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TITLE: Layer-by-Layer Bioprinting of Stem Cells for Retinal Tissue Regeneration

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Layer-by-Layer Bioprinting of Stem Cells for Retinal Tissue Regeneration

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We aim to investigate a Layer-by-Layer (LBL) bioprinting process using stem cells for retinal tissue regeneration. The LBL nature of the bioprinting process matches nicely with the native, multilaminar anatomy of the retina. This research will bioprint retinal stem cells (RSCs) and appropriate growth factors (GF) encapsulated in a biomaterial (e.g., hyaluronic acid, HA). We will investigate the mechanical, chemical, and biological properties of the hydrogel materials. We will study the cell viability and analyze the biological functions of the 3D printed retina tissue.

Unclassified

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1. **Introduction**

We report on the project - “Layer-by-Layer Bioprinting of Stem Cells for Retinal Tissue Regeneration” for FY 2014-2015. We have developed the hydrogel biomaterials with comparable mechanical properties that are 3D printable using light-based polymerization process. We have demonstrated that these 3D-printed hydrogel materials are also biocompatible for retinal cell growth.

2. **Keywords**

retinal tissue regeneration, 3D bioprinting, Layer-by-Layer, hydrogel biomaterials.

3. **Accomplishments**

**What were the major goals of the project?**

The major goal of this project is to investigate a Layer-by-Layer (LBL) bioprinting process using stem cells for retinal tissue regeneration. Our specific aims are as followings:

**Specific Aim 1:** Develop and optimize a 3D bioprinting method with encapsulated RSCs,

**Specific Aim 2:** Layer-by-layer bioprinting of *in vitro* retina PRs/RPE/Bruch’s membrane tissues.

**What was accomplished under these goals?**

1) Major activities: There are two major tasks for Year 1: a) Biomaterial Development, b) Layer-by-layer Bioprinting

2) Specific objectives: a) Synthesis of hyaluronic acid - glycyl methacrylate hydrogel; b) Mechanical Testing of the hydrogel; c) Bioprinter development and optimization; d) Layer-by-layer printing using hyaluronic acid - glycyl methacrylate hydrogel; e) Layer-by-layer printing encapsulating retinal stem cells

3) Significant results or key outcomes:

a) For the synthesis of HA-GM, 200 mg of hyaluronic acid (Lifecore Biomedical, MN) was added into 25ml of 50% acetone solution. The solution was mixed overnight before adding 1.8ml (20-fold molar excess) of triethylamine and glycyl methacrylate. The reaction took 8 hours before dialysis and lyophilization. The methacrylation was confirmed by $^1$H-NMR characterization (Joel 500) by the peaks at 5.6 and 6.0 ppm, as shown in **Fig. 1a**. The degree of methacrylation (DM) is determined by integration of the methacrylation group over that of the methyl groups in hyaluronic acid at 1.7, 1.8 and 1.9 ppm, as shown in **Fig. 1b**. For example the DM of the sample in Fig. 1a is 23%. The relation between the initial molar ratio of reagents and the degree of methacrylation of the product is shown in Table 1.

![Figure 1](image1.png)

**Figure 1.** The $^1$H-NMR results of HA-GM showing: a) the methacrylate group; b) the
integration of methacrylate group over the methyl groups of hyaluronic acid to calculate the degree of methacrylation

Table 1. Degree of methacrylation of HA-GM synthesized by different initial stoichiometric ratio

