Multiscale Biomaterials for Cell and Tissue Engineering

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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The Ohio State University
2017

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Abstract

The aim of tissue engineering is to fabricate functional tissue constructs for treating diseases. Contemporary approach for tissue engineering is to embed cells in homogenous three-dimensional (3D) macroscopic scaffolds for mimicking the function of extracellular matrix (ECM) in tissues. However, the native ECM is usually not homogenous and these macroscopic scaffolds are suboptimal due to the limited diffusion length of oxygen and nutrients in cellularized tissues. In this dissertation work, cell microencapsulation and microfluidic technologies are utilized to fabricate multiscale heterogeneous biomaterials for resolving these issues.

First, the development of a novel non-planar microfluidic flow-focusing device for high-throughput encapsulation of mouse embryonic stem cells (mESCs) in a liquid core of microcapsules with an alginate hydrogel shell is reported. Using the non-planar microfluidic device, mESCs can be encapsulated in the core with high viability to form uniform sized aggregates, to mimic the growth of totipotent-pluripotent stem cells in early pre-hatching embryos. Further, it is shown that mESCs cultured in the biomimetic microcapsules have higher pluripotency and differentiation potential than the cells cultured under traditional two-dimensional (2D) condition. Utilizing the same microfluidic device, biomimetic ovarian microtissue consisting of an ovarian follicle embedded in microcapsules with a collagen core and alginate hydrogel shell is fabricated. This miniaturized 3D culture of early secondary preantral follicles facilitate their development
to the antral stage. The study revealed the crucial role of mechanical heterogeneity in the mammalian ovary in regulating follicle development and ovulation. It is also demonstrated that the proliferation, differentiation, and development of mESCs and preantral follicles can be modulated by changing the composition of ECM in the core of the microcapsules.

Next, a bottom-up approach for fabricating 3D vascularized human breast tumor model with the core-shell microencapsulation technology is developed. Microtumors (i.e., 3D aggregates of cancer cells) are generated in core-shell microcapsules and used together with human endothelial cells and human adipose derived stem cells (hADSCs) as building blocks to self-assemble into vascularized tumor in collagen hydrogel. The utility of the platform in drug screening is further demonstrated. It is shown that vascularization can render increased cancer resistance to chemotherapy. This vascularized tumor system may be valuable for in vitro drug screening to better predict the drug efficacy in cancer patients. Lastly, a multiscale system for efficient co-delivery of cells and proteins/growth factors in vivo is developed to address the issue of low cell survival associated with cell delivery in vivo. The multiscale delivery system is comprised of therapeutic agents-laden nanoparticles encapsulated in microcapsules (nano-in-micro), hADSCs, and collagen hydrogel. The nano-in-micro system enables sustained release of therapeutic proteins to interact with their receptors on the hADSCs in the system which significantly improves the survival and proliferation of the hADSCs after implantation. This is shown to greatly facilitate the tissue regeneration in an ischemic disease model.

To conclude, this dissertation work demonstrates how microscale encapsulation of cells via microfluidics provides a powerful suite of tools to engineering the cellular microenvironment at micro and macro scales. The technologies and systems described here
could potentially help in building tissue engineering constructs that enable treatment of a myriad of human diseases.
Dedicated
To

My grandparents (nana and nani) for making me who I am, and my complete family for supporting me all the way!
Acknowledgements

My experience at The Ohio State University has been unique and very rewarding, which helped me to prosper personally and scientifically. This was not possible without the love, support, and the guidance of many people. I would like to express my extreme gratitude to my parents, Mukund and Shikha for their love, sacrifice, and all of their efforts to shape my character. Dr. Xiaoming He, my Ph.D. advisor, has been the one graciously helping me with the thesis and all of my academic endeavors throughout these years. I would like to thank him for his mentorship, support, and patience to train me as a scientist. I would like to express my respect and admiration for his knowledge, vision, management, dedication, and hard work that goes into running the lab.

I would like to thank the committee members, Dr. Keith Gooch, Dr. Gunjan Agarwal, and Dr. Yi Zhao for their advice, perspective, and generosity with their time. All the insights and advice they offered tremendously improved my research. Also, I would like to thank Dr. Zhao for generously letting me use their lab equipment.

I would also like to thank all my fellow lab members, especially, Dr. Hai Wang, Dr. Wujie Zhang, Dr. Wei Rao, Dr. Jiangshen Xu, Dr. Shuting Zhao, Dr. Haishui Huang, Mingrui Sun, and Jenna Dumbleton. Your friendship means a lot to me. I admire your character, knowledge, and passion for science. Similarly, I would also like to thank Dr. Qian Wang and Dr. Kang Wei from Zhao lab for teaching me microfabrication. Thanks to Leming Sun and Xinghua Jia from Prof. Mingjun Zhang’s lab for their friendship.
Throughout these years, I had the opportunity to work with many undergraduate and high school students; I want to thank them all, especially, Pete Bielecki and Joe Jeffery. I would also like to thank my Indian friends, Prashant, Mukul, Ashish, Pavandeep, Sai, Amit, Sanjay, Balaji, and many others who have been like family to me throughout these years. Lastly, I would like to express my gratitude to my previous advisors and mentors: Dr. Samuel Sia, Dr. Cheul Cho, and Dr. Brian Gillette. You all have been inspirational.

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Major Field: Biomedical Engineering
Statement of Contributions

This dissertation represents the accumulated doctoral research of the present author and other collaborators. Prof. Xiaoming He, as the principal investigator, contributed to all the work the present author has with the designs of experiments, data analysis, and preparations of journal articles and this dissertation. A brief statement of contributions made by the author and other collaborators are provided below:

Chapter 1: The present author performed the literature review, edited figures, and wrote the main text in this chapter.

Chapter 2: This chapter was reproduced from a paper published as: Agarwal, P., Zhao, S., Bielecki, P., Rao, W., Choi, J.K., Zhao, Y., Yu, J., Zhang, W., and He, X. One-step microfluidic generation of pre-hatching embryo-like core-shell microcapsules for miniaturized 3D culture of pluripotent stem cells. Lab on a Chip 2013; 13: 4525-4533. PMID: 24113543. The present author carried out all the experiments and analyzed the data with inputs from all other co-authors.

Chapter 3: This chapter was reproduced from a paper published as: Choi, J.K.*, Agarwal, P.*, Huang, H.*, Zhao, S., and He, X. The crucial role of mechanical heterogeneity in regulating follicle development and ovulation with engineered ovarian microtissue. Biomaterials 2014; 35: 5122-5128. (*equal contribution). PMID: 24702961. The present author fabricated microfluidic devices and conducted encapsulation of preantral follicles and characterized biomaterials together with Huang, H. Choi, J.K.
performed animal care, isolation of preantral follicles, the culture of encapsulated follicles, and analysis of the maturation of the follicles. Zhao, S., helped in the preparation of oxidized alginate. All authors analyzed the data and helped in the preparation of the manuscript.

Chapter 4: This chapter was reproduced from a paper published as: Agarwal, P.*, Choi, J.K.*, Huang, H., Zhao, S., Dumbleton, J., Li, J., and He, X. A biomimetic core-shell platform for miniaturized 3D cell and tissue engineering. *Particle & Particle Systems Characterization* 2015; 32: 809-816 (*equal contribution). PMID: 26457002. The present author carried out all biomaterial characterization, encapsulation experiments, and RT-PCR. Choi, J.K. performed IVM, ELISA, and other animal experiments. All authors contributed to data analysis and reviewed the manuscript.

Chapter 5: This chapter is reproduced from a paper prepared as: Agarwal, P.*, Wang, H.*, Huang, H., Gooch, K.J., Zhao, Y., Liu, X., and He, X (* equal contribution). Bottom-Up Tissue Engineering to Fabricate 3D Vascularized Mammary Tumor for Drug Screening. The present author carried out all the experiments. Wang, H. assisted in *in vivo* experiments. All the authors contributed to the data analysis and reviewed the manuscript.

Chapter 6: This chapter was reproduced from a paper submitted as: Wang, H.*, Agarwal, P.*, Xiao, Y.*, Peng, H., Zhao, S., Liu, X., Liu, Z., He, X. (* equal contribution). A Multiscale System for Injectable Co-delivery of EGF and Stem Cells to Treat Ischemic Diseases. Agarwal, P., conducted encapsulation experiments, fabricated the multiscale hydrogel, performed rheological experiments, and wrote the methods of these experiments for the manuscript. Wang, H. fabricated nanoparticles, conducted drug/protein release studies, performed histological and immunochemical analyses, and participated in all other
experiments. Xiao, Y. helped to perform animal surgery and perfusion measurements. All other authors contributed to discussion of the results and review of the manuscript.

Chapter 7: The present author provided the concise conclusion and future perspective of the studies presented in this dissertation.
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Chapter 1: Introduction to multiscale biomaterials for cell and tissue engineering

In this chapter, the status quo and remaining challenges in cell and tissue engineering is thoroughly reviewed. A brief overview of current techniques to culture cells and common biomaterials are described. Further, the need for new cell microencapsulation technology utilizing a microfluidic method is illustrated. Finally, the most recent applications of cell-laden microcapsules in tissue engineering are summarized. Lastly, the chapter is concluded with an outline of the rest of the thesis.

1.1. Overview of tissue engineering and its challenges

Tissue engineering utilizes the cells in a variety of ways to restore, maintain, and improve the function of tissues and organs [1, 2]. Tissue engineering envisions to build organs from scratch in the laboratory, ready to be transplanted into the patients. The potential impact of tissue engineering is immense: (1) engineered tissue could reduce the need for organ replacement, (2) engineered tissues could accelerate the development of novel drugs, access the cytotoxicity of available drugs, thus eliminating the need for organ transplant completely [3-5].

However, engineering a living tissue in vitro is a complex process, cells are typically cultured on a bioactive degradable scaffold which provides biochemical cues to guide them to become a functional 3D tissue [3-5]. Furthermore, the time scale of these
events can range from seconds to several weeks [6]. Inducing cells to form functional tissues is currently a major engineering design and biological challenge which must be accomplished with high fidelity and low cost [7]. The other technical challenges that are needed to be overcome to create “off-the-shelf” tissues include (1) availability of adequate source of healthy expandable cells, (2) optimization of scaffolds, (3) creation of large scale bioreactors that mimic the native microenvironment of tissues, and (4) long-term preservation of engineered tissues [7]. Additional key aspects of tissue engineering and its challenges are provided in Table 1.1 [3, 7-14].

**Table 1.1. Key aspects of tissue engineering**

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Unlimited availability of embryonic stem cells, adult stem cells or differentiated cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold design requirement</td>
<td>Injectability. Ability to encapsulate cells. Ability to mimic the biomechanical microenvironment of the native tissue. Degradation at a rate fast enough to allow the in-growth of surrounding tissue.</td>
</tr>
<tr>
<td>Cell surface interactions</td>
<td>Ability to manipulate the interaction of cells to the surface.</td>
</tr>
<tr>
<td>Growth factor and cellular delivery</td>
<td>Increased efficiency of loading physiological amounts of growth factors. Controlled release of the growth factors. High survival and viability of transplanted cells.</td>
</tr>
<tr>
<td>Assessment</td>
<td>Development of clinically relevant models to assess the functionality of tissue engineered products.</td>
</tr>
<tr>
<td>Scale up or tissue vascularization</td>
<td>Ability to use large tissue engineered products where diffusion limitation may limit the viability of encapsulated cells.</td>
</tr>
</tbody>
</table>

### 1.2. Sources of cells

The production of successful tissue engineering products utilizes the cells to proliferate within the scaffolds. Recently, more attention has been given to stem cells, including embryonic stem (ES) cells and bone marrow derived stem cells (BM-MSCs). Stem cells are pluripotent and can be differentiated into all different cell types. However, there is **two critical steps for the use of stem cells in tissue engineering applications**, (1)
maintenance of pluripotency for extend culture period in the laboratory and (2) the ability to control the differentiation of these cells to the desired tissue lineage [15-17]. It is also equally important to be able to engineer cells in order to produce/secrete small biological molecules or desired function for therapeutic purposes. Controlling the cell aggregation (stem or non-stem cells) and stiffness of the supporting matrix is known to modulate the functional capabilities of cells. Moreover, in the case of stem cell-based therapies, well-planed strategies are needed to deliver cells in vivo that ensures high retention and survival post transplantation [15-17]. Other applications of controlled cell aggregation are drug discovery/cytotoxicity screening. Therefore, in this dissertation, we will focus on the application of different biomaterial systems that may affect the proliferation, development, and survival of various types of cells.

1.3. Native tissue microenvironment

The function and regeneration of any tissue are a result of intricate coordination between numerous individual cellular processes, which are induced by different signals originated from extracellular matrices. For example: complex biochemical and biophysical signals, communicated from outside microenvironment are combined via intracellular signaling, ultimately converge to regulate gene and protein expression which establishes cell/tissue function and phenotype. The extracellular microenvironment surrounding the cells consist of insoluble macromolecules (fibrillary protein such as collagen, laminin, fibronectin), soluble molecules (growth factors, cytokines, chemokines), and proteins on the surface of the adjacent cells (Figure 1.1) [18].
1.4. Current techniques for culturing cells (2D vs. 3D culture system)

Typically, in in vitro experiments, cells are cultured on a stiff plastic surface which does not capture properties of in vivo biology (Figure 1.2a). Monolayer culture allows all cells
to receive the homogeneous amount of nutrients and growth factors. They mostly consist of proliferating cells since necrotic cells detached from the surface. Furthermore, the morphology of cells in 2D are flatter and stretched compared to *in vivo* settings [19, 20]. This abnormal growth condition influences cell proliferation, differentiation, and gene/protein expression. In contrast to 2D cultures, cells in 3D system are grown into aggregates either on the matrix or embedded within the scaffold/matrix (*Figure 1.2b-c*). Cells in 3D can also be grown in scaffold-free microenvironment as shown in *Figure 1.2d*. Cells in these 3D conditions more closely mimic the natural microenvironment. They have cell-matrix and cell-cell interactions [19, 20]. In addition, cells in 3D coexist in different stages such as outer proliferative, hypoxic, and necrotic cells. They have nutrient and oxygen gradient as present in *in vivo*. Furthermore, the proliferation of cells in 2D and 3D cell culture is different, as it is dependent on ECM. This 3D culture system has also been utilized for the co-culture of different type of cells as well to gain invaluable information. For example: tumor stromal cells have been co-cultured with mammary cancer cells and has been shown to regulate tumor growth, metastasis, and even drug response [19, 20].

*Figure 1.2. Schematic diagram of traditional 2D monolayer culture and typical 3D cell culture systems. (A) 2D cultured cells, (B) cell spheroids/aggregates grown on matrix, (C) cell embedded within matrix or (d) scaffold-free cell spheroids in suspension [19].*
In addition to the morphological changes between 2D and 3D culture systems, it has been shown that 3D cell cultures differ in gene and protein expression profiles compared to 2D cultured cells. For example, mouse embryonic stem cell cultured in 3D scaffolds (Cytomatrix) have significantly higher expression of ECM related genes, as well as genes that regulate cell growth, proliferation, and differentiation [21]. Various cancer cells grown in 2D and 3D cultures often display differential gene expression of genes involved in proliferation, angiogenesis, migration, invasion, and chemosensitivity. In one study, it was shown that cell-ECM interactions in 3D culture (on matrigel) of prostate cancer cells can modulate morphology and upregulate CXCR4 and CXCR7 expression [22]. Loessner et al. reported that ovarian cancer cells grown in 3D culture system had increased expression of integrin and metalloprotease MMP9 compared to traditional 2D culture system [23]. Altogether, these observations suggest that 3D microenvironment of the cell is important to maintain the gene and protein expression to the most in vivo like level.

<table>
<thead>
<tr>
<th>Property</th>
<th>Two dimensional (2D)</th>
<th>Three-dimensional (3D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Flat and stretched</td>
<td>Natural shape in spheroids and aggregates</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Very proliferative compared to in vivo</td>
<td>Proliferation depends on cell type and matrix</td>
</tr>
<tr>
<td>Gradients</td>
<td>Cells are exposed to equal concentration of medium and growth factors</td>
<td>Spheroids have concentration gradients. High concentration on the outer surface</td>
</tr>
<tr>
<td>Cell Stage</td>
<td>Typically, cells are in similar stage</td>
<td>Contains proliferative, quiescent, apoptotic, and necrotic zones</td>
</tr>
<tr>
<td>Gene/Protein expression</td>
<td>Different compared to in vivo</td>
<td>More similar to in vivo conditions</td>
</tr>
<tr>
<td>Drug sensitivity</td>
<td>Cells are typically more sensitive</td>
<td>Cells are more resistant to drugs compared to 2D cultured cells</td>
</tr>
</tbody>
</table>

Table 1.2. Key differences between two-dimensional and three-dimensional culture systems.
Cell bases assays are critical for drug and cytotoxicity screening. Most of the drug screening assays utilize 2D cell culture tests, followed by animal model tests, to clinical trials [19]. However, the information obtained from 2D experiments sometimes provide misleading or non-predictive data in drug screening. For example: numerous studies have shown that cells cultured in 3D models are more resistant to anticancer drugs compared to 2D cultures. Karlsson et al., investigated the drug sensitivity of colorectal cancer cells HCT-116 in 3D spheroids and 2D monolayer cultures to melphalan, 5-FU, oxaliplatin, and irinotecan; their results showed that all drugs were less active on 3D spheroids in contrast to 2D monolayer cells [24]. It has been shown that high drug resistance in 3D cultured cells is due to increased cell-cell interactions between neighboring cells, limited diffusion, and hypoxia [19]. Another possible explanation could be altered gene and protein expression in 3D cultured cells as compared to 2D cells [19]. This phenomenon observed in 3D spheroids is similar to in vivo as well. Key differences between 2D and 3D culture systems are provided in Table 1.2.

Altogether, it is evident that 3D cell culture models are better compared to 2D culture systems since it closely resembles the in vivo architecture. It holds a great promise for the application in drug discovery, cancer cell biology, and stem cell research. However, there are still many hurdles that must be overcome before they can be widely utilized. (1) Currently, they are more expensive for large scale studies and high throughput assays. (2) Some methods generate spheroids that differ greatly in size which results in high variability in results. (3) 3D models still lack vasculature which plays a vital role in tissue proliferation, survival, and drug delivery [12].
1.5. Biomaterials for 3D cell culture

Definition of Biomaterials: According to the National Institutes of Health Consensus Development symposium biomaterial may be defined as “Any substance (other than a drug) or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body.

1.5.1. Types of biomaterials being used for tissue engineering

Biomaterials in the form of grafts (sutures, joint replacements, bone plates, etc.) and medical devices (pacemakers, artificial organs, blood tubes, etc.) are broadly used to replace and/or restore the function of damaged organs and thus improve the patient outcomes. The most important requirement for the selection of the biomaterial is its acceptability/compatibility by the human body. A biomaterial used for transplantation should have some important qualities in order to long-term usage in the body without any kind of immunological rejection [25]. Initially, various types of natural products such as wood, resin, rubber, and tissues from living, and synthetic materials such as iron, gold, silver and glass were used as biomaterials. The host immunological responses to these materials/natural products were tremendously varied. Polymers are suitable candidates as scaffold materials for tissue engineering since they can be tailored to have desired sizes, shape and properties (e.g. mechanical properties, geometrical shapes, biocompatibility and least toxicity, etc.) and, be degraded at the same rate as new tissue is formed [26]. Here I will introduce some commonly used natural and synthetic biopolymers being used as biomaterials in 3D culture, as well as the biochemical functions.
Natural Polymers: Natural products based biomaterials possess perfect bioactivity and biodegradability in vivo. Proteins, peptides and other molecules of those biopolymers could be very similar to native ones, so cells could attach, recognize and respond. Biopolymers derived from natural materials such as collagen type-I, fibrin, alginate, chitosan, chondroitin sulfate, and HA has been used to fabricate hydrogel scaffolds [27-30]. Hydrogels of naturally derived biopolymers have the benefit of rapid biodegradability and resemble the natural ECM. However, some of them have also their disadvantages. Collagen hydrogels can be immune responsive while fibrin hydrogels could produce insoluble fibrin peptides aggregates and can be linked with a certain degree of shrinkage when used as matrices for cell encapsulation [31].

Collagen: Collagen is considered as a perfect choice for tissue engineering biomaterials because it is the main fibrous protein in the extracellular matrix (ECM), and offers strength and structural integrity to various types of connective tissues including bone, skin, cartilage, blood vessels, tendons, and ligaments. Collagen biopolymers show the biocompatibility, high porosity & permeability, hydrophilicity, biodegradability, but less mechanical strength for bone tissue engineering in weight bearing applications [32]. Although, the decomposition rate, compressive and tensile strength can be increased by various physical and chemical cross-linking methods.

