cm thick water filter was placed between the mirror and sample to block harmful infrared radiation and heat from the projector. A syringe pump (Kent Scientific, Torrington, CT, USA) was utilized to dispense the bioink before printing each layer.

The working principle of the SLA bioprinting is illustrated in Figure 1C. Inside the beam projector, there is a digital micromirror device (DMD) in which one micromirror represents a pixel. The DMD can control the angle of each mirror and define the brightness of a light passing through the mirror. Thus, the DMD is able to control the light intensity across a small area of the projected beam. In the DMD shown in Figure 1C, the green colour represents pixels with high intensity while the black colour represents pixels with low intensity. The light beam generated by the DMD passes through a lens system and is focused onto the hydrogel pre-polymer solution. Areas irradiated by high-intensity light have the photoinitiator receive sufficient photons to trigger polymerization, while areas irradiated by little or no light do not undergo polymerization. In this way, spatial crosslinking and SLA bioprinting are realized.

C. Cell Culturing and Bioprinting

NIH-3T3 fibroblasts cells were cultured at 37℃ and 5% CO_2. The cell media consists of Dulbecco's Modified Eagle Medium (Lonza, Basel, Switzerland) with 10% v/v heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 1% v/v penicillin-streptomycin (VWR) and 1% v/v amphotericin B (VWR). The cell media was changed every two days. EY-GelMA hydrogel pre-polymer was mixed with cells ($8x10^6$ cells/mL) before the bioprinting process. Then 1 mL of the bioink was evenly added to the (3.5-cm-diameter) petri dish before bioprinting to form a uniform and thin layer of hydrogel. The petri dish was then exposed to the patterned light for 4 minutes to form a bottom layer of the pattern. For each subsequent layer, 200 µL of the hydrogel pre-polymer solution was added into the petri dish and exposed to the light for 2 minutes. This process was repeated until the pattern was completed. Immediately after printing, the sample was washed by PBS solution twice, and 3 mL of fresh media was added to the petri dish. At the end, the petri dish with the printed pattern was placed into an incubator for culturing.

D. Immunostaining and Microscopy

Cell adhesion of the bioprinted sample was examined at day 5 via nuclei/F-actin staining. Briefly, we fixed the samples using 3.7% v/v paraformaldehyde (Sigma) in PBS and treated the samples by 0.5% v/v Triton-X 100 in PBS to make the cell membrane permeable. Subsequently, a working stock of Acti-stain™ 488 phalloidin (Cytoskeleton, Denver, CO, USA) was added and the samples were incubated for 60 minutes to stain the F-actin of NIH-3T3 cells. In the end, the samples were washed with PBS and fixed by mounting media (Fluoroshield with DAPI, Sigma-Aldrich). The samples were examined immediately under a fluorescence microscope (DMi8, Leica, Wetzlar, Germany).

III. Results and Discussion

To ensure proper functioning of the beam projector and EY-based photoinitiator, we measured the light spectrum of the projector using a spectrometer (CCS200, Thorlabs, Newton, NJ, USA) positioned in the printing plane. As shown in Figure 2A, the lamp of beam projector has significant light intensity (> 20% of its peak intensity) between 480 nm and 600 nm. In addition, we characterized the absorption spectra of the EY in GelMA pre-polymer solution using a UV-Vis spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). A blank solution sample, only containing 15% w/v GelMA, was used as a spectra reference. As shown in Figure 2B, the EY-based photoinitiator has a major absorption peak at 522 nm and a minor absorption peak around 315 nm. Therefore, the major absorption peak of the EY-based photoinitiator fits into the light spectrum of the beam projector. It is concluded that the beam projector and EY-based photoinitiator are an effective combination for SLA bioprinting.