<table>
<thead>
<tr>
<th>Initial molar ratio of glycidyl methacrylate to hyaluronic acid</th>
<th>Degree of methacrylation of final HA-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:1</td>
<td>23%</td>
</tr>
<tr>
<td>10:1</td>
<td>11%</td>
</tr>
<tr>
<td>5:1</td>
<td>3%</td>
</tr>
</tbody>
</table>

b) For mechanical testing, the hydrogel sample was prepared by a 3D printing apparatus developed in the lab. A rectangular hydrogel sheet with a dimension of 5mm x 10mm x 1mm was made with the synthesized HA-GM. The elastic modulus of the 3D-printed sheets using HA-GM was determined by tensile tests using a thermomechanical analysis machine (TMA, Perkin Elmer). Briefly, the sample was clamped at two ends. The force applied on the sample started from 3mN with a constant increment of 5mN/min while the elongation of the sample was measured. A stress vs. strain curve is obtained and the elastic modulus is calculated as the slope of the curve. Samples made by HA-GM with methacrylation ratio lower than 23% could not be characterized by this method due to softness. Such softer hydrogels will be measured in the future using atomic force microscopy (AFM). The test result of 2% hydrogel made by HA-GM with 23% methacrylation is shown in Fig. 2. The elastic modulus of the hydrogel is about 30±5 kPa. Previous work suggested that the elastic modulus of native retina is about 20 kPa. Compare to other materials used for retina replacement, such as poly (glycerol-sebacate) which has an elastic modulus of 600kPa, HA-GM has closer mechanical property as native retina.

Figure 2. Stress vs. strain curve of HA-GM hydrogel with 23% methacrylation ratio
c) We have established a strategy to print the HA-GM hydrogel into dome shapes to resemble the native environment for retina development. To improve the cell adhesion within the hydrogel, we added another printing material, methacrylated gelatin, GelMa, into the HA-GM hydrogel. As the hydrolysis product of collagen, gelatin could facilitate the adhesion of cells due to its chemical structure. We used lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as the free radical generator. LAP has a local absorbance maximum at approximately 375 nm and significant absorbance at 365 nm. The pre-polymer resin included HA-GM, GelMa and LAP. Cell could be mixed into the resin if encapsulation is needed for year 2 tasks.

Figure 3 shows the 3D printing apparatus developed for this project. Briefly, a coherent light source (Omnicure S2000, 365nm) is used to provide the UV light for photopolymerization. Patterns are generated by Adobe Photoshop and transferred to a digital-mirror array device (DMD) by in-house software. The DMD chip is used as an optical mask for projecting patterns onto the pre-polymer resin to allow the resin to polymerize. Once the light passes through the lenses, it is collimated to form a precise image on the resin. The resin is positioned on the stage. The stage could move in all three dimensions, dictated by the image from the computer. By changing the height of the stage, a 3D device can thus be fabricated. The printer enabled us to construct complex structures with the biomaterials. For example, we could print a dome structure with softer material as the core, followed by a harder material as a shell, as shown in Figure 4. Briefly, HA-GM was printed as the first layer in a cylindrical shape with thickness of 125 μm and diameter of 1,500 μm. The shell was printed with HA-GM and GelMA mixture. It was printed in the same shape as the first layer with a thickness of 250 μm and diameter of 2,500 μm. With our printer and biomaterial, we could deliver a layer-by-layer printing strategy for retina regeneration in vitro.
d) We developed the layer-by-layer (LBL) bioprinting system for the encapsulation of living cells, particularly retinal stem cells (RSCs). In the previous quarters, we were able to use the LBL system to print three-dimensional (3D) structures with hydrogel materials, such as hyaluronic acid-glycidyl methacrylate (HA-GM) and gelatin methacrylate (GelMa). During this quarter, we succeeded to incorporate living cells in the LBL bioprinting process, and the cell-loaded structures were cultured in vitro to evaluate the cell viability and investigate the interaction between cells and the microenvironment created by bioprinting.

Briefly, the RSCs were mixed with the monomer solution made of HA-GM and GelMa by gentle pipetting. The cell-loaded solution was then loaded to the stage for bioprinting. A core-shell structure was printed to mimic the curved layer-by-layer fashion of the native retina tissues (Figure 5). This design was also expected to introduce a gradient of nutrition and growth factor diffusion from the outside to the inside of the dome structure, which could guide the orientation of the cells to mimic the highly ordered retinal cells including photoreceptors, ganglion cells and bipolar cells. As shown in Figure 5, we were also able to vary the cell density in each layer to investigate the optimal condition for the differentiation of the RSCs into the cells of different layers in the retinal tissue. The cell density in the core part was 30 million per ml and the cell density in the shell was 15 million per ml.