Chitosan: Chitosan, derived from chitin, is a linear polysaccharide biopolymer comprising copolymers of β (1-4)-glucosamine and N-acetyl-D-glucosamine moiety. Chitin occurs in the exoskeleton of crustaceans (such as scorpion, crabs, and shrimps), cuticles of insects and cell walls of bacteria: bacilli. Chitosan has attained much attention from scientists because of its biocompatibility, less toxicity, biodegradability, controllable mechanical and
structural features, and capability of being managed in several forms, sizes, and shapes. But pure chitosan as a biopolymeric tissue engineering scaffold is inadequate because of their weak mechanical properties and inconsistent behavior with seeded cells. Although, chitosan could be physically and chemically altered, and produce materials with a broad range of properties [33].

**Alginate**: Alginate is a brown sea algae-derived linear polysaccharide. It belongs to a family of linear block polyanionic copolymers containing (1-4)-linked β-D-mannuronic acid and (1-4)-linked α-L-Guluronic acid residues. Alginate can produce stable and well-categorized hydrogels in the presence of divalent cations (e.g. Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$). In order to prevent immunological responses after implantation, alginate must undergo wide purification. Alginate shows various properties like biocompatibility, hydrophilicity, less-toxicity, biodegradability, and is also relatively less expensive. But some limitations have restricted their uses in tissue engineering such as weak mechanical properties, less cell adhesion (for highly hydrophilic nature) and uncontrollable decomposition [34].

**Hyaluronic Acid**: HA is a naturally derived polysaccharide which is present in ECM of delicate connective tissues and body fluids. With regards to its mechanism of synthesis, its size and physio-chemical properties, HA is exclusive among other glycosaminoglycans. HA could interact with other molecules (e.g. proteins) and, in order to participate in regulating the cell behavior during various morphogenic, restorative, and pathological developments in the human body. The role of HA in diseases, such as several types of malignancies, arthritis, and osteoporosis, is leading to new impulse in research. Although, the fabrication of the safest and well-organized HA-based biomaterials for theranostic medicine for any type of damages, regardless their surface, remains a challenge [35].
**Synthetic Polymers:** Many synthetic polymers have been used to produce biomaterials because of their several benefits as a scaffold material and also due to their availability. Synthetic polymers can be biodegradable and non-biodegradable. Synthetic polymeric materials can be synthesized with a personalized architecture and properties (e.g. porosity, degradability, and mechanical features), as per their applications. Polymers such as Poly (lactic-co-glycolic) acid (PLGA), PEG and poly(ethylene glycol) (PEG) have been broadly worked on because of their relatively simple alteration to fabricate gels with required mechanical and physical properties [36].

**Poly (lactic-co-glycolic) acid (PLGA):** PLGA is fabricated by random ring-opening copolymerization of glycolic acid and lactic acid monomers. PLGA possess unique features including extraordinary mechanical strength, excellent biocompatibility, less toxicity and immunogenicity, and adaptable degradation kinetics [37]. The properties of PLGA is tailored by altering the monomer ratios and molecular weight [37].

**Poly (ethylene glycol) (PEG):** PEG, the utmost commercially used polyethers, also known as polyethylene oxide (PEO) or polyoxyethylene (POE) based on its molecular weight, refer to an oligomer or polymer of ethylene oxide. PEG has some important properties including good biocompatibility, hypoinmunogenicity, and resistance to protein adsorption and cell adhesion for which it has been a central hydrophilic polymer in biomedical applications including bioconjugation, surface alteration, drug delivery, and tissue engineering [37].
1.5.2. Properties of Ideal biomaterials

Ideal biomaterials must be non-toxic, non-carcinogenic, hypo-immunogenic, chemically inert, stable, and mechanically durable to withstand the repeated forces of a lifetime [38]. Irrespective of the tissue type, a number of key factors are important when designing or determining the appropriateness of a biomaterial for use in tissue engineering:

(i) Biocompatibility: The first criteria of any biomaterial to be used for tissue engineering are that it must be biocompatible; cells must adhere, and drift onto the surface and eventually through the scaffold and begin to multiply before laying down the new matrix. After implantation, the biomaterial or tissue engineered construct must induce a negligible immunological response in order to prevent it causing such a severe inflammatory response that it might decrease healing or cause rejection by the body [38, 39].

(ii) Biodegradability: The main goal of tissue engineering is to allow the body’s own cells/tissues, over time, to ultimately replace the grafted biomaterial or tissue engineered construct. Biomaterials and tissue engineering constructs are not envisioned as permanent implants. The biomaterial must, therefore, be biodegradable so as to allow cells to produce their own ECM. The by-products of this degradation should also be non-toxic and able to exit the body without interference with other organs [38, 39].

(iii) Mechanical properties

Ideally, the biomaterial should possess mechanical properties consistent with the anatomical site into which it is to be grafted and, from a practical viewpoint, it must be strong enough to permit surgical handling during implantation [38, 39].
(iv) Biomaterial architecture

The architecture of biomaterials applied for tissue engineering is of crucial importance. Biomaterial should possess an interconnected pore architecture and high porous structure to ensure cellular penetration and suitable diffusion of nutrients to cells within the construct and to the ECM made by these cells. Moreover, a porous interconnected structure is required to allow diffusion of waste products/toxic agents out of the biomaterial, and the products of scaffold degradation should be able to exit the body without interfering with surrounding tissues [38, 39]. The concern of core degradation, arising from less vascularization and waste removal from the center of tissue engineered constructs, is of major apprehension in the field of tissue engineering [38, 39].

1.5.3. Manufacturing technology

In order for a particular biomaterial or tissue engineered construct to become clinically and commercially applicable, it should be less expensive and it should be promising to scale-up from manufacturing one at a time in a research laboratory to significant batch production in industry. The progress of ascendable manufacturing procedures to good manufacturing practice standard is crucially important in ensuring effective translation of tissue engineering methods to the clinical applicability. Another important factor is estimating how a product will be conveyed and made available to the clinician. This will determine how either the biomaterial or the tissue engineered construct will be stored.
1.6. Cell encapsulation in micro-hydrogels for tissue engineering

Encapsulation of cells in tiny hydrogels offer numerous advantages for tissue engineering. It is easy to handle and provides 3D microenvironment of cell and tissue growth. Furthermore, many features of the micro-hydrogels such as swelling, mechanical properties, degradation, biochemical, and diffusion can be modulated through a myriad of processing conditions [40-42]. The encapsulation of cells can be performed using macro-

![Figure 1.3](image)

**Figure 1.3. Schematic diagram of major methods utilized to encapsulate cells.** Cell encapsulation prevents immune response once the encapsulated cells are transplanted into the host system. The small size of material allows facile transport of nutrients, metabolic wastes, oxygen and therapeutic agents. The main methods to encapsulate cells include macro platforms, micro molding, and microfluidic based systems to produce beads and fibers [43].

platforms, micro-molding techniques, and microfluidic based technologies (Figure 1.3) [43]. Among these technologies, encapsulation of cells in small size microbeads/microcapsules are very attractive. Encapsulation of cells in microcapsules composed of the semi-permeable membrane can prevent direct contact of encapsulated
cells and immune cells after *in vivo* implantation in the host system [44]. Furthermore, the small size of microcapsules allows facile diffusion of nutrients, oxygen, metabolic waste, and therapeutic agents secreted by encapsulated cells. Since, the free diffusion limit is \(~200 \mu m\) [12], the ideal size of microcapsules to encapsulate cells is \(~400 \mu m\) which ensures that cells buried in the core of microcapsules will obtain sufficient nutrients and oxygen. Cell encapsulation is advantageous for 3D *in vitro* cell culture. Single or multiple cells can be encapsulated to proliferate in microcapsules which serve as a micro-bioreactor. Furthermore, microcapsules can also be used as a carrier to deliver bioactive agents or growth factors. Depending on the application, microcapsules can be fabricated using poly(ethylene glycol), polysaccharides (alginate and hyaluronic acid), and proteins (collagen, fibrinogen, and gelatin). In addition, various crosslinking mechanisms of biomaterials to form hydrogels must be considered. Generally, photo-cross linkers, ions, and heat are utilized to form particles [40-42].

**1.6.1. Core-shell microcapsules for cell encapsulation and its advantages**

Alginate beads have been generally used for the encapsulation of cells owing to its excellent biocompatibility and biodegradability, low toxicity, and rapid gelation in the presence of divalent cations such as Ca\(^{2+}\) and Ba\(^{2+}\) [45, 46]. Even though the solid alginate beads has been shown to encapsulate cells for culture, the homogenous mixing of cells with alginate is not ideal [46]. This is because alginate does not adhere to the mammalian cells. Furthermore, solid beads are ineffective to form spherical aggregates of cells. Another disadvantage of solid beads is that cell or cell aggregate can grow near the edge of beads which may lead to inadequate immune protection. In order to effectively use microencapsulation of cell culture and allied applications, it is desirable to use tissue-
specific ECM with desired biomechanical and biochemical properties. The present methods generate microcapsules does not effectively solve above said problems. To this end, we propose core-shell microcapsules which have all the advantages of alginate beads. In our design core-shell microcapsules are composed of an alginate shell and core ECM. The system is not only an improvement for cell encapsulation, but also enables greater freedom to change core materials for survival enhancement of encapsulated cells or to improve the biocompatibility with cells. Furthermore, such a system will allow recreating 3D microenvironment that mimics the natural tissue.

1.7. Cell encapsulation via droplet microfluidics

Droplet microfluidics has been emerged as an important approach to fabricate microcapsules [47-51]. Using the microfluidic device, high-throughput production of microcapsules with low polydispersity can be achieved. Furthermore, a microfluidic system for droplet formation provides more control over the droplet size. For example: other methods for preparing microparticles such as electrospray and emulsification using

Figure 1.4. Concept of core-shell microcapsules for recapitulating natural cellular microenvironment in artificial ECM. The in vivo microenvironment is composed of multiple cell/cell types, an ECM which represents adhesion ligands to interact with the cell surface receptors. Furthermore, biophysical composition can also influence cellular proliferation and development. Autocrine and paracrine signaling molecules also affects cellular function. All these components can be included in core-shell microcapsules.
sonification generates particles with broad size distribution. In order to generate microparticles via microfluidics, two or more immiscible liquids are used which are independently controlled using syringe pumps (either pressure control or volumetric flow rate control). Various microfluidic geometries to produce microcapsules has been introduced over the years [52, 53]. For example: T-shaped junctions and flow focusing (FF) device are most common as shown in Figure 1.5. The two liquid phases meet at a junction at which the dispersed phase liquid forms a droplet. The geometry of the microfluidic device decides the flow or velocity field during the experiments. Ultimately,

![Diagram](https://example.com/diagram.png)

**Figure 1.5. Schematic of the two types of microfluidic device generally used to form droplets.** (A) Shows the T-shaped junction device and (B) shows the flow-focusing (FF) device.

the droplet pinches off from the dispersed phase due to the interfacial tension between the two phases [52, 53]. Continuous and uniform generation of droplets is dependent on the steadiness of the flow rates. The wettability of the microfluidic channels is also important for the successful generation of droplets [53]. For example: if the dispersed and continuous phase is aqueous and oil solutions, the channels of the microfluidic device must be hydrophobic. In other words, the continuous phase liquid must preferentially wet the walls of the microfluidic channels. Therefore, the selection of material for fabricating a microfluidic device is also important. To make water in oil droplets, microfluidic channels in polydimethylsiloxane (PDMS) is fabricated using soft lithography since PDMS is
hydrophobic. However, the surface properties of PDMS can be readily changed by chemical modifications in order to make oil in water droplets [54]. Otherwise, materials like silicon can be used which is hydrophilic. In addition, choice of fabricating material also depend on its strength and compatibility with fluids[53].

The size of continuous and dispersed phase channels, height and width, are important parameters which determine the flow field and ultimately the size of the droplets. Several important fluid properties that need to consider are the viscosities (\(\eta\)) and densities (\(\rho\)) of dispersed (\(d\)) and continuous (\(c\)) phase, and the interfacial tension between the two (\(\sigma\)) [53]. Key dimensionless parameters are used to analyze the important forces acting in the microfluidic system. Capillary number (\(Ca\)), represents the relative effect of viscous force and the surface tension [53]. The capillary number for droplet formation process is defined in terms of continuous/dispersed phase flow field as:

\[
Ca = \frac{\mu V}{\eta}
\]  

(1)

Where, \(\eta\) is the dynamic viscosity of the continuous/dispersed phase, \(V\) is the characteristic velocity, and \(\sigma\) is the surface tension. The typical values of \(Ca\) ranges from \(10^{-3}\) to \(10^{1}\). Another important parameter is the ratio of volumetric flow rate \(\varphi\), given by

\[
\varphi = \frac{Q_d}{Q_c}
\]  

(2)

The Reynold number \(Re\) is also another dimensionless quantity that is used to predict the flow pattern in the microfluidic device and is defined as the ratio of inertial forces to viscous forces,
\[ Re = \frac{\rho V D}{\eta} \]  

Where \( D \) is the hydraulic diameter of the channel. Typically for microfluidic experiments, \( Re \ll 1 \).

In a typical flow focusing device, as shown in Figure 1.5 B, continuous and dispersed phase flow coaxially in separate channels in a planar microfluidic device. Also, continuous flow channels flank dispersed phase channel on its both sides and they meet at the flow-focusing junction. As a result of the velocity field of continuous phase at the junction, the dispersed phase liquid breaks into droplets. Droplets in this device can be generated in dripping and jetting regimes which are based on the flow rates of flown liquids as well as on the capillary number [52, 53]. In dripping regime, dispersed phase immediately forms a droplet at the FF junction whereas in jetting elongation of dispersed phase occurs and droplets are formed downstream of the FF junction. In both regimes, the frequency of droplet formation and the droplet sizes are different. Furthermore, as the capillary number is increased or the flow rates of liquids are increased, the transition of dripping to jetting is observed.

In order to form hydrogel particles for cell culture in a FF microfluidic device, a suitable gelling/solidification mechanism should be included in the downstream channels. The solidification can occur by various chemical reactions. Mostly, chemical crosslinking (use of photoinitiators and UV light) or physical crosslinking (in presence of ions) are utilized [55]. For the cell encapsulation purposes, it is always desired to have a gentle crosslinking mechanism to ensure the high viability of encapsulated cells. Kim et al, demonstrated the use of microfluidic FF device to encapsulate embryonic carcinoma cells
to form aggregates in microcapsules with a liquid core and alginate hydrogel shell. Also, they compared the aggregate formation of cells in core-shell microcapsules and microbeads. They report the formation of several clusters of cells in microbeads whereas uniform size aggregates were formed in core-shell microcapsules (Figure 1.6 A)[56]. In this study, however, they utilized oleic acid with infused calcium chloride to gel the alginate which could be harmful to cells. Furthermore, the inclusion of glycerol in the core of microcapsules could also compromise the viability of the encapsulated cells. In another study by Leong and coworkers utilized a microfluidic device to encapsulate hepatocyte cells alginate-collagen and alginate microcapsules (Figure 1.6 B)[57]. The study showed that encapsulation of hepatocyte enhances its long-term performance.

![Figure 1.6. Application of core-shell microcapsules in 3D cell culturing.](image-url)
1.8. Modular tissue engineering for fabricating complex tissues

There is still a lack in a number of *in vitro* engineered tissues due to our current inability to mimic the tissue vasculature. *In vivo* all the tissues get nutrients and oxygen from blood vessels (larger blood vessels and microcapillaries). Since the free diffusion limit is ~200 µm, currently it is not possible to efficiently fabricate tissue engineered products larger than this without creating a vascular architecture[12].

There are two broad strategies which can be used to create hierarchical tissue structures: top-down and bottom-up approaches. In the top-down approach, cells are seeded on a biodegradable polymeric scaffold such as PLGA (synthetically manufactured

![Diagram of top-down and bottom-up tissue engineering](image_url)
vascularized scaffolds) and are expected to secrete ECM and recreate microarchitecture similar to the in vivo conditions. Typically, perfusion systems, growth factors, and mechanical stimulation are provided that aid the cellular growth. However, such a top-down system is often limited to mimic the intricate microstructural details of the desired tissue[58]. In bottom-up approaches, modular structures are first micro-engineered which can be used as building blocks to create larger tissues. These modules can be prepared using different ways: encapsulation of cells, cell sheet formation, or bioprinting. Since most of the tissues are made up of repeating units, bottom-up approaches are better to create more biomimetic engineered tissues [58]. Figure 1.7 shows the schematic diagram showing the difference between the two approaches.

As an example of the bottom-up approach, Takeuchi et al, seeded 3T3 fibroblast cells on the surface of collagen gels [59]. Later, they assembled a large number of beads to
generate a 3D hierarchical system. They formed an arbitrary shapes macroscopic object as shown in Figure 1.8 using a molding approach to form a dense tissue. Such a system could be used to form vascularized complex tissues because different micro-tissue can be easily prepared with tissue relevant cells. Furthermore, using core-shell microcapsules (the system we introduced in an earlier section) composed of tissue-specific biomaterials as an ECM can be utilized as a building block. This would potentially enable to replicate more intricate in vivo like structures needed for tissue engineering applications.

1.9. Scope of the thesis and organization
As described above, there are several critical challenges that need to be addressed so that tissue engineering can be used widely to treat the human diseases. The aim of this dissertation was to develop a microfluidic platform for high-throughput encapsulation of cells and tissues in biomimetic 3D core-shell microcapsules that recapitulate native cellular microenvironment for various applications including regenerative medicine, assisted reproduction technologies, and fabricating biomimetic structures as summarized below.

In Chapter 2, the development of a non-planar microfluidic device for creating core-shell microcapsules is described. Various parameters that may affect the size and shape of the core-shell microcapsules will be explained. Furthermore, the utility of 3D core-shell microcapsules will be validated by encapsulating and culturing mouse embryonic stem cells (mESCs) in microcapsules. In Chapter 3, the fabrication of biomimetic micro-ovaries is described using core-shell microcapsules for the 3D culture of mouse preantral follicles which is an important topic of research in assisted reproduction technologies. Next, in Chapter 4, fabrication of core-shell microcapsules is described to create different biophysical and biochemical microenvironments for 3D cell culture. Furthermore, the
effect of the biochemical and biomechanical microenvironment in regulating proliferation, differentiation, and development of encapsulated cells is studied. In Chapter 5, the utility and application of core-shell microcapsules in engineering tumor microenvironment to investigate tumor proliferation and metastasis is described. In addition, it is shown that these core-shell microcapsules can be used as building blocks to fabricate macro scale and complex 3D vascularized human breast tumor model. The utility of the system is described to investigate tumor biology and discover novel anticancer drugs. In Chapters 6, a multiscale hydrogel delivery system comprised of nano-encapsulated therapeutic protein, core-shell microcapsules, and human adipose derived stem cells (hADSCs) is described for co-delivery of protein and cells with high efficiency to treat ischemic diseases.
Chapter 2: Microfluidic generation of core-shell microcapsules for miniaturized culture of mouse embryonic stem cells

2.1. Introduction

Pluripotent stem cells such as embryonic stem (ES) and induced pluripotent stem (iPS) cells hold great potential for tissue regeneration and cell-based therapy because they are capable of both differentiation (into any somatic cells) and self-renewal (to retain pluripotency) under appropriate culture in vitro [60-65]. However, the difficulty to culture and produce a large number of cells with high pluripotency and purity in vitro has been one of the major hurdles to overcome before pluripotent stem cells can be widely used for treating diseases [3, 7, 66].

Pluripotent stem cells have been cultured both on two-dimensional (2D) substrates and in three-dimensional (3D) space. The former is non-physiological and can lead to altered gene and protein expression in cells [9, 21, 67-69]. On the other hand, 3D culture has been shown to be important in controlling proliferation and differentiation of pluripotent stem cells [70-76]. As they do in their native milieu in a pre-hatching embryo, these cells tend to self-assemble through cell-cell interactions into 3D aggregates up to a few hundred microns under in vitro culture. Therefore, they are desired to be cultured in an aqueous liquid environment with minimal resistance to better maintain their stemness [77-79]. Hanging drop, static or stirring suspension culture, and micropatterned features have been the most
commonly used techniques for culturing pluripotent stem cells [67, 76, 80-85]. However, these methods are limited in several aspects including cell damage due to shear stress, limited control of aggregate size and shape, and/or difficult to scale-up for clinical applications for which the capability of mass production of the cells are needed.