We printed a NIH-3T3 fibroblast-laden mesh pattern at

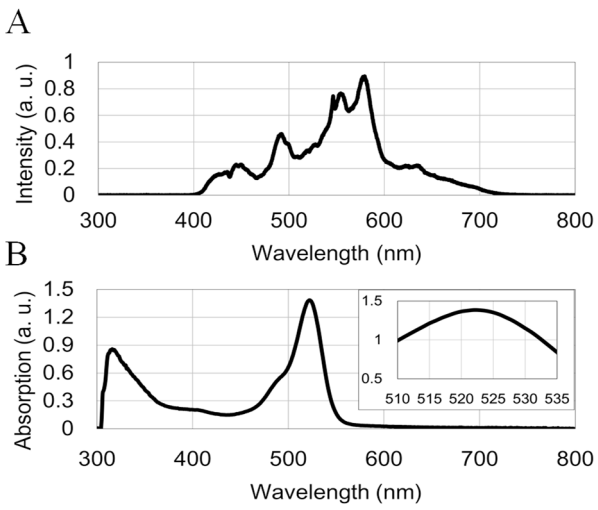

Figure 2. Spectrum of the projector and photoinitiator. (A) Spectrum of the light from the projector. (B) UV-Vis absorption spectrum of EY-based photoinitiation. The inset of (B) shows a closeup of the major absorption peak.

the millimeter scale to demonstrate SLA-based bioprinting. After printing, samples were cultured for five days and nuclei/F-actin staining was applied to visualize cell adhesion and morphology. We obtained fluorescent-labeled images of the samples under an inverted fluorescence microscope. Figures 3A and 3B shows representative fluorescent images at 10x magnification. The cells spread and proliferated well in the printed GelMA network, suggesting that cell adhesion of the bioprinted EY-GelMA scaffold is good.

Since gelatin is a hydrolysis product of collagen and contains many arginine-glycine-aspartic acid (RGD) sequences to allow for cell adhesion [19], the RGD sequences are maintained in GelMA (which is made from gelation by introducing methacrylate subsitutions). As shown in Figure 1A, the methacrylate subsitutions are directly introduced to reactive amine via reacting with methacrylic anhydride at 50℃. The degree of methacrylation can be tuned by regulating the amount of methacrylic anhydride. After this modification, GelMA contains abundant methacrylate groups and can be crosslinked by water soluble photoinitiators, such as EY-based photoinitiator, via free radical photopolymerization, see Figure 1B. The EY-based photoinitiation is a multi-reagent crosslinking method where EY, TEA and NVP are used as the photosensitizer, initiator and accelerator, respectively [20]. Once irradiated by green light, EY's spectral properties in polar solvent are changed to make the EY take reductive electrons from the electron donor, TEA. The TEA, after electron transfer, forms a TEA radical cation (TEA^+) which crosslinks methacrylate groups in the GelMA. Note that the methacrylic anhydride-based reaction only involves less than 5% of the amino acid residues in molar ratio [21]. As a result, GelMA still contains most of the functional amino acid motifs used for cell adhesion [22].

We further examined the cell morphology at 40x magnification. The results are shown in Figure 3C. As can be seen, the cells spread well inside the bioprinted sample and form a 3-D cell-cell network structure, which is very similar to the *in vivo* cellular microenvironment [23]. On the contrary, the NIH-3T3 fibroblasts remained round in shape after five days' culturing inside the blend of PEGDA and GelMA hydrogel [16]. This indicates that the cells cannot adhere to the hydrogel. Since cell adhesion is an essential process for tissue formation [17], the EY-GelMA bioink is deemed to be well suited for bioprinting and artificial organ generation. Future studies will systematically characterize the effects of EY and GelMA concentration on the mechanical and biological properties of printed hydrogels.