After 10 days of in vitro culture, we found that some RSCs were spreading in the hydrogel structure along the direction of the radius as well as the interface between the core and the shell (Figure 6). Immunofluorescence staining also showed the expression of photoreceptor-specific reporter IRBP-GFP (green) and recoverin (red), indicating photoreceptor induction with the help of differentiation medium (Figure 7).

Figure 4. A dome structure constructed by DMD based projection printing using biocompatible HA-GM and GelMA hydrogel. Green color comes from fluorescein isothiocyanate-dextran in HA-GM as the first layer; red color comes from the tetramethylrhodamine isothiocyanate-dextran in HA-GM and GelMA as the second layer. Scale bar = 500 µm.

Figure 5. Bioprinted core-shell structure encapsulated with RSCs. Scale bar: 500 µm.
Figure 6. Encapsulated cells spreading in the shell layer (left) and the interface between the core and the shell at day 10. Scale bar: 250 µm.

Figure 7. Characterization of RSC differentiation to photoreceptors at day 10. Green: photoreceptor-specific reporter IRBP-GFP. Red: recoverin. Scale bar: 250 µm.

In order to construct a multi-layered structure to facilitate the differentiation of RSCs, a single layered hRPE cells is desired. A uniform spreading of hRPE cells mimics the in vitro environment during the retinal formation, thus secreting the necessary growth factors and differentiation signals for the RSCs. We have prepared a single layered hRPE cells by encapsulating the cells into GelMa solution and printed a thin layer on the cover glass, as shown in Fig. 8. At day 2, the hRPE cells were still encapsulated in the gel without spreading. After 4 more days, the cells have detected the stiffer glass surface and migrated to form a single layer.
Figure 8. Formation of single layered hPRE cells: Left: 2 days after printing. Right: 6 days after printing.

What opportunities for training and professional development have the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
We have presented our research results in several invited talks in university seminars and professional meetings such as the Pre Retina Society Annual Meeting, Philadelphia, PA.

What do you plan to do during the next reporting period to accomplish the goals?
We plan to carried out research for Specific Aim 2 - Layer-by-layer bioprinting of in vitro retina PRs/RPE/Bruch’s membrane tissues. We will first print out three individual layers for PRs, RPE, and Brunch’s membrane separately and study cell functions. Then we will print a multilayer constructs with all three cell types.

4. Impact
What was the impact on the development of the principal discipline(s) of the project?
The layer-by-layer 3D bioprinting method will be a viable tool for retinal research. It could also be translational for future clinical uses in retina tissue regeneration. The hydrogel biomaterials will also be useful for creating retinal tissue constructs that could be used for basic research.

What was the impact on other disciplines?
The 3D bioprinting process is versatile in that it can be applied to other cell and tissue types. The 3D-printed retinal tissues could be used as in vitro models for early drug screening.

What was the impact on technology transfer?
We will file a provisional patent when we have enough results.
What was the impact on society beyond science and technology?
Development of artificial retina tissues will change the clinical landscape by eliminating the current dependency on retina donor tissue and by providing a new strategy for restoring vision that would otherwise be lost in soldiers with severe retina blindness. The proposed 3D bioprinting and stem cell engineering represent an integration of emerging technologies that are truly novel for retina repair and regeneration.

5. Changes/Problems
Nothing to report

6. Products
Publications, conference papers, and presentations:
Dr. Chen was invited to give the following seminars and presentation

Dr. Zhang was invited to give a seminar - “Genetics, Epigenetics, and Stem Cell Based Therapies for Blinding Eye Diseases”, Dept. of Bioengineering, UC San Diego, May 1, 2015.