To overcome the challenges, microencapsulation of pluripotent stem cells in biocompatible hydrogel matrices for culture is gaining more and more attention recently because it offers several advantages:[42, 86-91] First, the miniaturized culture in microcapsules allows efficient transport of oxygen, nutrients, and metabolites to ensure viability of all cells; second, the selective permeability of hydrogel matrix in microcapsules can protect cells from host’s immune response, which may eliminate the need for immunosuppressive drugs and improve transplantation outcome; and lastly, microencapsulation has been shown to promote cell survival post cryopreservation and banking of the cells for future use. Existing methods for cell microencapsulation commonly involve the use of synthetic or natural polymers to form hydrogels such as that of gelatin, agarose, alginate, and poly(ethylene glycol) and its derivatives [41, 91-94]. Typically, cells are suspended in solutions of the polymers and microcapsules are generated by emulsification, electrospray, air shear, or the conventional planar microfluidics, followed by polymerization that can be induced by ultraviolet (UV), temperature, chemical, physical, and ionic crosslinking [41, 91-94]. However, these methods are usually for producing microbeads with a cell-containing, solid-like hydrogel core that leads to the formation of cell aggregates of uncontrollable size and shape [86, 89, 95, 96]. To overcome this problem, microcapsule with a liquid core has been produced by liquefying the hydrogel core of alginate microbeads after coating with poly-l-lysine (PLL) or chitosan to promote
proliferation and formation of a spheroid aggregate with controllable size and shape [86, 89, 95]. However, this approach requires multiple steps of coating and liquefying that are laborious and often harmful to the encapsulated cells.

Although they have been utilized to contribute significantly to the understanding of stem cell biology, all the above-mentioned in vitro culture systems do not completely recapitulate the native milieu of ES cells in a pre-hatching embryo with a round hydrogel shell (the zona pellucida) and an aqueous liquid core containing embryonic cells (Figure 2.1)[97, 98]. A recent study has shown the potential to encapsulate embryonic carcinoma cells in microcapsules with a liquid core and alginate hydrogel shell using microfluidic device, which, however, may not be utilized for encapsulating the stress sensitive pluripotent stem cells because of the necessity of using cytotoxic chemicals such as oleic acid, methyl propanol, and high concentration of glycerol that have direct contact with cells during microencapsulation [56].

Figure 2.1. The native microenvironment of embryonic stem cells. After the fertilization of oocytes with sperm in ovaries, a zygote is formed. Zygote, at one cell stage develops into 2-cell, 4-cell, morula, and early blastocyst. In all these stages of development, embryos are covered by a semipermeable membrane hydrogel shell called zona pellucida [97,98].

In this study, we micro-fabricated a non-planar (3D)
microfluidic flow-focusing device to achieve one-step generation of core-shell microcapsules with an alginate hydrogel shell of controllable thickness and an aqueous liquid core of ES cells without using any cytotoxic chemicals or organic solvents. The core-shell architecture of the microcapsules resembles that of a pre-hatching embryo where ES cells reside naturally. Alginate was used to fabricate the microcapsule shell because of its superb biocompatibility and reversible gelation with divalent cations such as Ca\(^{2+}\) under mild conditions that are not harmful to living cells \[27, 34\]. Mouse ES cells encapsulated in the liquid core of the microcapsules were found to survive well (> 92%) and proliferate to form a single aggregate in each microcapsule within 7 days. Furthermore, the aggregated cells were found to have significantly higher expression of pluripotency marker genes compared to the ES cells cultured on 2D substrates and they could be efficiently differentiated into beating cardiomyocytes under the induction of a single small molecule (cardiogenol C) without the complex combination of multiple growth factors.

2.2. Materials and methods

2.2.1. Fabrication of non-planar microfluidic device

To fabricate polydimethylsiloxane (PDMS, Dow Corning) microfluidic device, silicon master with patterned microfluidic channels was prepared using a multilayer (3-step UV exposure) SU8 fabrication technique \[99\]. Briefly, photosensitive epoxy (SU-8 2025, MicroChem) was spun coated onto a 4-inch silicon wafer. The thickness of the first SU-8 coating was 60 µm. The wafers were then soft-baked at 95 ºC for 9 min and exposed to UV light through the first shadow mask printed with the core channel. After a post-exposure baking at 90 ºC for 7 min, an additional layer (50 µm) of SU-8 photoresist was spun coated,
soft baked, exposed with a different shadow mask to pattern shell channel. The third layer for oil channel was similarly patterned. All three exposures were aligned using an EVG620 automated mask aligner. The SU8 pattern on the substrate was developed in the SU-8 developer (MicroChem) for 10 min, rinsed with isopropyl alcohol, and then dried using nitrogen gas. PDMS pre-polymer was then poured on the silicon substrate and cured at 65 °C for 3 h to form PDMS slab. Thereafter, the PDMS slab embedded with microchannels (half-depth) was lifted off. Two PDMS slabs with the same channel design were then plasma-treated for 30 s using Harrick PDC-32 G plasma cleaner at 18 W and 27 Pa, wetted with methanol (to prevent instant bonding), and aligned and bonded together under a microscope to produce the final microfluidic device. A detailed schematic of the fabrication process is provided in Appendix. Assembled device was kept on a hotplate at 80 °C for ~10 min to evaporate residual methanol and further kept at 65 °C for 2 days to make it sufficiently hydrophobic for experiments.

2.2.2. Cell culture

R1 mouse ES cells (ATCC) were cultured in medium consisted of Knockout DMEM (Millipore) supplemented with 15 % (v/v) Knockout Serum (Millipore), 4 mM l-glutamine (Sigma), 100 μg ml⁻¹ antibiotics (Invitrogen), 1000 U ml⁻¹ Leukemia Inhibitory Factor (LIF) (Millipore), 10 μg ml⁻¹ gentamicin (Sigma), and 0.1 mM mercaptoethanol (Sigma) on a gelatin-coated tissue culture flask at 37 °C in a humidified 5% CO₂ incubator. When reaching 70% confluence, cells were detached using trypsin/EDTA (Invitrogen) and gently pipetted to break aggregates. The cells were then centrifuged, resuspended, and counted for further passaging or experimental use.
2.2.3. Encapsulation of cells in core-shell microcapsules for miniaturized 3D culture

The fluids in the core and shell microchannels were 1% sodium carboxymethylcellulose (Sigma) and 2% purified sodium alginate (Sigma) in 0.3 M $d$-mannitol (Sigma) solution, respectively. All the solutions were sterile and buffered with 10 mM HEPES to maintain pH 7.2 before use. To make mineral oil infused with calcium chloride for flowing in the oil channel, stable emulsion of mineral oil and 0.7 g ml$^{-1}$ calcium chloride solution (volume ratio: 3:1 with the addition of 1.2 % SPAN 80) was prepared by sonication for 1 min using a Branson sonifier. Water in the emulsion was then removed by rotatory evaporation for ~2 min at 90 ºC. All solutions were injected into the microfluidic device using syringe pump at room temperature to generate microcapsules that were collected in 4 ºC medium in 50 ml centrifuge tube. Microcapsules were separated from oil into the medium by gently centrifuging at 300 rpm. The microencapsulated cells were cultured in ES cell medium for up to 7 days. Further pluripotency marker and cardiac differentiation analyses were performed using the aggregated cells on day 7. Cell viability was determined using the standard Live/Dead Viability/Cytotoxicity assay kit (Invitrogen).

2.2.4. Pluripotency analysis

For pluripotency analysis, ES cell aggregates were released from the core-shell microcapsules on day 7 by incubating in 55 mM sodium citrate solution for ~ 5 min. Aggregates were then washed with 1x PBS and centrifuged. For quantitative RT-PCR (qRT-PCR) analysis, RNAs were extracted from the aggregated cells using RNeasy plus mini kit (Qiagen) following the manufacturer’s instruction. Next, reverse transcription was carried out to generate complementary DNA (cDNA) using the iScript™ cDNA synthesis
kit (Bio-Rad) and GeneAmp 9700 PCR system. The qRT-PCR was conducted with the superfast SYBR Green mix (Bio-Rad) using a Bio-Rad CFX96 real time PCR system. Relative gene expression was calculated using the $\Delta \Delta C_T$ method built in the Bio-Rad software. Pluripotency genes including Oct-4, Sox2, Nanog, and Klf2 were studied with GAPDH being used as the housekeeping gene. Primer sequences of GAPDH and the 4 pluripotency genes are given in Table 2.1.

Table 2.1. List of primers for qRT-PCR studies

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-4</td>
<td>5'-GAAGCCCTCCCTACAGCAGA-3'</td>
<td>5'-CAGAGCAGTGACGGGAACAG-3'</td>
</tr>
<tr>
<td>Sox-2</td>
<td>5'-GCATGTCCTACTCGACGACG-3'</td>
<td>5'-GCTGATCATGTCCCCGAGGGT-3'</td>
</tr>
<tr>
<td>Nanog</td>
<td>5'-CCCCACAAGCCTTGGAATTA-3'</td>
<td>5'-CTCAAATCCCAGACAACCACA-3'</td>
</tr>
<tr>
<td>Klf2</td>
<td>5'-CTGCTGGAGGCCAAGCCCAA-3'</td>
<td>5'-AGGTGGTGGACCTGGAGAA-3'</td>
</tr>
<tr>
<td>Brachyury (T)</td>
<td>5'-CTCTAATGTCTCCCTTGTTGCC-3'</td>
<td>5'-TGCGATATGTCTTGGCTTGG-3'</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>5'-GATGGGAAAAGCTCCCCTATG-3'</td>
<td>5'-GAGACACCAGGCTACGCTAATA-3'</td>
</tr>
<tr>
<td>cTnT</td>
<td>5'-GAAGTTGCGACCTCAGGAAA-3'</td>
<td>5'-TTCCCCACAGTGTGGAGAC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CTCTGGCTCAGAGGTTTGG-3'</td>
<td>5'-ACAGAAACCAGTGAGGCTTTGA-3'</td>
</tr>
</tbody>
</table>

For immunohistochemical staining of two pluripotency marker proteins including Oct-4 and SSEA-1, cell aggregates were fixed in 4% paraformaldehyde, washed 3 times with 1x PBS, and permeabilized with 0.25% Triton X-100 (Sigma). The aggregates were then incubated in 3% BSA in 1x PBST (1x PBS and 0.05% Tween 20) at room temperature for 1 h to block non-specific binding, followed by overnight incubation at 4 °C with primary antibodies (Abcam) targeting Oct-4 or SSEA1. The aggregates were then washed 3 times and incubated in dark at room temperature for 45 min with secondary antibodies (Abcam) including DyLight 488 conjugated rabbit IgG for Oct4 or DyLight 550 conjugated mouse IgM for SSEA-1. All the antibodies were diluted in 3% BSA in 1x PBST. The
aggregates were then washed and further stained for nuclei using Hoechst for examining with an Olympus FV 1000 confocal microscope.

### 2.2.5. Cardiac differentiation analysis

For cardiac differentiation, ES cell aggregates were released from the core-shell microcapsules on day 7 by incubating in 55 mM sodium citrate solution for ~ 5 min. Released aggregates were transferred into gelatin coated 6-well plates for culturing in differentiation medium consisted of DMEM (Invitrogen), 20% FBS (Hyclone), 0.25 µM cardiogenol C (Sigma), and 100 µg ml⁻¹ antibiotics for 3 days. Thereafter, the cells were cultured up to day 13 in DMEM supplemented with 20% FBS and 100 µg ml⁻¹ antibiotics. Culture medium was changed every other day. The number of beating foci (see Movie S3) was counted daily from the day of initiating differentiation. On average, more than 100 aggregates per experiment were analyzed to calculate beating percentage. In addition, cardiac differentiation using the conventional method to form embryoid body (EB) in hanging drops was conducted as a control. In short, EBs were formed by suspending 1500 single ES cells in 20 µl hanging drop of ES cell culture medium without LIF and cultured for 4 days. The EBs were then plated on gelatin coated 6-well plate for further differentiation in medium containing DMEM, 20% FBS, and 100 µg ml⁻¹ antibiotics with medium changed every other day.

For differentiated marker gene analysis using qRT-PCR, cells were harvested using trypsin/EDTA on day 12 of differentiation, processed using the same procedure described in section 2.4, and analyzed for cardiac and mesodermal differentiation markers including cardiac troponin T (cTnT), Nkx2.5, and Brachyury (or T) genes. Primer sequences for 3
differentiated genes are also provided in Table 2.1. In addition, immunohistochemical staining and flow cytometry were conducted, for which cells were processed as that described in section 2.4 and treated with primary antibodies (Abcam) targeting cTnT, connexin 43, and α-actinin. Secondary antibodies used were Dylight 488-conjugated rabbit IgG (Abcam) for connexin 43 and α-actinin and rhodamine 555-conjugated mouse IgG (Invitrogen) for cTnT. The aggregates were then washed and further stained with Hoechst for nuclei and examined with an Olympus FV 1000 confocal microscope. Flow cytometry analysis was conducted using a BD LSR II Flow Cytometer. To estimate the percentage of cTnT positive cells, un-stained differentiated cells were used as a control to eliminate the background fluorescence. The flow cytometry data were further analyzed using the FlowJo software.

2.2.6. Statistical analysis

All the experiments were conducted for at least three times independently. Results are reported as mean ± standard deviation (SD). Two-tailed student t-test was performed to determine statistical significance ($p < 0.05$).

2.3. Results and discussion

2.3.1. Non-planar microfluidic flow-focusing device

An overview together with a schematic illustration of the 3D design of the non-planar microfluidic device is shown in Figure 2.2 A-C. The device was made by plasma bonding two PDMS slabs fabricated with the same channel design (Figure 2.2 B) to form the
rectangular core, shell and oil channels with three inlets (I-1, I-2, and I-3 for core, shell, and oil flows, respectively) and one outlet (O-1). At the flow-focusing junction that is indicated by the dashed box in Figure 2.2 B and further illustrated schematically in Figure 2.2 C, the height (or depth) and width of the core (H1 x W1), shell (H2 x W2), and oil (H3 x W3) channels are 120 × 120, 220 × 50, and 320 × 200 µm, respectively. As shown in the inset of Figure 2.1 C, the height (or depth) of core channel (H1: 120 µm) is the smallest, followed by the shell (H2: 220 µm) and oil (H3: 320 µm) channels. After the flow-focusing junction, a serpentine design was used to attain a total channel length of ~ 130 mm for sufficient gelation of alginate in the device.

2.3.2. Generation of core-shell microcapsules from two (core and shell) aqueous flows in one step

To generate pre-hatching embryo-like microcapsules with an aqueous hydrogel shell and an aqueous liquid core in one step in the microfluidic device, 1% cellulose solution either with or without ES cells, 2% sodium alginate solution, and mineral oil infused with calcium chloride were injected through the core, shell, and oil channels, respectively. Cellulose and mineral oil were used because of their excellent biocompatibility for cell culture [100-102].

At the non-planar flow-focusing junction (Figure 2.2 C), the shell flow was pushed by the crossing oil flow to fold over the parallel core flow and both the aqueous core and shell flows were sheared by the crossing oil flow into droplets as a result of the immiscible nature (i.e., high surface tension) between water and oil. Because the two parallel aqueous flows have direct contact, mixing between them must be minimized to retain the core-shell configuration. This was achieved by using 1% cellulose in the core flow to significantly raise its viscosity (alginate in the shell flow was highly viscous already). Moreover, we
infused mineral oil (cell culture grade) with calcium chloride that could dissolve and diffuse into the aqueous shell flow to crosslink alginate there into hydrogel before the droplets exited the device. As a result, the core-shell configuration could be stabilized to encapsulate cells for extended culture. It is worth to note that a non-planar flow-focusing design with varied channel depth (Figure 2.2 C) is necessary for making core-shell microcapsules from two parallel aqueous flows by allowing the viscous shell flow to fold over and envelope around the viscous core flow. A non-planar channel design is even important for the efficient production of core-shell emulsions of immiscible fluids [103]. At an oil flow rate of 4 ml h$^{-1}$, the folding and enveloping of the aqueous shell flow (220 µl h$^{-1}$, made visible by adding 0.5% 500 kD FITC-dextran to give the yellow appearance)

Figure 2.2. Non-planar microfluidic flow-focusing device for one-step generation of core-shell microcapsules. (A), an overview of the microfluidic device with reference to a U.S. quarter coin; (B), a schematic view of the microchannel system: I-1, I-2, and I-3 are the inlets of core, shell, and mineral oil flows, respectively, and O-1 is the outlet; (C), a zoom-in look of the non-planar design at flow-focusing junction (the dashed box region in B); and (D), typical images showing gradual coverage of the core fluid by the shell fluid that was stained yellowish with 0.5% high molecular weight (500 kD) dextran-FITC.
over the aqueous core flow (110 μl h\(^{-1}\)) are shown in Figure 2.2 D of a top view of the flows in the region of flow focusing, where a white band (indicated by asterisk) of the core flow was clearly seen on droplet 1, diminished on droplet 2, and disappeared on droplet 3. The observation is a result of the gradual displacement of oil on top (and at bottom) of the core flow by the folding-over viscous shell flow, as illustrated schematically in 3D by the droplets in the microchannel after flow focusing in Figure 2.2 C. The non-spherical appearance of the droplets in Figure 2.2 D was due to their relatively large size (compared to the microchannel) to block most of the flow in the microchannel, which resulted in high enough pressure difference along the droplets to deform them. Smaller and thus more spherical droplets were produced under the same flow rates when there was no dextran in the core flow (lower viscosity).

Moreover, a spherical core-shell architecture is clearly observable in phase contrast images of the microcapsules after exiting the device (Figure 2.3 A-C), which suggests the highly elastic nature of the alginate hydrogel in the microcapsule shell that enables the microcapsules to take a spherical shape (driven by minimization of surface tension) after exiting the microchannel when the pressure difference across them is gone. The inner boundary of the shell does not appear as smooth as the outer boundary, which is not surprising because the interfacial surface tension between the two aqueous flows on the inner boundary is negligible compared to that between the aqueous shell and oil flows on the outer boundary. In addition, the shell thickness and microcapsule size can be easily varied by adjusting the flow rates of the three (core, shell, and oil) flows as shown in Figure 2.3 A (core: 110 μl h\(^{-1}\), shell: 150 μl h\(^{-1}\), and oil: 4 ml h\(^{-1}\)), B (core: 110 μl h\(^{-1}\), shell: 220 μl h\(^{-1}\) and oil: 4 ml h\(^{-1}\)), and C (core: 110 μl h\(^{-1}\), shell: 150 μl h\(^{-1}\), and oil: 8 ml h\(^{-1}\)).
microcapsules in Figure 2.3 A and C are ~ 240 µm and 190 µm on average, respectively, with a wall thickness of ~ 10 µm while in Figure 2.3 B, they are ~ 260 µm on average with a wall thickness of ~ 50 µm. The data under the various flow conditions are further shown in Figure 2.3 D and E to illustrate the effect of the three flow rates on the core and overall microcapsule size. Clearly, the core size is mainly determined by the core flow rate and is not significantly affected by the change in the shell (from 150 to 220 µl h⁻¹) and oil (from 4 to 8 ml h⁻¹) flow rates. The overall microcapsule size is significantly decreased with the increase in oil flow rate, but it is not significantly affected by the change in shell flow rate. Lastly, it is worth to note that the non-planar microfluidic device could be easily utilized to produce microcapsules with a collagen (and potentially many other biomaterials)
core and an alginate hydrogel shell, as shown in Figure 2.4.

Figure 2.4. Confocal images showing the presence of collagen in the core. Microcapsules with a collagen core and alginate hydrogel shell generated using the non-planar microfluidic device in one step: (A), typical phase contrast image showing the core-shell morphology of microcapsules with collagen being labeled with FITC in the core; (B), the corresponding fluorescence image showing collagen in the core of microcapsules in panel A; and (C), a merged view of the images in panels A and B showing the colocation of the fluorescence collagen and the visible core of microcapsules.

2.3.3. Encapsulation of ES cells cultured in pre-hatching embryo-like microcapsules

All ES cell encapsulation experiments were conducted using the same flow rates as that used for generating microcapsules shown in Figure 2.3 B. Concentration of ES cells in core solution was 5 million cells/ml. Typical phase contrast and fluorescence images of ES cells immediately after encapsulation showing high viability (> 92%) are given in Figure 2.5A and B, respectively. A total of 20 ± 6 cells were successfully encapsulated in the aqueous liquid core of each microcapsule. The high cell viability is attributed in part to the use of biocompatible mineral oil and cellulose [100-102]. Also, the gelation of alginate in the shell shortly after contacting calcium infused in oil should improve cell viability by confining cells in the aqueous core so that they are not exposed to oil and the damaging shearing stress near the interface between the aqueous shell and oil flows.

Phase contrast images of the encapsulated ES cells at day 3, 5, and 7 demonstrating their proliferation are given in Figure 2.5 C, D, and E, respectively. The cells were
observed to form multiple small aggregates within 3 days that further merged to form a single aggregate in each microcapsule on day 5-7, depending on the number of cells initially encapsulated in the core. The aggregates were 163 ± 29 µm, which was similar to the diameter of the liquid core in the microcapsules. High viability of cells in the aggregates is clearly seen in Figure 2.5 F where no dead cell (red stain) is observable at all. Therefore, using this non-planar microfluidic encapsulation technology, it is possible to form ES cell aggregates of high cell viability and fairly uniform size.