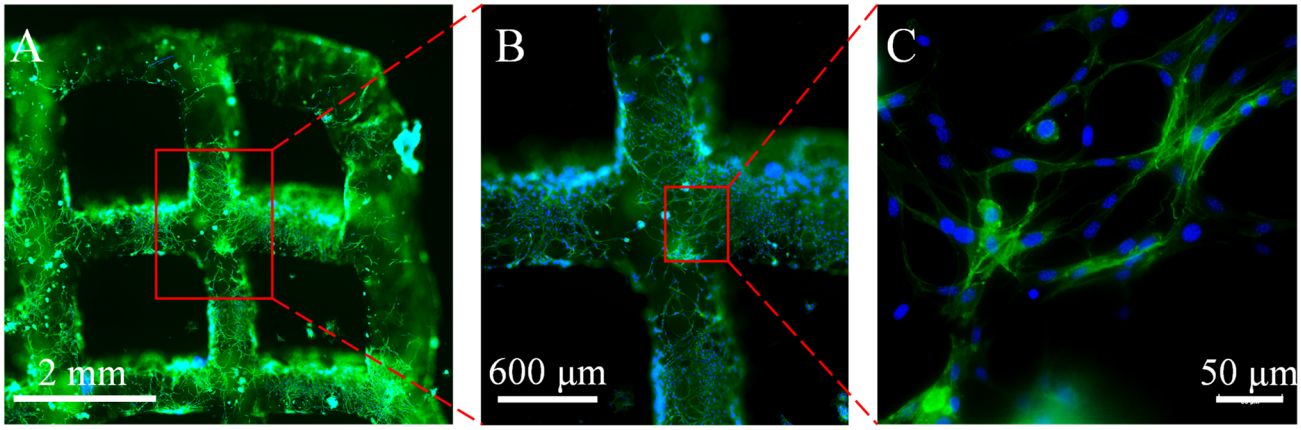

Figure 3. A fluorescently labeled image of NIH-3T3 cell-laden bioprinted mesh pattern sample at day 5. Cell morphology was visualized by the staining (A) 10x tilescanned image (B) 10x deconvoluted image (C) 40x single layer image.

IV. Conclusion

In this paper, we present a visible light crosslinkable and cell-adhesive bioink based on EY photoinitiation and gelatin-derived GelMA hydrogel. The EY-GelMA bioink can be crosslinked by light from a beam projector, the characteristics of which were defined by spectral measurements. We performed bioprinting and culturing of the

bioprinted samples. Verifications via F-actin staining showed that most cells adhered to the bioink after five days' culturing. Ultimately, the new bioink offers significant improvement over prior non-cell-adhesive bioinks and thus has potential for future bioprinting and tissue engineering applications.

ACKNOWLEDGMENT

This work was supported by Natural Sciences and Engineering Research Council of Canada Discovery Grant and Canada Foundation for Innovation John R. Evans Leaders Opportunity Fund. The authors thank Dr. Sanjoy Ghosh at the UBC Irving K. Baber School of Arts and Sciences for providing the NIH-3T3 fibroblast cells.

REFERENCES

[1] U. S. D. of H. & H. Services, "United Network for Organ Sharing: OPTN report," 2015.

[2] J. P. Vacanti and R. Langer, "Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation.," *Lancet*, vol. 354 Suppl, p. SI32-I34, 1999.

[3] M. W. Tibbitt and K. S. Anseth, "Hydrogels as extracellular matrix mimics for 3D cell culture," *Biotechnol. Bioeng.*, vol. 103, no. 4, pp. 655–663, 2009.

[4] Z. Wang, R. Samanipour, and K. Kim, "Organ-on-a-Chip Platforms for Drug Screening and Tissue Engineering," in *Biomedical Engineering: Frontier Research and Converging Technologies*, Springer International Publishing, 2015, pp. 209–233.

[5] C. Mandrycky, Z. Wang, K. Kim, and D. H. Kim, "3D bioprinting for engineering complex tissues," *Biotechnol. Adv.*, vol. 34, no. 4, pp. 422–434, 2016.

[6] D. B. Kolesky, R. L. Truby, A. S. Gladman, T. A. Busbee, K. A. Homan, and J. A. Lewis, "3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs.," *Adv. Mater.*, vol. 26, no. 19, pp. 3124–30, May 2014.

[7] X. Cui, K. Breitenkamp, M. G. Finn, M. Lotz, and D. D. D'Lima, "Direct Human Cartilage Repair Using Three-Dimensional Bioprinting Technology," *Tissue Engineering Part A*, vol. 18, no. 11–12. pp. 1304–1312, Jun-2012.

[8] G. Gao, T. Yonezawa, K. Hubbell, G. Dai, and X. Cui, "Inkjet-bioprinted acrylated peptides and PEG hydrogel with human mesenchymal stem cells promote robust bone and cartilage formation with minimal printhead clogging," *Biotechnol. J.*, vol. 10, no. 10, pp. 1568–1577, 2015.

[9] F. Xu, J. Wu, S. Wang, N. G. Durmus, U. A. Gurkan, and U. Demirci, "Microengineering methods for cell-based microarrays and high-throughput drug-screening applications.," *Biofabrication*, vol. 3, no. 3, p. 34101, 2011.

[10] Z. Wang, B. Calpe, J. Zerdani, Y. Lee, J. Oh, H. Bae, A. Khademhosseini, and K. Kim, "High-throughput investigation of endothelial-to-mesenchymal transformation (EndMT) with combinatorial cellular microarrays," *Biotechnol. Bioeng.*, vol. In press, Dec. 2015.