Dr. Zhang and Nature Publishers are organizing a Nature Conference on Tissue Engineering and Regenerative Medicine in 2016.

Website(s) or other Internet site(s)
Dr. Chen’s lab website updates news and publications: http://schen.ucsd.edu/lab/index.html
Dr. Chen’s lab website updates news and publications: http://zhanglab.ucsd.edu

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report
7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name</th>
<th>Project Role</th>
<th>Nearest person month worked</th>
<th>Contribution to Project</th>
<th>Funding Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaochen Chen</td>
<td>PI</td>
<td>1</td>
<td>Supervised the project, designed the experiments, and advised the graduate students</td>
<td>Partially from this grant.</td>
</tr>
<tr>
<td>Kang Zhang</td>
<td>Co-PI</td>
<td>1</td>
<td>Co-supervised the project, co-designed the experiments, and co-advised the graduate students</td>
<td>Partially from this grant.</td>
</tr>
<tr>
<td>Wei Zhu</td>
<td>Graduate Student</td>
<td>6</td>
<td>Carried out the experiments, and analyzed the results</td>
<td>Partially from this grant.</td>
</tr>
<tr>
<td>Pengrui Wang</td>
<td>Graduate Student</td>
<td>10</td>
<td>Carried out the experiments, and analyzed the results</td>
<td>Partially from this grant.</td>
</tr>
</tbody>
</table>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

New grants:

- CMMI1547005 (Chen) 10/1/2015–9/30/2017 1%
  NSF $39,734
- EAGER/Cybermanufacturing: Cloud-based, Rapid, Microscale 3D Bioprinting
  The major goal of this project is to develop a cyber-manufacturing system for 3D printing using cloud-based computational approach.
  Our specific objectives are to: 1) Create and characterize the mCOP bioprinting platform for digital, projection 3D printing, 2) Demonstrate the use of mCOP for creating complex structures in hydrogel, and 3) Create live, functional 3D scaffolds with cell encapsulation.
  POC: Dr. Bruce Kramer, (703) 292-5348, bkramer@nsf.gov
The goal of this project is to develop a 3D bioprinting platform using hESC for the direct-write of 3-dimensional functional cardiac tissue constructs.

Specific Aims are: 1) Develop and optimize a 3D printing technique to create biomimetic 3D micro-architectures using HA-based biomaterials and hESC-derived cardiomyocytes, 2) Create an advanced vascularization technique for 3D pre-vascularized cardiac tissues with precise control of spatial organization, 3) Test the in vivo functionality of the 3D bioprinted pre-vascularized cardiac tissue in Hu-mice and rat MI models.

POC: Dr. Ryan Wells, 415-396-9124, rwells@cirm.ca.gov

Completed projects / grants

**R01EB012597** (Chen, Khademhosseini) 08/30/2010 – 08/31/2014 8%
NIH/NIBIB $240,000
A Microfabrication Platform for Direct Printing Vascularized Functional Tissue Constructs
The major goal of this project is to develop a microfabrication platform using projection printing for 3D scaffold fabrication and create vascularized functional tissue constructs.
Specific Aims are to: 1) develop and optimize the PSL system for the fabrication of 3D microstructures using HA with Arg-Gly-Asp (RGD) and matrix metalloproteinase (MMP), 2) direct-write 3D HA scaffolds encapsulating cardiomyocytes, 3) create vascularized structures in a 3D scaffold and analyze vasculature functions.
POC: Dr. Rosemarie Hunziker, 301-451-1609, hunzikerr@mail.nih.gov

**CMMI 1130894** (Chen) 08/01/2011 – 07/31/2014 5%
National Science Foundation $53,841
Collaborative Research: Nano/Femtosecond Laser Processing of Gas Impregnated Polymer for Biomedical Applications
The major goals of this project are to develop a micro-manufacturing process by integrating a nanosecond laser with a femtosecond laser for polymer processing.
Our objectives are to: 1) study the heating and ablation mechanisms of the nano-/femtosecond lasers in gas impregnated polymers, 2) study the bubble nucleation and growth process under nanosecond laser heating, 3) evaluate the fabricated porous scaffolds for organotypic 3D cell culturing.
POC: Dr. Mary Toney, (703) 292-7008, mtoney@nsf.gov