The proliferation of ES cells in the core-shell microcapsule mimics that of embryonic cells (including ES cells) during early (pre-hatching) embryo development from a few cells to a few tens of cells and later a single merged cell aggregate (i.e., blastocyst) in the hydrogel shell of each pre-hatching embryo (i.e., zona pellucida), which should promote self-renewal of the ES cells. Indeed, as shown in Figure 2.5 G, further quantitative RT-PCR (qRT-PCR) studies of the expression of four pluripotency genes (Oct-4, Sox2, Nanog, and Klf2) show significantly higher expression of 3 (Sox2, Nanog, and Klf2, p < 0.05) out of the 4 genes in the aggregated ES cells from miniaturized 3D culture, compared to the cells grown on 2D surface. All the 4 genes are key transcription factors involved in maintaining stemness or self-renewal of pluripotent stem cells [60-65]. To further examine the pluripotency at the protein expression level, immunohistochemical staining was performed for two pluripotency marker proteins: Oct-4 and SSEA-1. As shown in Figure 2.5 H, both ES cell-specific marker proteins are highly expressed in the aggregated ES cells.

These results demonstrate that the miniaturized 3D culture can maintain both high viability and high pluripotency of the encapsulated ES cells. This may be due to efficient
transport of oxygen, nutrients, and metabolic wastes in the miniaturized liquid core of the pre-hatching embryo-like microcapsule, which allows better survival and proliferation of all encapsulated cells [42, 86-91]. Moreover, the alginate shell can force the encapsulated
cells to stay together and may prevent the dilution into the bulk medium of endogenous metabolites produced by the encapsulated ES cells to maintain their pluripotency. All these should render the miniaturized 3D culture to better recapitulate the native milieu of ES cells in pre-hatching embryos compared to the open 2D or even bulk 3D culture [77-79].

2.3.4. Cardiac differentiation of aggregated ES cells

To further ascertain their stemness, we performed cardiac differentiation using cardiogenol C as the single inducer on the ES cell aggregates formed on day 7 (aggregate differentiation). Cardiogenol C is a small molecule (296.75 D) that has been reported to be promising for inducing cardiogenic differentiation of both pluripotent and multipotent stem cells [104-106]. As a control, we also performed cardiac differentiation using the conventional method with the embryoid body (EB) obtained in hanging drops (EB differentiation). As shown in Figure 2.6, spontaneously beating foci were first observed on day 7 for the aggregate differentiation while it was not observed until day 8 for EB differentiation. The cumulative percentage of beating foci reached the maximum (60.4 ± 5.1%) on day 12 for the aggregate differentiation and was significantly higher than that for

![Figure 2.6. A typical image of the aggregated cells on day 12 of differentiation showing a beating focus.](image-url)
EB differentiation. The maximum cumulative percentage of beating foci for EB differentiation was 25.3 ± 9.3%, which is comparable to what has been reported previously [107]. A typical bright field image of the differentiated cells from the aggregate differentiation on day 12 is shown in Figure 2.6 (the boundary of a spontaneously beating}

Figure 2.7. Comparison of efficiency of cardiac differentiation using the 3D aggregated ES cells and the conventional embryoid body (EB) method. (A), cumulative percentage of beating foci as a function of time after initiating cardiac differentiation; (B), qRT-PCR data showing expression of markers of cardiomyocytes (cTnT), cardiac progenitor cell marker (Nkx2.5), and mesodermal cell marker (Brachyury or T) in the differentiated cells; (C), flow cytometry data of unstained control cells and cTnT stained cells showing expression of cardiomyocyte specific marker (cTnT) protein in ~ 38% of the cells differentiated from the 3D aggregated ES cells on day 12 of differentiation; and (D), immunohistochemical staining for cardiomyocyte specific marker proteins including cTnT (red), α-actinin (green), and connexin 43 (Cx 43, green) in the cells differentiated from the 3D aggregated ES cells on day 12 after initiating differentiation together with Hoechst (blue) staining of cell nuclei and differential interference contrast (DIC) images showing the aggregate morphology, demonstrating high expression of all the differentiated marker proteins. *: Statistically significant (p < 0.05)
focus is labeled with solid line).

Further qRT-PCR analyses (Figure 2.7 B) show significantly higher expression of early mesoderm marker gene brachyury or T (~ 9 folds) and cardiac progenitor marker gene Nkx2.5 (~ 3.4 folds) in the cells from aggregate differentiation compared to that from EB differentiation. The expression of cardiac troponin T (cTnT), a marker gene specific of cardiomyocytes, is also higher (although less significant, \( p = 0.11 \)) for aggregate differentiation than EB differentiation. In addition, the differentiated cells from aggregate differentiation show significantly higher levels of brachyury (~ 45 folds) and Nkx2.5 (~ 40 folds) compared to the aggregated ES cells before differentiation and this difference is even much bigger (~ 1200 folds) for cTnT (Figure 2.8).

Translation of the cTnT gene to cTnT protein in cardiomyocytes is required for the cells to beat and function. Therefore, further flow cytometry analysis was conducted to quantify intracellular cTnT protein. As shown in Figure 2.7 C, the cardiomyocyte specific protein was expressed in ~ 38% of the differentiated cells from aggregate differentiation. The expression of the cTnT protein in the differentiated cells was further confirmed by
immunohistochemical staining (Figure 2.7 D). Also shown in Figure 2.7 D are the highly positive staining of α-actinin (a protein in the z line of cardiac sarcomere) that is also important for cardiomyocytes to beat and function and connexin 43 (Cx 43, a protein in the gap junction between cardiomyocytes) that is indispensable for synchronized beating of multiple cardiomyocytes.

These results confirm that ES cell aggregates formed in the pre-hatching embryo-like core-shell microcapsules are indeed capable of being differentiated directly into functional cardiomyocytes. Of note, the result of ~ 38% of cTnT protein-positive cells in the differentiated cell population is close to what has been reported in the literature for cardiac differentiation of ES cells [108]. Moreover, it is close to that (~ 30% on average) of cardiomyocytes in the heart [109, 110]. Therefore, the differentiated cell aggregates may closely mimic native cardiac tissue and may be transplanted directly to treat cardiac diseases such as myocardial infarction, which warrants further investigation in future studies.

Considering that thousands of microcapsules can be easily produced using the non-planar microfluidic device in a few minutes and only a few tens of cells are required in each microcapsule to form ES cell aggregate of controllable size within 7 days as a result of the miniaturized 3D culture in the pre-hatching embryo-like microcapsules, the microencapsulation technology developed in this study is superior to the commonly used, labor-intensive hanging-drop method of culturing ES cells. For the latter, at least a few hundred cells in each hanging drop are usually needed in order to form pluripotent stem cell aggregates or embryoid bodies (EBs) in a few days [80, 85]. In addition, many of the microfluidic channels could be fabricated to run in parallel to further increase the
throughput of the technology.

2.4. Conclusion

In summary, we successfully fabricated a non-planar microfluidic flow-focusing device to generate pre-hatching embryo-like microcapsules with an aqueous liquid core of ES cells and an alginate hydrogel shell with controllable size. The core-shell microcapsule system was shown to be excellent for the miniaturized 3D culture of pluripotent stem cells to promote their proliferation and aggregation and to maintain higher pluripotency compared to the conventional 2D culture. Moreover, this microencapsulation technology can be used to easily produce thousands of microcapsules in a matter of minutes, which is highly desirable for the clinical applications of pluripotent stem cells where a large number of cells are usually desired. Therefore, the novel microencapsulation technology and miniaturized 3D culture system developed in this study are of importance to facilitate in vitro culture and expansion of pluripotent stem cells for their ever-increasing use in modern cell-based medicine.
Chapter 3: The crucial role of mechanical heterogeneity in regulating follicle development and ovulation with engineered ovarian microtissue

3.1 Introduction

The ovarian follicle consisting of a centrally located oocyte, an inner layer of granulosa cells, and an outer layer of theca cells is the fundamental functional tissue unit of mammalian ovary. During development as shown in Figure 3.1, granulosa cells proliferate to generate additional layers followed by differentiation into cumulus cells and mural granulosa cells. An antral stage follicle is characterized by the presence of cumulus cell-oocyte complex (COC) inside a fluid-filled antral cavity (also called antrum).

Anatomy of COC and ovarian follicles at the preantral and antral stages. (A) A schematic illustration showing that an early secondary preantral follicle consists of a single oocyte, few layers of granulosa cells, and an outer rim of theca cells. During development, granulosa cells proliferate to generate additional layers followed by differentiation into cumulus cells and mural granulosa cells. An antral stage follicle is characterized by the presence of cumulus cell-oocyte complex (COC) inside a fluid-filled antral cavity (also called antrum). An in vitro culture of ovarian follicles to obtain oocytes has been proposed as an attractive strategy for
restoring infertility of women who are born with ovarian disorder and for preserving fertility of women who may want to delay childbearing or lose gonadal function due to aggressive medical treatment (e.g., chemotherapy), exposure to environmental/occupational biohazards[111-113]. Both two and three-dimensional approaches have been developed for in vitro culture of ovarian follicles[114, 115]. For two-dimensional (2D) culture on the surface inside culture dishes, both the theca and granulosa cells easily detach from the follicles, spread out, and attach on the surface of culture dishes, leading to a diffused morphology that is non-physiological[102]. Moreover, endogenous paracrine and autocrine factors produced by granulosa and theca cells are easily diluted into the bulk culture medium, which can negatively affect follicle development[116]. On the other hand, three-dimensional (3D) approaches involving the use of a homogeneous hanging drop or hydrogel encapsulation (in millimeter-sized alginate hydrogel) can better preserve the native 3D follicular architecture during in vitro culture[117-121]. Although these approaches have been successfully used for in vitro culture of follicles from inbred mouse model and contribute significantly to the understanding of follicle biology[117-121], they are far away from being used as an assisted reproductive technology (ART) to restore infertility or preserve fertility for humans. This is because most of the culture systems developed using inbred mouse have been shown not to be directly applicable to even outbred mouse and primate models[102], not to mention humans. Moreover, both the 2D and homogeneous 3D (alginate hydrogel or liquid hanging drop) microenvironment used to culture follicles are non-physiological because it does not recapitulate the heterogeneous nature of the extracellular matrix (ECM) in the ovary with the ECM of medulla (inner region) being much softer than that in the cortex (outer region). The ECM
is believed to not only provide a 3D network to support the ovarian tissue architecture but also regulate (together with soluble endocrine, paracrine, and autocrine factors) cell-ECM and cell-cell interactions that are important for follicle development[116, 121-123].

To overcome these challenges, we employed outbred deer mice as the model to study the effect of culturing microenvironment on follicle development and ovulation with particular emphasis on mechanical heterogeneity that has been barely explored in the field of assisted reproduction. Deer mice are indigenous rodents in North America and believed to be more suitable than inbred mice for research aimed for medical applications due to their outbred nature as with humans[102, 116, 121-124]. Moreover, we engineered the in vitro culturing microenvironment of follicles by mimicking their native 3D milieu including the mechanical heterogeneity in ECM of medulla versus cortex and its 3D distribution. With the biomimetic microtissue, we identified the crucial role of mechanical heterogeneity in the ovary for follicle development from early preantral to the antral stage. Moreover, the biomimetic microtissue makes it possible to achieve ovulation in vitro under 3D culture for the first time ever in the absence of luteinizing hormone (LH) and epidermal growth factor (EGF), both of which are conventionally believed to trigger and facilitate ovulation[125-131]. Collectively, the biomimetic ovarian microtissue together with the microfluidic technology developed in this study should be valuable for preserving fertility as a better in vitro culture system and for understanding the role of mechanobiology in regulating follicle development and ovulation to facilitate the search of cures to ovarian disorders to restore infertility.
3.2 Materials and methods

3.2.1. Animals and materials

*Peromyscus maniculatus bairdii* (BW stock) deer mice were purchased from the *Peromyscus* Genetic Stock Center at the University of South Carolina, Columbia, SC and were maintained on a 16-8 h light-dark cycle. All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and every effort was made to minimize animal suffering. A total of 15 animals were used in this study to ensure that follicles from at least 3 animals were used for each experimental condition except the 2D culture condition for which 2 animals were used. L-15 Leibovitz-glutamax, α-MEM-glutamax medium, and fetal bovine serum (FBS) were purchased from Invitrogen and Hyclone, respectively. Alginate was purchased from Sigma and purified by washing in chloroform with charcoal and dialyzing against deionized water, followed by freeze-drying. Unless specifically noted otherwise, all other chemicals were purchased from Sigma.

3.2.2. Isolation of early secondary preantral follicles

Early secondary preantral follicles (100-130 μm) were isolated from ovaries of female deer mice of 12 to 16-week old using a mechanical method. In short, the ovaries were placed in 2 ml Leibovitz L-15 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin at 37 °C in 5% CO₂ air. Preantral follicles were obtained by using two 30 G needles to mechanically break the extracellular matrix between follicles in the ovarian tissue.
3.2.3. Fabrication of non-planar microfluidic device

To fabricate polydimethylsiloxane (PDMS, Dow Corning) microfluidic device, silicon master with patterned microfluidic channels was prepared using a multilayer (3-step UV exposure) SU-8 fabrication technique. In short, photosensitive epoxy (SU-8 2025, MicroChem) was spun coated onto a 4-inch silicon wafer. The thickness of the first SU-8 coating was 60 µm. The wafers were then soft-baked at 95 ºC for 9 min and exposed to UV light through the first shadow mask printed with the core channel. After a post-exposure baking at 90 ºC for 7 min, an additional layer (50 µm) of SU-8 photoresist was spun coated, soft baked, exposed with a different shadow mask to pattern shell channel. The third layer for oil channel was similarly patterned. All three exposures were aligned using an EVG620 automated mask aligner. The SU-8 pattern on the substrate was developed in SU-8 developer (MicroChem) for 10 min, rinsed with isopropyl alcohol, and then dried using nitrogen gas. PDMS pre-polymer was then poured on the silicon substrate and cured at 65 ºC for 3 h to form PDMS slab. Thereafter, the PDMS slab embedded with microchannels (half-depth) was lifted off. Two PDMS slabs with the same channel design were then plasma-treated for 30 s using Harrick PDC-32 G plasma cleaner at 18 W and 27 Pa, wetted with methanol (to prevented instant bonding), and aligned and bonded together under microscope to produce the final microfluidic device. A detailed schematic of the fabrication process is provided in Appendix. Assembled devices were kept on hotplate at 80 ºC for ~ 10 min to evaporate residual methanol and further kept at 65 ºC for 2 days to make it sufficiently hydrophobic for experiments.
3.2.4. Rheological characterization of hydrogel materials for making ovarian microtissues

Rheological measurements were carried out using a TA instrument AR-1000N rheometer. For alginate and collagen (type I, BD Biosciences) hydrogels, 40 mm parallel plate and plate-cone geometries were used, respectively. Stress sweeps at a constant frequency of 1 Hz were first performed to obtain the linear viscoelastic region for collecting subsequent data. Frequency sweeps were performed in the linear viscoelastic regime to determine values of the storage ($G'$) and loss ($G''$) modulus. The data at 1 Hz are reported for comparison. More rheology data showing the shearing rate (or frequency) dependence of the moduli of the various materials are given in Electronic Supplementary Information. The 2% and 0.5% alginate hydrogels were prepared on a mold using calcium infused mineral oil for 30 min, followed by washing with mannitol solution and transferring onto the rheometer plate. Collagen gels were prepared directly on the rheometer, for which 0.5% collagen solution was placed on the rheometer plate at 4 °C and gelled by raising the rheometer temperature to 37 °C for 30 min. To determine the time-dependent mechanical properties, the samples were prepared in the same way and further incubated in the basal culture medium of follicles at 37 °C in 5% CO₂ incubator till measurement at the desired times.

3.2.5. Encapsulation of early secondary preantral follicles to produce ovarian microtissues

The fluid entering the core microchannel via I-1 (Figure 3.2) was 0.5% collagen or sodium alginate (non-oxidized by default) solution. The dispatching fluid was the same as the core fluid. The fluid entering the shell microchannel via I-2 was 2% sodium alginate or a mixture
of 1% sodium alginate and 1% purified sodium alginate with oxidization using a reported protocol[132, 133]. For convenience, the mixture is called oxidized alginate (o-alginate) in this work. To increase the viscosity in the core solution, which was found to be necessary for formation of core-shell structure, 1% sodium carboxymethylcellulose (Sigma) was included. For the extraction channel, 1% sodium carboxymethyl cellulose solution was used which was necessary for forming stable interface between oil and aqueous phases. All the solutions were sterile and buffered with 10 mM HEPES to maintain pH at 7.2 before use. Further, osmolality of all the solutions were maintained at 300 mOsm by the addition of d-mannitol (Sigma). To make mineral oil infused with calcium chloride for flowing in the oil channel, stable emulsion of mineral oil and 0.7 g ml⁻¹ calcium chloride solution (volume ratio: 3:1 with the addition of 1.2% SPAN 80) was prepared by sonication for 1 min using a Branson 450 digital sonifier. Water in the emulsion was then removed by rotatory evaporation for ~2 min at 55 °C. All solutions (except collagen that was kept at 4°C) were injected into the microfluidic device using syringe pump at room temperature to generate microcapsules in oil phase and then extract them into aqueous phase. Flow rates for core, dispatching, shell, oil, and aqueous extracting fluids were 50 µl hr⁻¹, 30 µl hr⁻¹, 120 µl hr⁻¹, 2 ml hr⁻¹, and 4 ml hr⁻¹, respectively. Outlets of the device were connected to a 50 ml centrifuge tube containing M2 medium (Millipore) to collect microtissues at room temperature.

3.2.6. Preparation of ovarian cell-conditioned medium

To prepare ovarian cell-conditioned medium, ovarian cells were first isolated by following a protocol reported previously[125-131, 134]. In short, the ovaries of 12-14 week-old of
deer mice were collected and chopped after the removal of adherent tissues such as a fat pad. The specimens were incubated initially for 30 minutes in a dissociation medium consisting of a 50:50 (v:v) mixture of 0.25% (v/w) trypsin-EDTA (ethylenediaminetetraacetic acid) and DMEM (Dulbecco’s modified eagle’s medium) supplemented with 750 units/ml type I collagenase and 0.03% (v/v) fetal bovine serum at 37 °C in 5% CO₂ air. The dissociated cells were filtered through a 40 μm filter and subsequently centrifuged at 390×g for 4 minutes. The collected cells were further cultured in a 60 mm culture dish in 5 ml DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. After 20 h of culture in the dish, the DMEM-based medium was removed and the cells were washed once using 1x PBS. A total of 5 ml (non-conditioned) α-MEM–glutamax medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin solution was then added into the dish. The cells were incubated with the non-conditioned medium at 37 °C in 5% CO₂ air for two days and the resultant conditioned medium (CM) was collected and the procedure was repeated once to eventually make a total of 10 ml conditioned medium supplemented with 5 μg ml⁻¹ insulin, 5 μg ml⁻¹ transferrin, 5 ng ml⁻¹ selenium, and 100 mIU mL⁻¹ recombinant human follicle stimulating hormone (FSH) for further use.

3.2.7. In vitro culture of early secondary preantral follicles encapsulated in microtissues

For 2D culture, the early secondary preantral follicles were placed singly in 10 μl drops of ovarian cell conditioned culture medium overlaid with mineral oil in 60 mm culture dishes[132-134]. The preantral follicles encapsulated in microtissues were cultured singly in each well with 100 μl of ovarian cell conditioned culture medium in a 96-well plate. On
the following day, 10 μl and 100 μl of fresh medium were added to each drop and well, respectively. Starting from day 3, half the medium (10 and 100 μl) was replaced with fresh medium every other day till day 13. To study the influence of epidermal growth factor (EGF) and pituitary luteinizing hormone (LH) on follicle growth and ovulation, 2.5 IU ml⁻¹ LH and 5 ng ml⁻¹ EGF were added in the medium of some microencapsulated follicles once they developed to the antral stage. Ovulated cumulus-oocyte complexes (COCs) were further cultured in the same medium 18 hours. Afterward, the COCs were incubated in M2 medium containing 200 IU ml⁻¹ hyaluronidase at 37 °C for up to 3 min to remove cumulus cells and then washed twice in fresh M2 medium to obtain clean oocytes. The nucleus of the obtained oocyte was stained by incubating it with Hoechst 33342 (1 μg ml⁻¹) for 5 minutes at 37 °C in 5% CO₂ air.