[11] G. Gao, A. F. Schilling, K. Hubbell, T. Yonezawa, D. Truong, Y. Hong, G. Dai, and X. Cui, "Improved properties of bone and cartilage tissue from 3D inkjet-bioprinted human mesenchymal stem cells by simultaneous deposition and photocrosslinking in PEG-GelMA," *Biotechnol. Lett.*, vol. 37, no. 11, pp. 2349–2355, 2015.

[12] W. Liu, Y. S. Zhang, M. A. Heinrich, F. De Ferrari, H. L. Jang, S. M. Bakht, M. M. Alvarez, J. Yang, Y.-C. Li, G. Trujillo-de Santiago, A. K. Miri, K. Zhu, P. Khoshakhlagh, G. Prakash, H. Cheng, X. Guan, Z. Zhong, J. Ju, G. H. Zhu, X. Jin, S. R. Shin, M. R. Dokmeci, and A. Khademhosseini, "Rapid Continuous Multimaterial Extrusion Bioprinting," *Adv. Mater.*, pp. 1–8, 2016.

[13] Z. Wang, X. Jin, R. Dai, J. F. Holzman, and K. Kim, "An ultrafast hydrogel photocrosslinking method for direct laser bioprinting," *RSC Adv.*, vol. 6, no. 25, pp. 21099–21104, 2016.

[14] R. Gauvin, Y.-C. Chen, J. W. Lee, P. Soman, P. Zorlutuna, J. W. Nichol, H. Bae, S. Chen, and A. Khademhosseini, "Microfabrication of complex porous tissue engineering scaffolds using 3D projection stereolithography.," *Biomaterials*, vol. 33, no. 15, pp. 3824–34, May 2012.

[15] U. P. Kappes, D. Luo, M. Potter, K. Schulmeister, and T. M. Rünger, "Short- and long-wave UV light (UVB and UVA) induce similar mutations in human skin cells.," *J. Invest. Dermatol.*, vol. 126, no. 2004, pp. 667–675, 2006.

[16] Z. Wang, R. Abdulla, B. Parker, R. Samanipour, S. Ghosh, and K. Kim, "A simple and high-resolution stereolithography-based 3D bioprinting system using visible light crosslinkable bioinks," *Biofabrication*, vol. 7, no. 4, p. 45009, 2015.

[17] B. M. Gumbiner, "Cell adhesion: The molecular basis of tissue architecture and morphogenesis," *Cell*, vol. 84, no. 3, pp. 345–357, 1996.

[18] C. Cha, J. Oh, K. Kim, Y. Qiu, M. Joh, S. R. Shin, X. Wang, G. Camci-unal, K. Wan, R. Liao, and A. Khademhosseini, "Micro fluidics-Assisted Fabrication of Gelatin-Silica Core − Shell Microgels for Injectable Tissue Constructs," *Biomacromolecules*, vol. 15, pp. 283–290, 2014.

[19] Y. Liu and M. B. Chan-Park, "A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture," *Biomaterials*, vol. 31, no. 6, pp. 1158–1170, 2010.

[20] O. Valdes-Aguilera, C. P. Pathak, J. Shi, D. Watson, and D. C. Neckers, "Photopolymerization studies using visible light photoinitiators," *Macromolecules*, vol. 25, pp. 541–547, 1992.

[21] A. I. Van Den Bulcke, B. Bogdanov, N. De Rooze, E. H. Schacht, M. Cornelissen, and H. Berghmans, "Structural and rheological properties of methacrylamide modified gelatin hydrogels.," *Biomacromolecules*, vol. 1, pp. 31–38, 2000.

[22] K. Yue, G. Trujillo-De Santiago, M. Mois Es Alvarez, A. Tamayol, N. Annabi, and A. Khademhosseini, "Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels," *Biomaterials*, vol. 73, pp. 254–271, 2015.

[23] H. M. Langevin, N. A. Bouffard, G. J. Badger, J. C. Iatridis, and A. K. Howe, "Dynamic fibroblast cytoskeletal response to subcutaneous tissue stretch ex vivo and in vivo," *J. Anat.*, vol. 5405, pp. 747–756, 2005.