**What other organizations were involved as partners?**
Nothing to report

**8. Special Reporting Requirements**
The Quad Chart is shown below.
9. Appendices

Abstracts for presentation in university seminars and conferences
1) Corinne Bower Lecture, Pre Retina Society Annual Meeting, Philadelphia, PA, 2014

3D Bioprinting: Materials, Fabrication, and Tissue Engineering
Shaochen Chen, Ph.D.
Professor of NanoEngineering and Bioengineering Departments
Co-Director, Biomaterials & Tissue Engineering Center, Institute of Engineering in Medicine,
University of California, San Diego

Abstract

The goal of our laboratory is to develop micro- and nano-scale bioprinting techniques for the direct-write of 3D designer scaffolds used for tissue engineering and regenerative medicine. In this talk, I will present my laboratory’s recent research efforts in femtosecond laser nano-printing and projection 3D bioprinting to create 3D scaffolds using a variety of biomaterials. These 3D biomaterials are functionalized with precise control of micro-architecture, mechanical (e.g. stiffness and Poisson’s ratio), chemical, and biological properties. Design, fabrication, and experimental results will be discussed. Such functional biomaterials allow us to investigate cell-microenvironment interactions at nano- and micro-scales in
response to integrated physical and chemical stimuli. From these fundamental studies we can create both
in vitro and in vivo tissue models for precision tissue engineering and regenerative medicine.

2) (Distinguished Seminar) University of California at Davis, March 12, 2015

**3D Bioprinting: An Enabling Technology for Tissue Engineering and Regenerative Medicine**

Shaochen Chen, Ph.D.
Professor of NanoEngineering and Bioengineering Departments
Co-Director, Biomaterials & Tissue Engineering Center
Institute of Engineering in Medicine
University of California, San Diego

**Abstract**

My laboratory aims to develop micro- and nano-scale bioprinting techniques for the direct-write of 3D designer scaffolds used for tissue engineering and regenerative medicine. In this talk, I will present my laboratory’s recent research efforts in 3D nano-printing and rapid 3D bioprinting to create 3D scaffolds using a variety of biomaterials. These 3D biomaterials are functionalized with precise control of micro-architecture, mechanical (e.g. stiffness and Poisson’s ratio), chemical, and biological properties. Design, fabrication, and experimental results will be discussed. Such functional biomaterials allow us to investigate cell-microenvironment interactions at nano- and micro-scales in response to integrated physical and chemical stimuli. From these fundamental studies we can create both in vitro and in vivo tissue models for precision tissue engineering and regenerative medicine.


**Nano and Microscale 3D Bioprinting: An Enabling Technology for Personalized Regenerative Medicine**

Shaochen Chen, Ph.D.
Professor of NanoEngineering and Bioengineering Departments
Co-Director, Biomaterials & Tissue Engineering Center, Institute of Engineering in Medicine,
University of California, San Diego

**Abstract**

In my laboratory, we develop micro- and nano-scale bioprinting techniques for the direct-write of 3D designer scaffolds used for tissue engineering and regenerative medicine. In this talk, I will present my laboratory’s recent research efforts in femtosecond laser nano-printing and projection 3D bioprinting to create 3D scaffolds using a variety of biomaterials. These 3D biomaterials are functionalized with precise control of physical, mechanical, chemical, and biological properties, aiming for personalized medicine. Design, fabrication, and experimental results will be discussed. Such functional biomaterials allow us to investigate cell-microenvironment interactions at nano- and micro-scales in response to integrated physical and chemical stimuli. From these fundamental studies we can create both in vitro and in vivo tissue models for precision tissue engineering and regenerative medicine.