3.3. Results and discussion

3.3.1. Design and materials of biomimetic ovarian microtissue

As illustrated schematically in Figure 3.2 A, all primordial and primary follicles stay within the rigid cortex. Once some of them are activated to grow at the beginning of each estrous cycle, they move from the cortex towards the medulla where profuse blood supply is available to provide the necessary nutrients and supplements for them to develop into antral follicles, followed by ovulation to release cumulus-oocyte complex (COC)[112, 135, 136]. To mimic the mechanical heterogeneity in the mammalian ovary in vitro, we propose to produce ovarian microtissue by encapsulating early secondary preantral follicles in microcapsules consisting of a softer, biodegradable collagen (0.5%) hydrogel core and a harder, slowly degradable alginate (2%) hydrogel shell (Figure 3.2 B). This miniaturized
3D culture ensures effective transport of oxygen and nutrients to all cells in the fast growing follicles[46, 89, 116, 137]. Alginate was used because of its excellent biocompatibility and mild gelation condition using divalent cations such as Ca\(^{2+}\) that are not harmful to living cells[27, 30, 138, 139]. As shown in Figure 3.2 C-D, the mechanical properties including both storage (G’, representing elastic effect) and loss (G’’, representing viscous effect) modulus of 2% alginate (Alg(2)) for shell/cortex are much higher than that of 0.5% collagen (Col(0.5)) for core/medulla. For comparison, we also used 2% oxidized alginate (O-alg(2)) that degrades fast (Figure 3.2 C) as the shell materials and 0.5% alginate (Alg(0.5)) with higher modulus than 0.5% collagen (Figure 3.2 D) as the core materials to understand how the change in mechanical cue in the ovarian microtissue could affect the development and ovulation of the encapsulated early secondary preantral follicles.

### 3.3.2. Microfluidic fabrication of biomimetic ovarian microtissue

The ovarian microtissues were produced using a non-planar microfluidic flow-focusing device by injecting collagen (or alginate) core solution with follicles, alginate shell solution, and mineral oil infused with aqueous calcium chloride solution from the I-1, I-2, and I-3 inlets into the device, respectively (Figure 3.3 A). At the flow-focusing junction, the core and shell solutions were pinched into droplets by the oil emulsion flow as a result of the Rayleigh-Plateau instability[140, 141]. Alginate in the shell solution was crosslinked to form calcium alginate hydrogel when it met the mineral oil-calcium chloride emulsion[46, 142]. The aqueous core flow is arranged in the center of alginate shell flow both horizontally and vertically, to ensure that the core flow can be encompassed by the shell flow (Figure 3.3 A-B). However, in some cases, two or more follicles could be

(1)
so close in the core channel immediately before flow focusing that they would be encapsulated in one microtissue. This is undesired because the core of the microtissue is too small to accommodate more than one fully-grown follicle. To overcome this problem, we designed a dispatching channel shortly upstream of flow focusing on increasing the distance between neighboring follicles by injecting the same solution as the core flow.

**Figure 3.2. Design and materials of biomimetic ovarian microtissue.** (A) A schematic illustration of mouse ovary that consists of two mechanically distinct tissue layers: the more rigid cortex and the softer medulla. (B) A schematic illustration of the in vitro engineered biomimetic ovarian microtissue. (C) The storage ($G'$, representing elastic effect) and loss ($G''$, representing viscous effect) moduli of materials for making the microtissue shell. Two different harder hydrogels, 2% alginate (alg(2)) and 2% alginate with oxidization (o-alg(2)), were used for making the shell (cortex). The moduli of o-alg(2) decrease by >16 times after ~7 days incubation in culture medium at 37 °C. (D) The two moduli of materials for making the microtissue core. Two different softer hydrogels, 0.5% alginate (alg(0.5)) and 0.5% collagen (col(0.5)), were used for making the core (medulla).
through I-4 inlet (Figure 3.3 A-B). For the Stokes laminar flow in a microchannel, microparticles (or follicles) can be taken to flow along the flow streamlines [143, 144]. As a result of mass conservation, the ratio of the distance between two follicles before dispatching to that after dispatching can be calculated as follows:

\[
\frac{d'}{d} = \frac{q + \lambda q}{q} = 1 + \lambda
\]

Where \(d\) and \(d'\) are the distances between follicles before and after dispatching,

![Figure 3.3](image_url)

**Figure 3.3.** Non-planar microfluidic flow-focusing device for encapsulating early secondary preantral follicle in core-shell microcapsules to produce the biomimetic ovarian microtissue. (A) A schematic view of the microchannel system (top) together with a zoom-in view of the nonplanar design of the flow-focusing junction (bottom) where \(W_1xH_1 = 200x200 \mu m\), \(W_2xH_2 = 80x300 \mu m\), and \(W_3xH_3 = 200x400 \mu m\). (B) Typical image of the boxed regions in panel A showing the dispatching and flow-focusing areas. (C) Typical image of the boxed regions in panel A showing the entrance of the extraction channel. (D) Typical image of the boxed regions in panel A showing the exit of the extraction channel. I-1, I-2, I-3, I-4, and I-5 are the inlets of core, shell, mineral oil emulsion, dispatching, and extraction flows, respectively. O-1 and O-2 are outlets for the aqueous (containing microtissues) and oil emulsion flows, respectively.

respectively, \(q\) is the flow rate of the follicle-loaded core solution, and \(\lambda\) is the ratio of the dispatching to core flow rate. The effectiveness of this dispatching channel (\(\lambda=3\)) in separating two adjacent microparticles was demonstrated using 125 \(\mu m\) polystyrene beads.
With dispatching at a much lower $\lambda$ (0.6) to minimize the effect of the dispatching flow on droplet formation, we were able to encapsulate one follicle in each microcapsule or microtissue with high efficiency (> 97%). To minimize the stay of microtissues in oil emulsion and collect them in aqueous culture medium, we further incorporated an extraction channel downstream of the serpentine channel in the microfluidic device to efficiently extract microtissue from the oil emulsion into an isotonic aqueous phase. Since the Reynolds Number for our flow conditions is much less than one (~ 0.1), the movement of microparticles is dictated by viscous force and the microtissue would travel with oil emulsion if undisturbed. Therefore, we used an expansion design for the extraction channel that is narrow (400 µm) at the beginning part (Figure 3.3 C) and wide (800 µm) at the end (Figure 3.3 D). Because the diameter of the microtissue is ~350 µm, they were forced to penetrate through the oil-water interface once the oil and aqueous solutions meet at the entrance of the extraction channel and further extracted from the oil into aqueous phase as a result of surface tension force (Figure 3.3 C). The expansion design at the exit should make it more convenient for collecting the microtissue by allowing them going straight into a 500 µm aqueous channel to exit from O-1 while the oil flow turns into a side channel to exit from O-2 (Figure 3.3 D). Thereafter, the microtissues were collected and cultured for up to 13 days (the typical time length of an estrous cycle of deer mice) to monitor their development and ovulation in vitro.

3.3.3. The crucial role of mechanical heterogeneity in regulating follicle development and ovulation

Typical micrographs showing an early secondary preantral follicle in the biomimetic ovarian microtissue with a collagen core and alginate (non-oxidized, by default) shell on
day 0 and its development into the antral stage are shown in Figure 3.4 A. A total of ~28% (17/60) of the preantral follicles in the biomimetic microtissue could develop to the antral stage, typically on day 9 (Figure 3.4 B and Table 3.1). This percentage dropped drastically to 0% (0/42) when oxidized alginate was used to make the shell of the microtissue even though the core was the same (0.5% collagen). The hydrogel shell of oxidized alginate degrades in 5-7 days with much reduced (by >16 times) mechanical strength thereafter compared to the hydrogel shell of alginate without oxidization (Figure 3.2 C). These data indicate the critical role of mechanical heterogeneity of the cortical and medullary tissue in the ovary in regulating follicle development. This is supported by the low percentage of development to the antral stage (~6%, 3/47) when the shell was the same (2% alginate) and the collagen (0.5%) core was replaced with the harder alginate (0.5%). Lastly, it is not surprising that 0% (0/40) of the early secondary preantral follicle developed to the antral stage.
stage from 2D culture since it is non-physiological at all and could not maintain the 3D architecture of the follicles during culture (Figure 3.4 B and Figure 3.5).

Table 3.1. *In vitro* culture of early secondary preantral follicles using different methods including 2D culture in dishes and 3D culture in microtissues with a core of either 0.5% collagen (Col(0.5)) or 0.5% alginate (Alg(0.5)) and a shell of either 2% alginate (Alg(2)) or oxidized alginate (O-alg(2)). COC: cumulus-oocyte complex.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Core  (%)</th>
<th>Shell (%)</th>
<th>No. of preantral follicles</th>
<th>No. (%) of antral follicle</th>
<th>Culture condition for ovulation (No. of antral follicles used)</th>
<th>No. of COC ovulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miniaturized 3D culture</td>
<td>Col(0.5)</td>
<td>Alg(2)</td>
<td>60</td>
<td>17 (28)</td>
<td>LH+EGF+ (6)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O-alg(2)</td>
<td>Alg(2)</td>
<td>42</td>
<td>0 (0)</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>Alg(0.5)</td>
<td>Alg(2)</td>
<td>47</td>
<td>3 (6.4)</td>
<td>LH+EGF+ (3)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2D culture</td>
<td></td>
<td></td>
<td>40</td>
<td>0 (0)</td>
<td>N/A</td>
<td>0</td>
</tr>
</tbody>
</table>

As shown in Figure 3.2 A, the event following the development of follicles to the antral stage *in vivo* is ovulation, a delicate reproductive process that results in the release of a cumulus-oocyte complex (COC) from each follicle. Although the exact mechanisms that

![Figure 3.5. Typical micrographs showing the development of early secondary preantral follicles of deer mice under 2D culture.](image)

regulate the ovulation process are still not fully understood, it is well accepted in the contemporary literature that ovulation is triggered by the surge of luteinizing hormone (LH) from the pituitary gland. This LH surge activates a cascade of epidermal growth factor (EGF) mediated signaling pathways to induce cumulus cell differentiation (from granulosa cells) and expansion in the follicle, resulting in the escape of the COC out of the follicle. Guided by the literature on the critical role of LH and EGF in initiating ovulation, we
treated 6 out of the 17 antral follicles obtained by culturing early secondary preantral follicle in the microtissue with a collagen core and alginate shell (Figure 3.4 B and Table 3.1) with LH and EGF (LH^+EGF^+), leaving the remaining 11 antral follicles without LH and EGF treatment (LH^-EGF^-). In other words, we used less antral follicles for the LH^+EGF^+ group because we thought that according to the conventional theory, the probability of ovulation from this group should be much higher compared to the LH^-EGF^- group. In contrast to our anticipation, only 1 out of the 6 antral follicles treated with LH and EGF ovulated while ovulation was observed for all the 11 antral follicles without LH and EGF treatment (Figure 3.6 A). Typical micrographs of ovulation showing a released COC and a corpus luteum-like tissue complex remaining in the alginate shell are shown in

Figure 3.6. *In vitro* ovulation of antral follicles obtained by culturing early secondary preantral follicles in the collagen core of biomimetic ovarian microtissues with an alginate shell. (A) Quantitative data (pooled, the number of antral follicles n=6) showing the effect of luteinizing hormone (LH) and epidermal growth factor (EGF) on ovulation. (B) A typical micrograph of in vitro ovulation showing a cumulus-oocyte complex (COC) was released leaving behind a corpus luteum-like tissue complex in a biomimetic microtissue cultured in the presence of LH and EGF (LH^+EGF^+). (C) A typical micrograph of in vitro ovulation from a biomimetic microtissue cultured in the absence of LH and EGF (LH^-EGF^-). (D) A typical phase contrast image showing the oocyte morphology. (E) A typical fluorescence image showing the nucleus of the oocyte in panel D. Scale bar, 100 µm in panels B-C and 50 µm in panels D-E.
**Figure 3.6 B and C** for the LH⁺EGF⁺ and LH⁻EGF⁻ groups, respectively. Typical images showing the morphology and nucleus of the ovulated germinal vesicle (GV) oocytes are given in **Figure 3.6 D-E**. Interestingly, only 1 out of 3 antral follicles obtained from culturing early secondary preantral follicles in microtissue with an alginate core and alginate shell ovulated when they were cultured in the absence of LH and EGF (**Figure 3.6 A**). These data suggest that mechanical heterogeneity in the biomimetic ovarian tissue and ovary regulates follicle ovulation and the LH surge might even suppress ovulation of many antral follicles to allow few of them being ovulated during each estrous cycle, as occurs naturally. In this study, we cultured the ovarian microtissue with the commonly used dose of LH and EGF [126-131]. Future research is warranted to determine the critical level of LH and EGF that begins to inhibit ovulation under various mechanical microenvironments.

### 3.4. Conclusion

In summary, we have developed a microfluidic microencapsulation technology that allows us to engineer ovarian microtissue that recapitulates the 3D mechanical, physiological, and anatomical milieu in the ovary. With these biomimetic microtissues, we unraveled the important role of mechanical heterogeneity in the ovary in regulating follicle development and ovulation for the first time ever. It has been reported that disruption of the normal physical environment in the ovary may result in ovarian disorders such as premature ovarian failure (POF) and polycystic ovary syndrome (PCOS) [136, 145]. Our biomimetic ovarian microtissue may be valuable for identifying the mechanisms associated with these ovarian diseases and serve as a better *in vitro* culture system for preserving fertility and restoring infertility for women.
Chapter 4: Effects of extracellular matrix composition on cell/tissue proliferation and development

4.1. Introduction

Cells and tissues interact dynamically with the physical and chemical cues present in their microenvironment including the insoluble extracellular matrix (ECM) and soluble endocrine, paracrine, and autocrine cytokines, which determines the morphology, differentiation, proliferation, development, and function of the cells and tissues\[146-151].

A number of methods have been developed to control the cell and tissue microenvironment in vitro by patterning adhesive proteins/peptides (e.g., fibronectin/RGD) on two-dimensional (2D) substrates (e.g., glass and plastic) with various physical and chemical properties\[152-154]. Although it has contributed significantly to the understanding of cell/tissue biology, 2D culture differs substantially from the in vivo microenvironment of most cells and tissues where they are intricately associated with various adherent ligands present on three-dimensional (3D) ECM\[68, 146, 155-157]. Therefore, studies have been reported to encapsulate/embed cells and tissues in homogeneous 3D ECM made of one or more natural/synthetic polymers such as alginate, collagen, hyaluronic acid, polyethylene glycol (PEG), and polycaprolactone for in vitro culture\[8, 32, 35, 156, 158-160]. However, most of the reported 3D ECMs are macroscale (at least in millimeters) scaffolds where many cells/tissues may suffer hypoxia and/or deprivation of nutrients because the diffusion
length of oxygen and nutrients in cellularized tissue is usually less than ~200 \( \mu \text{m} \). To overcome this problem, encapsulation of cells/tissues in sub-millimeter constructs (e.g., microcapsules or sheets) for 3D culture is attracting more and more attention. However, current effort in this regard has been focused on producing homogeneous microscale constructs, which does not recapitulate the heterogeneous nature of most cells/tissues/organs that usually have a central core for performing specific function(s) and an outer shell/wall that physically isolates the core from the surroundings. For example, the plasma membrane of a cell helps to maintain intracellular concentrations of proteins and ions; the epidermis of skin helps to retain moisture in the tissues and organs semi-enclosed in it; the pericardium envelops the heart from the adjacent organs; and the zona pellucida that houses totipotent-pluripotent stem cells in pre-hatching embryos during early embryo development. In other words, both the central core and outer shell probably serve their purposes to maintain homeostasis in the cells/tissues/organs. Indeed, our recent studies show that a liquid core semi-enclosed in a hydrogel shell is better than the conventional 2D culture on a substrate and 3D culture in homogeneous microscale hydrogel for maintaining stemness. We also showed that ovarian preantral follicles cultured in a softer core semi-enclosed in a harder shell could develop better than the follicles under the conventional 2D and homogeneous 3D culture conditions. However, the effect of the core ECM on the proliferation and development of the cells/tissues under the miniaturized 3D culture has not been rigorously studied. Here, we describe the encapsulation of mouse embryonic stem cells (mESCs) and ovarian preantral follicles (with vastly different ECMs in their native niches in vivo) in the bio-inspired microcapsules comprised of a protein or protein-based (instead of liquid) core and hydrogel
shell, which allows precise control of the microenvironment to modulate the cell/tissue proliferation and development under the miniaturized biomimetic 3D culture.

We used naturally occurring alginate and type I collagen to fabricate the hydrogel shell and core of the microcapsules, respectively. Alginate was used due to its excellent biocompatibility and reversible gelation with divalent cations such as Ca$^{2+}$ or Ba$^{2+}$ under gentle conditions[124, 138, 165, 166]. The alginate shell acts as an outer/surface layer that provides sufficient mechanical strength/structural support to maintain the integrity of the softer core ECM. Further, the nano-porous structure (pore size on the order of tens of nanometers[132, 168]) of alginate shell may help to retain important autocrine and paracrine cytokines produced by encapsulated cells and tissues in the core ECM (i.e., reducing their dilution in the bulk medium). In brief, this biomimetic miniaturized 3D platform 1) ensures effective transport oxygen and nutrients due to their small size, 2) facilitates cell-cell contacts and autocrine and paracrine signals as a result of the miniaturized and semi-enclosed space, and 3) enables control of cell-ECM interactions by tuning the properties of core ECM.

4.2 Materials and methods

4.2.1. Fabrication of non-planar microfluidic device

The device used in this study was fabricated using the same method as described in Chapter 2 and Chapter 3.
4.2.2. Animals

*Peromyscus maniculatus bairdii* (BW stock) deer mice were purchased from the *Peromyscus* Genetic Stock Center at the University of South Carolina, Columbia, SC and bred. CD1 mice for isolating mouse embryonic fibroblasts (MEFs) cells were purchased from Charles River. Both deer mice and CD1 mice were maintained on a 16-8 h light-dark cycle before the experiments. All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and every effort was made to minimize animal suffering.

4.2.3. Cell culture

R1 mouse ESCs from ATCC (Manassas, VA) were cultured in medium consisted of Knockout DMEM (Millipore, Billerica, MA) supplemented with 15% (v/v) Knockout Serum (Millipore), 4 mM L-glutamine (Sigma, St. Louis, MO), 100 μg ml⁻¹ antibiotics (Invitrogen) and 1000 U ml⁻¹ leukemia inhibitory factor (LIF) (Millipore), 10 μg ml⁻¹ gentamicin (Sigma) and 0.1 mM mercaptoethanol (Sigma) on a gelatin-coated tissue culture flask at 37 °C in a humidified 5% CO₂ incubator. When reaching desired ~70% confluence, cells were detached using trypsin/EDTA (Invitrogen, Carlsbad, CA) and gently pipetted to break aggregates. Cells were centrifuged, resuspended, and counted for further passaging or experimental use.

4.2.4. Isolation of preantral follicles

Preantral follicles (100-135 μm) were isolated using mechanical methods from ovaries of female deer mice of 12 to 16-week old as reported by us previously. In brief, the ovaries
were placed in 2 ml Leibovitz L-15 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen) and 1% (v/v) penicillin-streptomycin. Preantral follicles were obtained by using two 30 G needles to mechanically break the extracellular matrix between follicles in the ovarian tissue and then immediately used for encapsulation. Encapsulated preantral follicles were co-cultured with MEFs in follicle culture medium consist of α-minimum essential medium-glutamax supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) with 5 μg ml⁻¹ insulin, 5 μg ml⁻¹ transferrin, 5 ng ml⁻¹ selenium, and 100 mIU mL⁻¹ recombinant human follicle stimulating hormone (FSH).

4.2.5. Preparation of mouse embryonic fibroblasts (MEFs)

MEFs were isolated by following a protocol reported elsewhere. In brief, E13.5 mouse embryos were dissected and the brain, limbs, and internal organs were removed to ensure a pure fibroblastic population. Embryos were minced with a sterile razor blade into small pieces and they were placed in 37 °C in 5% CO₂ air for 10 min. The samples were then pipetted up and down using 1 ml pipette to further break up the tissue, followed by culturing in 100 mm culture dish coated with 0.1% gelatin at 37 °C in 5% CO₂ air. MEFs were then expanded and frozen for future use. For experiments, MEFs (passage 3) at 2 × 10⁴ cells/well were seeded in 96-well plates followed by treatment with 10 μg ml⁻¹ mitomycin for 3 h. Afterward, MEFs were washed twice with 1x PBS and 100 μl of follicle culture medium with encapsulated preantral follicle was added.
4.2.6. Encapsulation of cells and follicles

A neutralized collagen solution (2x) was prepared according to manufacturer’s instruction (BD Bioscience) by mixing the stock collagen solution with appropriate volumes of NaOH, 10x PBS, and DI water. The solution was kept at RT for 10 min to initiate pre-gelling. After 10 min, cells and 2% sodium carboxymethyl cellulose were added to the collagen solution on ice. Final collagen concentration and cell density in the core solution were 0.5-3.0 mg ml\(^{-1}\) and \(5 \times 10^6\) cells ml\(^{-1}\), respectively. A similar method was applied to encapsulate preantral follicles except that the concentration of collagen in the core solution was 1.0-5.0 mg ml\(^{-1}\). In addition, in some cases we added 5.0 mg ml\(^{-1}\) alginate to the core solution to further tune the mechanical properties of the core ECM. Encapsulated cells/follicles were collected and cultured in their respective medium at 37 °C in a humidified 5% CO\(_2\) incubator.

4.2.7. In vitro culture of encapsulated mESCs and pluripotency analysis

Encapsulated mESCs were cultured in their culture medium (see Supplementary Methods for medium composition) for 10 days. On day 10, mESC aggregates were released from the core-shell microcapsules with 75 mM sodium citrate (~5 min to dissolve alginate), followed by 1000 units ml\(^{-1}\) type I collagenase (30 min at 37 °C to remove the collagen ECM). Aggregates were then washed with PBS, pipetted several times to make them single cells and then centrifuged. RNAs were extracted from the aggregated cells using RNeasy plus mini kit (Qiagen, Valencia, CA) following the manufacturer’s instruction. Next, reverse transcription was carried out to generate complementary DNA (cDNA) using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) and GeneAmp 9700 PCR system.
Quantitative RT-PCR was conducted with the superfast SYBR Green mix (Bio-Rad) using a Bio-Rad CFX96 real-time PCR system. Pluripotency genes Oct-4, Sox2, Nanog, and Klf2 were studied with GAPDH being used as the housekeeping gene. Primer sequences of GAPDH and the pluripotency genes are same as provided in Chapter 2.

4.2.8. *In vitro* culture of encapsulated preantral follicles and analysis of hormone release

Encapsulated preantral follicles were cultured (one in each well) in 100 μl of follicle culture medium (see Supplementary Methods for follicle culture medium composition) on MEFs feeder (cell density: $2 \times 10^4$ cells/well) in a 96-well plate. On the following day, 100 μl of fresh medium was added to each well. Starting from day 3, half of the medium (100 μl) was replaced with fresh medium every other day till day 10. The replaced medium (100 μl) was collected and used to analyze estradiol (E2) with an estradiol EIA Kit according to the manufacturer’s instruction (Cayman). The colorimetric analysis was conducted with a microplate reader (PerkinElmer, Waltham, MA) at 405 nm.

4.2.9. *In vitro* maturation (IVM) of antral follicles and developmental competence of MII oocytes

For IVM, antral follicles on day 10 after liquefying alginate hydrogel using 75 mM sodium citrate were incubated for 48 h in 500 μl of α-minimum essential medium-glutamax medium supplemented with 1000 IU ml$^{-1}$ mouse leukemia inhibitory factor (LIF), 5 μg ml$^{-1}$ epidermal growth factor (EGF), 10 mg ml$^{-1}$ streptomycin sulfate, 75 mg ml$^{-1}$ penicillin G, and 5% (v/v) heat-inactivated fetal bovine serum (FBS) covered with 250 μl of mineral oil in a 4-well plate at 37 °C in 5% CO$_2$ air. After maturation, the cumulus–oocyte complex
(COC) in each antral follicle was retrieved mechanically by follicular puncture. Oocytes were then released from the COC by incubating in hyaluronidase (200 IU mL\(^{-1}\)) for 2 min. The quality of the metaphase II (MII) oocytes was evaluated first by the extrusion of the first polar body and was stained to check meiotic spindle of microtubules. For the latter, MII oocytes were fixed in 4% formaldehyde/PBS for 30 min at room temperature. The oocytes were then permeabilized and blocked in 1x PBS containing 0.5% Triton X-100 and 3% fetal bovine serum for 1 h at room temperature, followed by overnight incubation with diluted (1:200) monoclonal anti-\(\alpha\)-tubulin antibody (Sigma) in the blocking solution at 4 °C. Samples were then washed for 1 h in blocking solution and incubated with 1:200 diluted AlexaFluor® 488 rabbit anti-mouse IgG (H + L) secondary antibody (Invitrogen) for 1 h at room temperature. Samples were further washed for 1 h in blocking solution and the cell nuclei stained for 20 min with Hoechst 333232 (1 µg ml\(^{-1}\)). After washing 3 times in 1x PBS, images were taken using a Zeiss (Oberkochen, Germany) microscope with a 40x oil objective. The quality of the MII oocytes was further assessed by their embryonic development after parthenogenetic activation using Ca\(^{2+}\)-free KSOM medium supplemented with 10 mM SrCl\(_2\) and 5 µg ml\(^{-1}\) cytochalasin B for 3.5 h.

4.2.10. Rheological characterization of core and shell materials

Rheological measurements were carried out using 40 mm cone geometry plate on a TA Instrument AR-1000N rheometer. Neutralized collagen solutions at different concentrations were prepared as aforementioned and placed directly on the rheometer at 4 °C. The temperature was then raised to 37 °C for 30 min to gel the collagen. Stress sweeps at a constant frequency of 1 Hz were first performed to obtain the linear viscoelastic
region for collecting subsequent data. Frequency sweeps were performed in the linear viscoelastic regime to determine values of the storage/elastic ($G'$) and loss/viscous ($G''$) modulus. Values at 1 Hz are reported in Figure 2 for comparison of the different core ECMs.

4.2.11. Statistical analysis

All the experiments were conducted for at least three times independently. Results are reported as mean ± standard deviation (SD). Two-tailed student t-test was performed to determine statistical significance ($p < 0.05$).

4.3. Results and discussion

4.3.1. Generation of core-shell microcapsules using a microfluidic device

We utilized high throughput microfluidic technique to encapsulate cells and tissues in the biomimetic core-shell microcapsules. Figure 4.1A shows a schematic illustration of the microfluidic device that consists of four inlets (I1, I2, I3, and I4) for injecting mineral oil emulsified with aqueous calcium chloride solution ($Ca^{2+}$ oil emulsion), sodium alginate solution, ice-cold collagen-based solution (with or without cells/tissues), and aqueous extraction solution, respectively. At the flow-focusing junction (Figure 4.1B), the alginate (shell) and collagen (core) solutions were pinched off into spherical droplets by the $Ca^{2+}$ oil emulsion as a result of interfacial tension. Alginate in the outer or shell layer of the spherical droplets was further gelled to form a hydrogel when flowing in the serpentine channels by $Ca^{2+}$ in the oil emulsion. Since an extended stay of microcapsules in the oil emulsion is harmful to the encapsulated cells (Figure 4.2, which demonstrates that on-chip
extraction of microcapsules significantly increases the cell viability), we incorporated an extraction channel in the device to extract the core-shell microcapsules from the oil phase into aqueous phase based on intrinsic interfacial tension between water and oil [169], as shown in Figure 4.1C. The aqueous phase containing microcapsules and the oil emulsion were collected from outlet O1 and O2, respectively. Microcapsules formed for the purpose of this study have a total and core size of 380.9 ± 33.98 µm and 285.05 ± 80.4 µm in diameter, respectively. The flow rates of solutions in the device can be easily varied to control the size of the microcapsules. The collected microcapsules were then incubated at 37 °C for 30 min to crosslink collagen in the microcapsule core and cells/tissues in the

Figure 4.1. A schematic illustration of the flow-focusing microfluidic device for encapsulating cells/tissues in the collagen-based core of microcapsules with an alginate hydrogel shell. (A) An overview of the microfluidic device showing Ca\(^{2+}\) oil, 2% sodium alginate, collagen (with or without cells), and aqueous extraction solutions were pumped into the device from inlets I1, I2, I3, and I4 respectively. The aqueous and oil phases exited the device from outlet O1 and O2, respectively. (B) A zoom-in view of the nonplanar flow-focusing junction where cells/tissues were encapsulated. (C) A zoom-in view of the channel where microcapsules in oil were extracted into aqueous phase. (D) Encapsulated cells/tissues were cultured for up to 10 days to form grown 3D microtissue.
microcapsules will be cultured for up to 10 days to monitor their proliferation and development (Figure 4.1D).

4.3.2. Core-shell microcapsules with different core ECMs

The 3D ECM can be tuned to modulate the cell proliferation, adhesion, and differentiation by using adhesion and signaling ligands and/or changing its structural/mechanical properties. We sought to investigate this by varying the concentration of collagen in the ECM between 0.5-5.0 mg ml\(^{-1}\). We also incorporated, in some cases, alginate to the core ECM to further increase the mechanical properties of the ECM. To visualize and examine the fibrous structure of the ECM, we utilized differential interference contrast (DIC) and confocal reflectance microscopy (CRM) techniques. Figure 4.3 A-D shows clearly the presence of collagen fibers in the core ECMs of the microcapsules. Furthermore, we quantified two structural parameters (fiber length and width) of different core ECMs in the microcapsules using CRM images. Our data indicate that as the concentration of collagen...
is increased, individual fibers become significantly thicker, longer, and sparser (Figure 4.4). We further measured the viscoelastic properties including both storage ($G'$, representing elastic effect) and loss ($G''$, representing viscous effect) modulus of core ECMs made of either single component (0.5-5.0 mg ml$^{-1}$ collagen) or two components (a mixture of 5.0 mg ml$^{-1}$ collagen and 5.0 mg ml$^{-1}$ alginate). As shown in Figure 4.4E, by increasing the collagen concentration in core ECM, we were able to regulate $G'$ between 0.48 and 429.74 Pa and $G''$ between 1.14 Pa and 68.45 Pa.

4.3.3. Encapsulation of mouse embryonic stem cells in different ECMs

After determining the effects of collagen concentration on fiber structure and mechanical properties of the core ECM in the core-shell microcapsules, we encapsulated mESCs in the different core ECMs to investigate their effect on the cell proliferation and pluripotency. Since stem cells naturally reside in a soft/liquid microenvironment, we chose 0.5 mg ml$^{-1}$ ($G' < G''$), 1.5 mg ml$^{-1}$ ($G' \approx G''$), and 3.0 mg ml$^{-1}$ ($G' > G''$) for our experiments. We suspended mESCs at a density of $5 \times 10^6$ cells ml$^{-1}$ in ice-cold collagen (0.5, 1.5, and 3.0 mg ml$^{-1}$) solution and injected the cell suspension into the core channel (I3) of the
microfluidic device (Figure 4.1) to form microcapsules. Collected microcapsules containing 48 ± 11 cells per microcapsule) were then cultured in stem cell culture medium for up to 10 days. Figure 4.5 shows the typical phase contrast and corresponding live/dead staining fluorescence images of stem cell aggregates formed on day 10 in different collagen ECMs and the corresponding quantitative data of the aggregate size are given in Figure 4.6. As it is evident from the data, we observed the formation of a single large aggregate in each microcapsule with ECM made of low concentrations of collagen (313.1 ± 75.8 and
293.61 ± 102.8 µm for 0.5 and 1.5 mg ml\(^{-1}\), respectively) and significantly smaller aggregates (128.1 ± 51.9 µm) in the ECM made of 3.0 mg ml\(^{-1}\)collagen. These data indicate that the mESCs exhibit high proliferation in a microenvironment with the low adhesion and soft ECM. This is possibly because the lower adhesion and softer ECM better mimics the permissive proteinaceous microenvironment in the core semi-enclosed in a hydrogel-like shell (known as the zona pellucida) of a pre-hatching embryo and enhances the pluripotency of the cells[138, 165], we further sought to investigate the effect of the different core ECMs of collagen on the cell stemness. To do this, we conducted quantitative RT-PCR studies for the expression of four pluripotency genes (Oct-4, Sox2, Nanog, and Klf2). The data shown in Figure 4.6 indicate that as the adhesion ligand/elastic modulus of the encapsulating ECM increases (from 0.5 mg ml\(^{-1}\) to 3.0 mg ml\(^{-1}\)), the expression of three pluripotency markers (Sox2, Klf2, and particularly Nanog) significantly decreases.

![Figure 4.5. Proliferation of encapsulated mESCs in different collagen core ECMs with varying structural and mechanical properties. Phase contrast and fluorescence (live/dead) images of the mESC aggregates on day 10 in different core ECMs. Massive aggregates were formed in softer core ECM with low collagen (0.5-1.5 mg ml\(^{-1}\)) compared to significantly smaller aggregates in more rigid core with high collagen (3.0 mg ml\(^{-1}\)). Scale bar: 100 µm.](image)
Possibly, culturing mESCs in liquid or soft ECM (G’< G’’) of 0.5 mg ml⁻¹ collagen facilitates e-cadherin mediated cell-cell interactions, which enhances the self-renewal capacity of mESCs (therefore bigger aggregates) through up-regulation of Sox2, Nanog, and Klf2[170, 171]. On the other hand, the increase in ligand density and stiffness of the ECM facilitates mechanical interactions between the ECM and mESCs via integrins on the cells, which favors differentiation as suggested by the down-regulation of pluripotent gene markers. Moreover, it is worth noting that although the aggregate size is not significantly different for the 0.5 and 1.5 mg ml⁻¹ collagen core ECMs, the gene expression could be significantly different. Therefore, proliferation alone may not be a good indicator of a culture condition for maintaining the cell properties. All in all, these data indicate that biological characteristics of mESCs can be easily regulated in this biomimetic culture system.

Figure 4.6. Quantitative data of the size of aggregates formed in microcapsules with different core ECMs. Quantitative RT-PCR data showing expression of pluripotency gene markers in the aggregated mESCs obtained from different core ECMs on day 10. * denotes $p < 0.05$. 

![Graph showing quantitative data](image-url)
4.3.4. Encapsulation of mouse preantral follicles in different ECMs

To encapsulate small tissues in this miniaturized system, we chose preantral follicles (at the early secondary stage and with a diameter of 100-135 µm) obtained from deer mice (deer mice are more suitable for research aimed for medical applications due to its outbred nature similar to humans) [167]. Follicles are the fundamental tissue unit of mammalian ovary and each preantral follicle consists of one single oocyte that is surround by layered granulosa and theca cells (See Figure 3.1 in Chapter 3). By providing necessary chemical and mechanical cues, the preantral follicle can be developed to an antral stage to obtain fertilizable oocytes, which may help to restore/preserve fertility in women. *In vivo* proliferation of preantral follicles occurs in the collagen-rich medullar region of the ovaries. To study the effect of ECMs on follicle development, we encapsulated the follicles in three types of core ECMs: 1.0 mg ml⁻¹ collagen, 5.0 mg ml⁻¹ collagen, and a mixture of 5.0 mg ml⁻¹ collagen and 5.0 mg ml⁻¹ alginate to significantly vary ligand concentration and mechanical properties ($G'$ between 1.42 and 429.74 Pa and $G''$ between 2.49 and 68.45 Pa).

![Figure 4.7. Bright field image of the encapsulated preantral follicles. Typical bright field image of deer mouse preantral follicles encapsulated in 5.0 mg ml⁻¹ collagen core ECM of microcapsules with a 2% alginate hydrogel shell on day 1. Scale bar: 200 µm.](image)
Pa) of the ECMs. Also, we chose 5.0 mg ml⁻¹ ECM core as a baseline based on the findings in a previous study. Typical bright field images at lower magnification showing the encapsulated early secondary preantral follicle in 5 mg ml⁻¹ collagen ECM are shown in Figure 4.7.

![Figure 4.7](image)

**Figure 4.7.** Images showing the proliferation of preantral follicles in core ECMs. Typical bright field images of encapsulated early secondary preantral follicles in 5 mg ml⁻¹ collagen ECM are shown in Figure 4.7.

![Figure 4.8](image)

**Figure 4.8.** Images showing the proliferation of preantral follicles in core ECMs. Typical bright field images of encapsulated early secondary preantral follicles in 5 mg ml⁻¹ collagen ECM are shown in Figure 4.7.

Typical images showing proliferation and development of the encapsulated early secondary preantral follicle to the antral stage in the three types of ECMs are given in Figure 4.8. The corresponding quantitative data showing proliferation (diameter of
follicles) of the preantral follicles (that did develop to the antral stage excluding those became degenerated) at 1, 6, 8, and 10 days of culture in the core ECMs are shown in Figure 4.9E, which indicates slightly (but insignificantly) higher proliferation in the 5.0
mg ml\(^{-1}\) collagen ECM compared to the other two. In our experiments, we observed 24.4% (11/45) development of preantral follicles to the antral stage after 10 days culture in the 5.0 mg ml\(^{-1}\) collagen core ECM (Figure 4.9A). By contrast, development of follicles to the antral stage remarkably decreased to 2.6% (1/38) when the concentration of collagen was lowered to 1.0 mg ml\(^{-1}\). Interestingly, when the structural and mechanical properties of the core ECM were changed while keeping the number of adhesion ligands the same (in 5.0 mg ml\(^{-1}\) collagen) by adding 5.0 mg ml\(^{-1}\) alginate, we also observed lower (10.8%) development of follicles into the antral stage (Figure 4.9A and Table 4.1). Representative DIC and live/dead images showing the morphology and high viability of cells in the antral follicle at day 10 are given in Figure 4.9B and C, respectively. Figure 4.9D shows the DIC image of an antral follicle released from the microcapsule together with the oocyte and fluid-filled antral cavity in the follicle. We further analyzed the production of estradiol in follicles (at days 1, 8 and 10) which is essential for the regulation of estrous and menstrual reproductive cycles of females[172]. The data (Figure 4.9F) indicate a significant increase in estradiol secretion at day 10 compared to day 8 in all the encapsulating ECMs. Moreover, we observed the significantly higher production of estradiol in 5.0 mg ml\(^{-1}\) collagen ECM than the other two ECMs. Higher estradiol concentration from antral follicles encapsulated in the 5.0 mg ml\(^{-1}\) collagen ECM indicates better quality of antral follicles. To affirm the quality of developed antral follicles, we performed in vitro maturation (IVM) of the cumulus-oocyte complex from the antral follicles. Although none of the oocytes in the antral follicles cultured in the other two ECMs (0/1 and 0/4, 0%) developed to the metaphase II (MII) stage, we obtained 5 MII oocytes out of the 11 antral follicles (45.5%) cultured in 5.0 mg ml\(^{-1}\) collagen core ECM.
We hypothesize that as the growing follicle expands inside the microcapsules, it experiences mechanical stresses from the surrounding core ECM that is communicated rapidly throughout the follicle. The ECM made of 5 mg ml\(^{-1}\) collagen probably provides an optimum mechanical and chemical microenvironment essential for proliferation, differentiation, and development of the encapsulated follicles. Other formulations of ECM provide minimal (1.0 mg ml\(^{-1}\) collagen) or excessive (5.0 mg ml\(^{-1}\) alginate in 5.0 mg ml\(^{-1}\) collagen) mechanical stresses that inhibit the development of follicles. The ECM of 1.0 mg ml\(^{-1}\) collagen could be insufficient in cell adhesion ligand density for the encapsulated follicle.

**Table 4.1.** *In vitro* culture of preantral follicles in different core ECMs of microcapsules with a shell made of 2% alginate hydrogel.

<table>
<thead>
<tr>
<th>Core ECM</th>
<th>Number of Antral follicles</th>
<th>Number (%) of antral follicles</th>
<th>Number (%) of MII oocyte</th>
<th>Number of two-cell embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col: 1.0 mg ml(^{-1})</td>
<td>38</td>
<td>1(2.6)</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Col: 5.0 mg ml(^{-1})</td>
<td>45</td>
<td>11(24.4)</td>
<td>5 (45.5)</td>
<td>1(^a)</td>
</tr>
<tr>
<td>Col: 5.0 mg ml(^{-1}) Alg: 5.0 mg ml(^{-1})</td>
<td>37</td>
<td>4(10.8)</td>
<td>0 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

Col: collagen. Alg: alginate. \(^{a}\)3 MII oocytes were used to obtain embryo by parthenogenetic activation.

To develop and proliferate either as the adhesion ligand density (involved in secretion of estradiol) and the mechanical properties of the ECM play an equally important role in regulating follicle development[136, 173]. In addition, these data together with that in **Figure 4.9E-F** indicate that proliferation alone may not be a good indicator of a culture condition for maintaining tissue properties. A typical DIC image of the MII oocytes obtained is shown in **Figure 4.9G**. To further confirm the developmental stage of the MII oocytes, we stained the meiotic spindles and nuclei by tubulin (in microtubules of the
spindles) antibody and Hoechst, respectively. Figure 4.9H shows the ordered attachment of meiotic spindles (green) to the chromosomes (blue) in the cytoplasm while the arrangement of tubulin and chromosomes is chaotic in the first polar body, which is characteristic of MII oocytes. It is worth noting that only MII oocytes could be further activated or fertilized into embryos for further embryonic development. Therefore, we used 3 of the MII oocytes to obtain early embryos by parthenogenetic activation, for which we successfully obtained one 2-cell stage embryo (Figure 4.9I). Overall, these data suggest that development of preantral follicles is sensitive to cell adhesion ligand density as well as the mechanical properties of the ECM under biomimetic 3D culture.

4.4. Conclusion

Cells and tissues respond to subtle changes in their microenvironment that is known to affect its proliferation, morphology, adhesion, migration, differentiation, and development. This paper describes a novel approach to engineer the cellular and tissue microenvironment and controls its biological impact by encapsulating cells/tissues in biomimetic 3D core-shell microcapsules where core and shell are composed of biocompatible/natural collagen and alginate, respectively. The collagen core can be easily replaced with other biocompatible synthetic or natural polymers. Our data show that proliferation alone is not sufficient to judge the quality of cells/tissues under biomimetic 3D culture. We anticipate this 3D biomimetic platform will find its broad applications in many fields including but not limited to regenerative medicine, developmental biology, and high-throughput drug screening.
Chapter 5: Bottom-Up tissue engineering to fabricate 3D vascularized mammary tumor for drug screening

5.1. Introduction
The role of the 3D microenvironment in tumor development has been extensively studied in recent years [6, 13, 174-178]. It has been reported that the surrounding extracellular matrix (ECM), mesenchymal cells (fibroblasts and adult stem cells), and supporting blood vessels all play a vital role in tumor growth, invasion, and metastasis [179, 180]. For example, the ECM (collagen, laminin, and fibronectin) provides structural support to the resident cells and acts as a reservoir of growth factors and cytokines [13, 175, 176]. Moreover, communications between cancer cells and stromal cells in the microenvironment enable tumor proliferation and distant colony formation [176]. Importantly, tumor microenvironment has received growing attention due to its role in chemoresistance, relapse, and metastasis [176]. Therefore, an *in vitro* 3D biomimetic model is essential that takes into account the complex interplay between different types of cells and their microenvironment for investigating tumor biology and drug screening applications.

A number of methods have been proposed lately to mimic the native microenvironment of the tumor. They include suspension culture to form multicellular
tumor spheroids and encapsulation of cells in porous scaffolds [13, 174, 176, 179, 181]. The former is simple and inexpensive. However, it is difficult for achieving high-throughput production and obtaining aggregates of uniform size [176, 179]. 3D tumor models have also been developed by introducing cells into porous synthetic scaffolds (e.g., polylactide or PLA, polyglycolide or PGA, and copolymers of PLA and PGA (PLGA)) and hydrogels (e.g., polyethylene glycol or PEG) [176, 179]. Although studies with these models have made significant contributions to tumor biology, the synthetic ECM does not mimic accurately that is present natural tumor microenvironment. The lack of vascularization is another fundamental limitation of most contemporary 3D tumor models where the diffusional restrictions of nutrients and oxygen in highly cellularized tissue (typically less than ~200 µm[176, 182-184]) may result in viable cells only in the surface layer of less than ~200 µm thick. Vascularization is also required for tumor metastasis. The differences between normal vasculature and vasculature in solid tumors [185-187](immature, tortuous, and hyperpermeable vessels) offer a unique target for anticancer therapies [188-190]. Moreover, the abnormal vasculature greatly affects the transport of anti-cancer drugs within the tumor tissues. Therefore, tumor vasculature is an important component of the tumor microenvironment that must be incorporated in the 3D tumor models. However, contemporary work on vascularization in vitro has been focused on either random assembly of endothelial cells in a homogeneous system (e.g., hydrogel or medium) to form new blood vessels (i.e., vasculogenesis) with no control on their distribution[88, 191-195], or the sprouting of existing or microfabricated vessels to produce new capillaries (i.e., angiogenesis)[185, 186, 191, 196, 197].
To mimic the vasculature of *in vivo* tissue, “bottom-up” tissue engineering principles could be utilized. Bottom-up approaches have the potential to construct vascularized tissues with defined properties including spatial and temporal control at the cellular level [6, 58]. We hypothesize assembling microscale (less than 200 µm, the diffusion limit of nutrients and oxygen in highly cellularized tissue) cell-containing building blocks or modules may allow for the fabrication of large viable complex tissues with the vasculature. Recent progress in encapsulating and culturing cells in core-shell microcapsules with a tumor relevant ECM core and an alginate hydrogel shell using a high-throughput microfluidic device is one of the ways to create these microscale modules[46, 198, 199]. To develop a vascular network, the cell-laden modules may be packaged with tumor-relevant ECM and supporting vascular and stromal cells for them to self-assemble and form vascularized tumor. Moreover, the microscale modules may provide geometric guidance to the vascular cells[195], enabling the formation of a complex 3D vascular network around the cancer cells in the modules to mimic the vasculature in the tumor *in vivo*.

To the best of our knowledge, this is the first report describing the fabrication of 3D vascularized human mammary tumor. This was achieved by first encapsulating and culturing MCF-7 cells in core-shell microcapsules (<~200 µm in radius) composed of type 1 collagen-rich ECM enclosed in a semipermeable alginate hydrogel shell using a high-throughput non-planar microfluidic device. We characterized the proliferation and gene expression of the encapsulated MCF-7 cells in different core ECMs with the different biophysical properties. Next, these engineered individual micro-tumors (µtumor) in core-shell microcapsules were assembled in a microfluidic device with endothelial and stromal
cells to form a millimeter-sized 3D vascularized breast tumor. The endothelial cells were guided by the microcapsules to form complex tortuous 3D vascular bed around the encapsulated µtumors, mimicking the vasculature of an *in vivo* tumor (Figure 5.1). Lastly, to illustrate the capability of our model for high-throughput drug screening, we investigated the effect of the tumor microenvironment on cancer drug resistance and demonstrated the use of nanotechnology to overcome the cancer drug resistance. Therefore, this biomimetic 3D model may be valuable for studying the effect of microenvironment on tumor progression, invasion, and metastasis, and for developing an effective therapeutic strategy to combat cancer.
5.2. Materials and methods

5.2.1. Fabrication of cell encapsulation and perfusion device

To fabricate the cell encapsulation non-planar microfluidic device, channels were patterned on a silicon wafer by multilayer photolithography technique. A 100 µm layer of SU8 2025 (MicroChem, MA) was first coated and then soft-baked followed by exposure to ultraviolet (UV) light through the first shadow mask (Cad/Art services, OR) printed with the core channel. After a post-exposure baking, an additional layer (50 µm) of SU-8 photoresist was spun coated, soft baked, exposed with a second shadow mask to pattern shell channel. The third layer for oil/separation channel was similarly patterned. All three exposures were aligned using an EVG620 automated mask aligner. In the end, the SU8 pattern on the substrate was developed in the SU-8 developer (MicroChem, MA), rinsed with isopropyl alcohol, and dried using nitrogen gas. To fabricate perfusion device, two layers of SU8 2100 were spun-coated on a silicon wafer to achieve a thickness of 500 µm followed by UV light exposure and development.

Microfluidic devices were made by replica molding of polydimethylsiloxane, PDMS (Sylgard 184, Dow Corning, MI). The mixture of crosslinker and PDMS prepolymer (1:10) was poured on a patterned silicon wafer. PDMS was cured at 65 °C for at least 3 h and then carefully peeled off the wafer. For cell encapsulation devices, two PDMS slabs with the same channel design were then plasma-treated for 30 s using a Harrick PDC-32G plasma cleaner at 18 W and 27 Pa, wetted with methanol, aligned and bonded together under a microscope to produce the final cell encapsulation device. Assembled device was kept on a hotplate at 80 °C for ~ 10 min to evaporate residual methanol and further kept at 65 °C for 2 days to make it sufficiently hydrophobic for experiments. Perfusion devices
were similarly bonded with a circular slab with holes for medium reservoirs before experiments.

5.2.2. Cell culture

MCF-7 human breast cancer cells (ATCC, Manassas, VA, USA) were cultured in EMEM medium supplemented with 10% FBS, 10 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary human umbilical vascular endothelial cells (HUVECs) (Angio-Proteomie, MA) were cultured on gelatin-coated flask in endothelial basal medium (EBM) supplemented with an EGM-2 bullet kit (Lonza, NJ). Primary human adipose-derived stem cells (ADSCs) (Lonza, NJ, USA) were cultured in ADSC basal medium (Lonza) supplemented with 10% fetal bovine serum (FBS), 5 ml of L-glutamine and 0.5 ml of gentamicin amphotericin. All the cells were cultured at 37 °C in humidified 5% CO₂ incubator. The medium was changed every other day. Primary cells were used between 3-7 passages.

5.2.3. Encapsulation of human breast cancer cells (MCF-7) in core-shell microcapsules and quantitative PCR

The fluids in the shell (from I2 entrance) and core (from I3 entrance) microchannels were 2% purified sodium alginate (Sigma) and neutralized rat tail collagen type I (BD Bioscience) solution with cells (density: \(5 \times 10^6\) cells/ml), respectively. In order to minimize the mixing of core and shell solutions during microcapsule formation, the viscosity of core solution was increased by adding 1% sodium carboxymethylcellulose (Sigma) to the collagen mix while keeping final collagen concentration between 0.5-3 mg/ml. In some cases, alginate was also added to the core solution with cells to increase
the stiffness of the encapsulating core ECM. For the separation channel (from I4 entrance), 1% sodium carboxymethyl cellulose solution was used which was necessary for stable interface between oil and an aqueous phase. All the solutions were sterile and buffered with 10 mM HEPES to maintain pH 7.2 before use. Further, the osmolality of all the solutions was maintained at 300 mOsm by the addition of D-Mannitol (Sigma). To make mineral oil infused with aqueous calcium chloride solution for flowing in the oil channel (from I1 entrance), stable emulsion of mineral oil and 1 g/ml aqueous calcium chloride solution (volume ratio: 5:1 with the addition of 1.2% SPAN 80) was prepared by sonication for 1 min using a Branson 450 Sonifier. Solutions (except collagen that was kept at ice temperature) were injected into the microfluidic device using syringe pump (Pump 11 Elite, Harvard Apparatus) at room temperature (RT) to generate microcapsules in the oil phase and then separate them into the aqueous phase. Flow rates for core, shell, oil and aqueous separation fluid were 100 µl/hr, 250 µl/hr, 6 ml/hr, and 4 ml/hr, respectively. Outlets of the device were connected to 50 ml centrifuge tube containing cell culture medium. Microcapsules were collected and further gelled at 37 °C for 30 min in the incubator. Encapsulated cells were then cultured for 10 days. On day 10, MCF-7 aggregates were released from the core-shell microcapsules by treating them with 75 mM sodium citrate (~5 min to dissolve alginate) followed by 1000 units/ml type I collagenase (30 min at 37 °C to remove the collagen ECM). Aggregates were then washed with PBS and centrifuged. RNAs were extracted from the aggregated cells using RNeasy plus mini kit (Qiagen, Valencia, CA) following the manufacturer’s instruction. Next, reverse transcription was carried out to generate complementary DNA (cDNA) using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) and GeneAmp 9700 PCR system. Quantitative RT-PCR was
conducted with the superfast SYBR Green mix (Bio-Rad) using a Bio-Rad CFX96 real-time PCR system. Expression of vimentin, e-cadherin, and CXCR4 were studied with GAPDH being used as the housekeeping gene.

5.2.4. Rheological characterization of core ECM materials

Rheological measurements were carried out using 40 mm cone geometry plate on a TA instrument AR-1000N rheometer. Neutralized collagen solutions at different concentrations were prepared as aforementioned and placed directly on the rheometer at 4 °C. The temperature was then raised to 37 °C for 30 min to crosslink the collagen. Stress sweeps at a constant frequency of 1 Hz were first performed to obtain the linear viscoelastic region for collecting subsequent data. Frequency sweeps were performed in the linear viscoelastic regime to determine values of the storage/elastic (G’) and loss/viscous (G’’) modulus. Values at 1 Hz are reported in Figure 1E.

5.2.5. Formation of 3D vascularized tumor tissue in microfluidic perfusion device

After formation of MCF-7 aggregates on day 10, core-shell microcapsules were collected and the excess medium was removed by passing them through 100 µm cell strainer. Thereafter prepolymer solution containing microcapsules, neutralized collagen, and cells was prepared on ice to produce a final collagen concentration of 1.5 mg/ml excluding the 40% volume of beads. The final density of HUVECs and ADSCs in collagen were 10 × 10^6 cells/ml and 1 × 10^6 cell/ml, respectively. The microcapsules-collagen-cell solution was carefully mixed for 1 min without introducing any air bubbles. Thereafter solution was
carefully introduced into the gel chamber in a sterile PDMS perfusion device using 100 µl positive displacement pipette (approximate volume of gel in each chamber ~10 µl). After injection, PDMS devices were immediately sealed with 0.15 mm thick cover glass and allowed to polymerize for 30 min in a 37 °C humidified incubator with 5% CO₂. Microfluidic devices were filled with cell culture medium maintaining 10 mm H₂O hydrostatic pressure drop to allow perfusion of the medium within the gel. To initiate cell-cell interactions between encapsulated MCF-7 aggregates and stromal cells, 75 mm sodium citrate (Sigma) was introduced in microfluidic devices for 5 min after day 1.

5.2.6. Imaging and immunocytochemistry

Fibrous collagen ECM in the core of microcapsules was visualized using scanning electron microscopy (SEM). Core-shell microcapsules were fixed by glutaraldehyde followed by dehydration through series of ethanol treatment and chemical dryer hexamethyldisilazane (HMDS). Samples were then mounted on aluminum stub using double stick carbon tape, coated with a very thin film of Au in a sputter coater, and imaged with an FEI NOVA nano400 scanning electron microscope. Assembled encapsulated MCF-7 aggregates with HUVECs and ADSCs were images every day using Zeiss Axio Observer Z1 microscope. Obtained images were analyzed using NIH ImageJ software to evaluate vessel lengths and width. On day 4, devices were washed with PBS and fixed with 4% paraformaldehyde for 20 min at RT. Post fixation, devices were washed 3 times with PBS and blocked and permeabilized with 3% bovine serum albumin (BSA) and 1% (v/v) Triton X-100 in 1X PBS buffer for 1 hr at RT. Following blocking, devices were incubated with the anti CD31 antibody (Abcam ab28364) at a ratio of 1:50 for overnight at 4 °C. Next day, the unbounded
antibody was removed by washing 3 times with PBS. Devices were incubated with secondary antibody at a ratio of 1:200 diluted with 1% BSA in PBS for 1 hr at RT. After 1 hr excess antibody was washed 3 times with PBS nuclei were stained using Hoechst. Devices were then imaged using Olympus FV1000 spectral confocal microscope.

5.2.7. Animal and in vivo tumor formation

Athymic female NU/NU nude mice were purchased from National Cancer Institute-Frederick Laboratory and were maintained on a 16:8 hr light-dark cycle. All procedures for animal usage were approved by Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and utmost care was taken to minimize suffering. To obtain xenograft model of the human mammary tumor, 3D ₿tumors with the microcapsules together with HUVECs-ADSCs in 1.5 mg/ml collagen solution (prepared similarly to in vitro experiments) were injected subcutaneously in mice. Control experiment with the equivalent number of 2D cultured MCF-7 cells (~ 1.3 x 10^6 cells) suspended in 1.5 mg/ml collagen were also performed. The mice were euthanized after 2 weeks. Tumor tissues were collected, formalin fixed, paraffin embedded, and hematoxylin & eosin (H&E) stained for further histological analysis. We analyzed 3 tumors (3 mice) per experimental group.

5.2.8. Preparation and characterization of drug loaded nanoparticles

Theranostics agents doxorubicin (DOX) and indocyanine green (ICG) were encapsulated in lipid-silica shell and fullerene core nanoparticles prepared using reverse microemulsion method as previously reported by our group. The nanoparticle size (diameter, nm),
polydispersity index (PDI), and surface charge (or zeta potential) were determined using a Brookhaven (Holtsville, NY, USA) 90 Plus/BI-MAS dynamic light scattering (DLS) instrument. The morphology of nanoparticles was characterized using standard sample preparation protocols for both transmission (TEM) and scanning (SEM) electron microscopy.

5.2.9. In vitro tumor destruction in 2D, 3D avascularized and 3D vascularized model using free and nano drug

For tumor destruction studies, the 2D MCF-7 cells, 3D MCF-7 aggregates in core-shell microcapsules, and assembled 3D vascularized tumor in microfluidic devices were incubated for 4 days with free DOX. 3D vascularized tumor in the devices were also treated with DOX and ICG encapsulated in nanoparticles (LC60S) in the same way. To determine the cell viability after 4 days, fresh medium with 10% WST-8 (Dojindo, DC) reagent was added to each sample and incubated for 4 hr at 37 °C. Thereafter, the absorbance at 450 nm was quantified using a Perkin Elmer Victor™X4 multilabel plate reader. Cell viability was calculated as the ratio of absorbance of each group to that of control.

5.2.10. Quantification of CD44⁺ CD24⁻ MCF-7 cell population

CD44 CD133 CSC subpopulation was quantified by flow cytometry. MCF-7 aggregates were released from the core-shell microcapsules by treating them with 75 mM sodium citrate (~5 min to dissolve alginate) followed by 1000 units ml⁻¹ type I collagenase (30 min at 37 °C to remove the collagen ECM). Aggregates were then washed with PBS and centrifuged. The dissociated MCF-7 cells were washed with 1X PBS and stained with CD44-FITC (Invitrogen, Carlsbad, CA) and CD24-PE (Miltenyi Biotec Ltd., Surrey, UK)
antibodies according to the manufacturer's instructions. Lastly, the stained samples were analyzed using a BD LSR-II Flow Cytometer together with BD FACS Diva software (Franklin Lakes, NJ).

5.2.11. Statistical analysis

All data are reported as the mean ± standard deviation of results from at least three independent runs conducted at three different times. Student's two-tailed t-test assuming equal variance was performed in GraphPad to determine the p-value for assessing statistical significance (P < 0.05).

5.3. Results

5.3.1. Microfluidic generation of cell-laden core-shell microcapsules to form µtumor modules

A schematic illustration of the core-shell microcapsule is shown in Figure 5.3A. The shell of the microcapsule was made of alginate, a biocompatible polymer derived from seaweeds that form a reversible viscoelastic hydrogel in the presence of divalent ions (Ca$^{2+}$ or Ba$^{2+}$) with controllable mechanical properties[167, 198, 200]. Fibrous type I collagen was used to form the core of the microcapsules since it is abundantly present in breast tumor stroma[201]. In addition, type I collagen may influence the growth and metastasis of resident cancer cells. The alginate shell acts as a surface layer providing sufficient mechanical strength to maintain the integrity of the otherwise easily deformed collagen ECM core. A schematic illustration of the microfluidic device used to encapsulate cells in core-shell microcapsules is shown in Figure 5.2A. The device is comprised of four inlets.
I, I2, I3, and I4 to inject mineral oil infused with aqueous calcium chloride solution (Ca\(^{2+}\) oil emulsion) for the oil flow, sodium alginate solution for the aqueous shell flow, ice-cold collagen solution for the aqueous core flow, and aqueous solution of carboxymethyl cellulose for extracting the microcapsules out of the oil flow, respectively. At the non-planar flow-focusing junction (Figure 5.2B), the aqueous solutions were pinched off into spherical droplets.
spherical core-shell droplets by the Ca$^{2+}$ oil emulsion as a result of Plateau-Rayleigh instability[169, 198]. Further, the Ca$^{2+}$ ions present in the oil flow crosslinks the alginate in the shell to form a hydrogel. After the gelation in the serpentine section of the device, microcapsules were extracted from oil phase to aqueous phase utilizing interfacial technique as previously reported[169, 198] (Figure 5.2C). Real images showing the flow focusing and the entrance and exit of the extraction microchannel are shown in Figure 5.2D.

Figure 5.2B shows the image of a typical microcapsule clearly depicting its spherical and core-shell morphology. Further, scanning electron microscopy (SEM) image revealed the fibrous morphology of the collagen ECM in the core. The microcapsules prepared for this study have total and core sizes of 387 ± 15 µm and 273 ± 21 µm in diameter, respectively (Figure 5.3C). Since the diffusion limit for oxygen and nutrients in vivo is less than ~200 µm, the small size of the core-shell microcapsules allows adequate mass transport for the encapsulated cells to survive and proliferate. We encapsulated human breast cancer (MCF-7) cells in the microcapsules by adding 5 x 10$^6$ cells/ml in the core collagen solution while forming microcapsules. Each microcapsule contained 33 ± 6 cells (Figure 5.3C). Figure 5.3D shows the morphology and proliferation of the MCF-7 cancer cells encapsulated and cultured in the microcapsules. The cells formed round colonies in the 3D collagen ECM consisted with past report[202]. Over the period of 10 days, the cells proliferated to form µtumors (i.e., avascular aggregates of cancer cells) with high viability.
5.3.2. Characterization of μtumor modules formed in core-shell microcapsules

Given the ability to efficiently encapsulate MCF-7 cells in the microcapsules, we next investigated the microenvironment critical to the growth and metastasis of the cancer cells.

We modulated the biomechanical properties of the core ECM in the microcapsules by

![Figure 5.3](image1.png)

**Figure 5.3. Characterization of encapsulated cells.** (A) Typical DIC image of a core-shell microcapsule and a SEM image to visualize the collagen fibers in the core. (B) Typical distribution of the total and core size of the microcapsules. Also shown is the distribution of number of cells in each microcapsule. (C) Typical phase contrast and fluorescent (live/dead) images of the encapsulated cells in 1.5 mg/ml collagen core microcapsules showing its proliferation from day 1 to day 10. (D) Elastic modulus of different collagen core ECMs used to encapsulate cancer cells. (E) Quantification of relative aggregate sizes on day 10 for the cells encapsulated in different collagen core ECMs. (F) Quantitative RT-PCR analysis showing the comparison of mRNA expression of 3D encapsulated cells in core-shell microcapsules for epithelial and mesenchymal phenotype markers responsible for tumor metastasis.
varying the collagen density in the core of the microcapsules from 0.5 to 3 mg/ml. In order to further increase the elastic properties of the encapsulating ECMs, in some case, we added 1-2% (w/v) of alginate together with the collagen. Rheological analysis of the bulk core ECMs shows that storage modulus (G’) can be tuned over ~1-15000 Pa (Figure 5.3D), spanning the range of stiffness of normal and malignant breast tissue[201, 203, 204]. At collagen concentration of 3 mg/ml (G’ = 22 Pa) in the core ECM, we observed small and

![Figure 5.4. Images of cancer cell proliferation in encapsulated cells. Time series micrographs of phase contrast and fluorescent images of human MCF-7 growth in different core ECMs. Single massive MCF-7 aggregates were observed in Col 0.5 and 1.5 mg/ml ECMs, whereas small and multiple aggregates were observed in other ECMs. Scale bar: 100 µm](image-url)
multiple aggregates in the core-shell microcapsules cultured over the span of 10 days. However, when we lowered the collagen concentration to 0.5 mg/ml or 1.5 mg/ml (G’=0.48 Pa and G’=3.68 Pa), significantly high proliferation (4.6 folds in 0.5 mg/ml and 5.9 folds in 1.5 mg/ml) of MCF-7 cells to form aggregates or µtumors were observed (Figure 5.3E and Figure 5.4). It is also worth noting that the µtumors formed in the compliant core collagen (0.5-1.5 mg/ml) mimic the architecture of the bulk tumors with an outer proliferative region and a gradient of oxygen and nutrients inside the aggregates. Next, to determine the role of stiffness alone on MCF-7 proliferation, we included alginate (1-2%) in addition to constant 1.5 mg/ml collagen in the core ECM. As shown in Figure 5.4 solely increasing the stiffness, significantly reduced the MCF-7 cell proliferation in the core-shell microcapsules.

Malignant transformation of tumors of epithelial origin including the mammary adenocarcinoma is often accompanied by changes in the tumor microenvironment[205]. Therefore, we investigated the effect of ECM stiffness on the malignant behavior of MCF-7 cells under the miniaturized 3D culture by examining the expression of two epithelial to mesenchymal transition (EMT) marker genes including vimentin and CXCR4 and one epithelial marker gene (e-cadherin). As the stiffness of the encapsulating ECM is increased (keeping the collagen density constant at 1.5 mg/ml), we observed significantly increased expression of vimentin (up to 10 folds) and CXCR4 (up to 3.6 fold) without significant change in e-cadherin (Figure 5.3F). These data suggest EMT is mediated by ECM stiffness under the miniaturized 3D culture, as also reported for cancer cells cultured under 2D and bulk 3D conditions[178, 203, 205]. Taken together, these results show the capability of engineering the tumor microenvironment within the core-shell microcapsules to study the
biological behavior of cancer cells under 3D culture, including interrogating the contribution of environmental cues to tumor progression and invasion.

5.3.3. Assembly of µtumor modules in a microfluidic device

After successful formation of µtumor modules in the core-shell microcapsules, we assembled them in a microfluidic device with endothelial cells and stromal cells to form 3D vascularized tumor. A schematic view of the polydimethylsiloxane (PDMS) microfluidic device is shown in Figure 5.5A. The device consisted of a gel-filling chamber (width x length x depth = 1 x 5 x 0.5 mm) connected to the two reservoirs through 4 pillars (width x length x depth: 0.8 x 0.8 x 0.5 mm). The composite hydrogel consisting of encapsulated µtumor modules in 1.5 mg/ml type I collagen with primary human umbilical vein endothelial cells (HUVECs) and human adipose-derived stem cells (hADSCs) was injected in the gel-filling space (~10-12 µl). We incorporated ADSCs in the system because it is now known that ADSCs are present in in vivo breast tumor stroma and can positively influence the vascularization by secreting pro-angiogenic growth factors[180, 206]. The gel was constrained within the region of the microfluidic device by the 4 micro-pillars. A total of 50-60 core-shell microcapsules encapsulated with the MCF-7 µtumor could be placed in the gel-filling region. We monitored the viability and growth of the assembled human breast tumor tissue with and without the perfusion of the medium through the channels. Without perfusion, no capillary formation was observed and many cells were dead by day 4. With perfusion by applying a hydrostatic pressure of 10 mm H₂O (~100 Pa) as depicted in Figure 5.5A, endothelial cells were live and exhibited elongated morphology, and vascular structures were observed by day 4 (Figure 5.5B). Perfusion of
the medium through the thick tissue allows sufficient transport of nutrients to all the cells, which is necessary for their survival. Thereafter, all the experiments were conducted under the flow condition with medium changed every 12 hours.

5.3.4. Formation of 3D vascularized breast tumor and its characterization

Next, we investigated the effect of the presence of the 3D modules of encapsulated MCF-7 cell aggregates in core-shell microcapsules on guiding the endothelial cells in the collagen gel in capillary morphogenesis around the microcapsules. We hypothesized that the encapsulated MCF-7 aggregates in 3D core-shell microcapsules would release the pro-angiogenic cytokines such as VEGF, bFGF, and IL-8 as depicted in Figure 5.1C to promote vasculogenesis[207]. Further, the core-shell microcapsules should provide a geometric guidance for the endothelial cells to form a complex 3D vascular system around the µtumors. Since the size of core-shell microcapsules is less than the diffusion limit (~200 µm), this enables adequate transport of nutrients and oxygen to all cells in the system including cells in the core of the µtumors. Indeed, high cell viability was seen in the system with perfusion (Figure 5.5B). After one-day culture, HUVECs displayed elongated morphology and encompassed the microcapsules. Formation of vacuoles and assembly of HUVECs into tube-like structures were observed on day 2. On day 4, a complex 3D capillary network occupying extended area of the gel region was observed, with representative phase contrast and fluorescent images taken at 10x and 4x magnification being shown in the first two columns of Figures 5.5D and Figure 5.6, respectively.
Figure 5.5. Assembly of microtumors. (A) An image of the microfluidic device made in PDMS used to form 3D vascularized breast tumor. (B) Fluorescence images showing live/dead staining images of the vascularized tumor on different days showing its high viability. Asterisk and arrow head represents microcapsules and cancer cell aggregates, respectively. (C) Time lapse micrographs of 3D vascularization with encapsulated MCF-7 aggregates in the microcapsules, empty microcapsules, and MCF-7 aggregates without the microcapsules at low magnification (D) Time lapse micrographs of vessel formation with encapsulated MCF-7 aggregates in the microcapsules (first two columns), empty microcapsules (third and fourth columns), and MCF-7 aggregates without the microcapsules (alginate hydrogel was dissolved after treating with 75 mM sodium citrate solution after day 1 (last two columns). Extensive vascularization was observed on day 3 and 4 when MCF-7 aggregates were present whereas little vascularization was observed with empty microcapsules. Scale bar: (B-D) 100 µm, (C) 200 µm
Because microcapsules act as a physical barrier to prevent direct cell-cell contact between the encapsulated µtumors and the HUVECs, contribution of the µtumors to vasculogenesis is likely through the secretion of vasculogenic factors by cells in the µtumors. We found that vasculogenic morphogenesis of HUVECs was highly dependent on the co-culture with the MCF-7 cells in the µtumors, since HUVECs without co-culture (i.e., mixed with empty microcapsules) failed to form interconnected vascular networks as shown in the third and fourth columns of Figures 5.5D and Figure 5.6.

Next, we investigated the effect of direct cell-cell contact between the µtumors and HUVECs on vascularization in addition to the cytokine effect. For this, we formed the tumor tissue in the microfluidic chip as explained above, but after the initial network was

![Figure 5.6. Images of vessel formation in different culture conditions. Time lapse micrographs of vessel formation with encapsulated MCF-7 aggregates in the microcapsules (first two columns), empty microcapsules (third and fourth columns), and MCF-7 aggregates without the microcapsules (alginate hydrogel was dissolved after treating with 75 mM sodium citrate solution after day 1 (last two columns). Scale bar: 200 µm](image-url)
formed after one-day culture, the alginate shell of the core-shell microcapsules was dissolved by treating it with 75 mM sodium citrate for 5 min. This allowed direct cell-cell interactions between the \( \mu \)tumors and the HUVECs. During the course of the following three days, we observed significantly aggressive vascularization within the device as shown in the fifth and sixth columns of Figures 5.5D and Figure 5.6.

To further verify that the vasculature engineered using the \( \mu \)tumors with the dissolution of the alginate shell after one-day culture, we tested it for its 3D structural integrity and the presence of characteristic CD31 markers. Immunofluorescent micrographs of the microvascular network exhibited the complex interconnectedness as
shown in Figures 5.7. Moreover, we observed the presence of open hollow lumens along the length of the vessels enclosed by HUVECs as shown in Figure 5.7.

5.3.5. *In vivo* culture of engineered 3D µtumors

To understand the effect of the miniaturized 3D culture on tumor formation *in vivo*, we injected engineered µtumors mixed with HUVECs and hADSCs in collagen subcutaneously in the athymic nude mice. Control experiments using conventionally used 2D cultured MCF-7 cells were also performed. Figures 5.8A show that the use of engineered µtumors could result in the formation of significantly much larger tumors than using the conventionally 2D-cultured cells. Figure 5.8B shows the quantitative data of the tumor volume in two conditions after 14 days of implantation. Light microscopy
examination of hematoxylin and eosin (H&E) stained slides of the tumors (Figure 5.8 A) shows higher heterogeneity in tumors formed with the µtumors than 2D-cultured cancer cells. To assess if the difference in size of the tumors formed under the two different conditions could be related to the difference in vascularity, we quantified blood vessel formation in the tumors. We observed a significantly higher number of blood vessels (RBCs indicated by arrows) in the tumors formed with the µtumors than 2D-cultured cancers cells. The average size of blood vessels is also larger in the tumors formed by injecting the µtumors than 2D cultured cells although the difference is not statistically significant. Taken together, these data suggest that 3D culture of MCF-7 cells and incorporation of supporting HUVECs and ADSCs have a significant impact on in vivo tumorigenicity compared to traditional 2D cultured cells.

5.3.5. In vitro drug testing for cancer destruction in different models

After successful fabrication and characterization of the avascular 3D µtumors and in vitro vascularized 3D tumors, we analyzed the responsiveness of the tumors to chemotherapy to illustrate their utility in high-throughput drug testing. The Food and Drug Administration (FDA) approved anticancer drug doxorubicin (DOX) for breast cancer was used[208]. We simultaneously compared the cytotoxicity of DOX on the conventionally 2D-cultured MCF-7 cancer cells, avascularized 3D µtumors, and in vitro vascularized 3D tumors. Doxorubicin was administered by incubating it with the cancer cells for 4 days at concentrations ranging from 0-100 µg/ml. The WST-8 cell viability assay was used to assess the drug response. As shown in Figure 5.9A, 2D-cultured MCF-7 cells were the most sensitive to DOX. The cancer cells in the engineered 3D (avascularized) µtumors
were significantly more resistant to the free DOX from 1-60 µg/ml than the 2D-cultured cells. Moreover, the cancer cells in the vascularized 3D tumors (cultured with perfusion) is significantly more resistant to the free drug from 10-60 µg/ml than the cancer cells in the 3D avascularized tumors. The inhibition concentration of free DOX to kill 50% of cancer cells (IC₅₀) was 0.24, 7.13, and 33.48 µg/ml on average for the 2D-cultured cells, cells in the avascularized 3D tumors, and cells in the vascularized 3D tumors, respectively. This differential drug sensitivity observed between 2D cultured cancer cells, 3D avascularized tumors and 3D vascularized tumors may be attributed to the decreased DOX availability.
or a reduced drug sensitivity of the two 3D-cultured cancer cells to the free drug. The former is ruled out by the homogeneous distribution of DOX in the cancer cells under all the three different culture conditions as shown in Figure 5.10. To investigate more on the possible mechanism of drug resistance in the 3D-cultured MCF-7 cells, we studied the expression of the putative breast cancer stem cell marker CD44 together with the differentiation marker CD24 using immunocytochemistry and flow cytometry. As shown in Figure 5.11, we observed significantly low expression of CD24 in the avascularized 3D tumors than the 2D-cultured cells. However, expression of CD44 was slightly but not statistically significantly increased in the 3D-cultured cells. The sub-population of
CD44⁺CD24⁻ cells (the putative mammary cancer stem cells) was also slightly albeit insignificantly increased compared to the 2D-cultured cells. We propose that there could be interplay among various factors (intracellular changes, paracrine signaling, and modification in the supporting matrix [209-212]) that may contribute to the reduced drug sensitivity in avascularized 3D tumors and in vitro vascularized 3D tumors, which

Figure 5.11. Microenvironment effects MCF-7 properties. (A) Differential interference contrast (DIC) and fluorescent images showing the expression of CD24 and CD44 marker proteins in cells cultured in 2D and 3D microenvironments. (B) Quantitative measurement of fluorescence intensity and percentage of CD44⁺ CD24⁻ cells cultured in 2D and 3D microenvironments using flow cytometry. 100 µm
warrants further investigation.

To show the utility of our novel 3D vascularized tumor model for anticancer drug discovery, we tested the efficacy of a novel nanoparticle-mediated combination therapy using the models. As schematically illustrated in Figure 5.9B, the LC60S nanoparticles used are composed of a phospholipid (L) outer membrane and a fullerene (C60) core embedded in a matrix of mesoporous silica (S), which is also called EukaCell due to its resemblance to the configuration (membrane, cytoskeleton, and nucleus) in a eukaryotic cell. The nanoparticles are ~60 nm with a round morphology (Figures 5.9C) and have a negatively charged surface as indicated by the negative surface zeta potential of approximately -22.3 mV. The former is excellent for cellular uptake and passive targeting of tumor in vivo while the latter is important for the blood stability of the nanoparticles [213]. More importantly, the nanoparticles are highly efficient for encapsulation of DOX and indocyanine green (ICG) to form the LC60S-DI nanoparticles to enable combined chemo, photothermal, and photodynamic therapies for tumor destruction. The release of the encapsulated DOX can be controlled by irradiation of near infrared (NIR, 800 nm) laser through the absorption of the laser energy by ICG for heating inside the nanoparticles. To check the anticancer capability of the LC60S-ID nanoparticles on the most drug-resistant 3D vascularized tumors, we perfused/incubated them in the microfluidic device with the nanoparticles for 12 hours when the tumors were irradiated with NIR (at 1.5 W/cm2 for 1 min). The tumors were further cultured for a total of 4 days. As shown in Figure 5.9E, the LC60S-DI nanoparticle-mediated combination therapy is significantly more effective than the chemotherapy of free DOX alone for killing the tumor in terms of the DOX dose. The cell viability at the DOX dose of 10 µg/ml in nanoparticles is significantly lower than that
of the DOX chemotherapy alone. As shown in Figure 5.9F, the IC$_{50}$ significantly dropped by more than 16 times to ~2 µg/ml with the combination therapy. Although it is difficult to correlate the drug resistance of in vivo tumors with our 3D vascularized tumor, this model may be valuable for prioritizing candidate compounds for further development with in vivo studies.

5.4. Conclusion

In summary, we demonstrate the ability to form µtumor modules in core-shell microcapsules using the high throughput microfluidic technology. Since the biomechanical properties of the core-shell microcapsules can be readily modulated, we tested its effect on the proliferation and gene expression of the encapsulated cancer cells. Next, using the bottom-up tissue engineering principles we fabricated 3D vascularized tumor by assembling the core-shell microcapsules with endothelial and stromal cells. The method closely mimics the intricate 3D architecture of the in vivo microenvironment present in the tumor. We characterized the vasculature of tumor in our model by quantifying vessel structures and protein markers. Next, we tested the anticancer efficacy of free DOX and DOX encapsulated in nanoparticles (LC60S). We found that 3D vascularized tumor shows higher resistance to the free DOX compared to other traditional 2D and 3D models but can be overcome by our novel nanoparticles. The results support the importance of tumor microenvironment on the effectiveness of anticancer therapies. We believe, our biomimetic model may provide a valuable tool to answer many biologically relevant questions, such as the role of the microenvironment, cell-cell, and cell-ECM interactions in cancer progression and may lead to the identification of novel therapeutic drugs in much lower cost and shorter time than xenograft animal models.
Chapter 6: Multiscale system for injectable co-delivery of EGF and stem cells to treat ischemic diseases

6.1. Introduction

Ischemic tissue disease is one of the major causes of morbidity and mortality worldwide[11, 214]. For therapy of the ischemic tissue, efficient restoration of blood perfusion is crucial to prevent tissue necrosis[215, 216]. Unfortunately, due to the limitations of current therapeutic approaches, there is an urgent need for effective and safe therapies to regenerate or repair ischemic tissue quickly[217]. One of the most promising approaches is stem cell-based therapy[16, 218]. Stem cells can be used to restore tissue/organ function either as integrated participants in the damaged tissue/organ (direct differentiation) or as vehicles that deliver complex signals to a target tissue (cytokine effect) without actually integrating into the tissue itself[219, 220]. However, the current therapeutic efficacy of stem cells still remains inefficient owing to the poor survival of the implanted stem cells[221-224]. Only ~20% of implanted stem cells could remain in the originally injected tissue after 24 h, and it may decrease to ~3% after 30 days[225-228].

To address this issue, a rational way is to deliver stem cells within hydrogels to maintain the stem cells at the originally injected tissue. To promote the survival/proliferation of stem cells, maintaining a certain concentration of growth factor in the environment is necessary[229]. However, growth factors (e.g., epidermal growth
factor, EGF) are not stable \textit{in vivo} in general and can be easily degraded within a few hours\cite{230}. Nanoparticles have been widely used as the carrier to protect and deliver proteins or nucleic acids \textit{in vivo}\cite{188, 231}. Unfortunately, nanoparticles are most suitable for intracellular delivery because cells can take up nanoparticles directly. This compromises the function of growth factors (e.g., EGF) that function by interacting with their receptors on the cell plasma membrane (Figure 6.1A). To resolve this problem, a nano-in-micro capsule was developed for encapsulating EGF to achieve the long-term sustained release of the growth factor (Figure 6.1B). The EGF was encapsulated inside the nanoparticles first, and the EGF-laden nanoparticles were then encapsulated in the

\textbf{Figure 6.1. Drawbacks of nanoparticle-based protein delivery methods.} (A) To promote the cell proliferation, maintained certain amount of growth factors (e.g., epidermal growth factor, EGF) are important. However, those growth factors are not stable and can be degraded easily. Although the nanoparticles can help to protect the EGF by encapsulating inside the nanoparticles (NP-E), the cells normally uptake the nanoparticles directly which block the chance of EGF to bind with EGF receptor (EGFR) on the cell membrane. (B) The nano-in-micro system can help to overcome the drawback and achieve both controlled drug release and extracellular release.
microcapsules. Due to their large size (~300 µm), the nano-in-micro capsules cannot be taken up by cells while the nanoparticles inside the capsules can protect the EGF to achieve sustained release. Finally, we integrated the EGF-laden nano-in-micro capsules and human adipose derived stem cells (ADSCs) into collagen hydrogel for implantation to treat the ischemic injury.

6.2 Materials and methods

6.2.1. Materials
PLGA (lactide:glycolide: 75:25, Mw: 4,000-15,000) and Pluronic F127 (PF127) were purchased from Sigma (St. Louis, MO, USA). Polyvinyl Alcohol (PVA, Mw: 100 kDa) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Curcumin (MW: 368.38 Da) from turmeric (Curcuma longa) with purity ≥65% was purchased from Sigma (St. Louis, MO, USA) and used without further purification. Human EGF was purchased from Shenandoah Biotechnology (Warwick, PA, USA). The CCK-8 cell proliferation reagent was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Human adipose-derived stem cells (ADSCs) and culture media were purchased from Lonza (Walkersville, MD, US). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless specifically mentioned otherwise.

6.2.2. Fabrication of microfluidic device
The method for fabricating the non-planar microfluidic device was detailed elsewhere. Briefly, microfluidic channels were patterned on a silicon wafer by multilayer
photolithography technique. A 100 µm of a layer of SU8 2025 (MicroChem, MA) was first coated and then soft-baked, followed by exposure to ultraviolet (UV) light through the first shadow mask (Cad/Art services, OR) printed with the core channel. After a post-exposure baking, an additional layer (50 µm) of SU-8 photoresist was spun coated, soft baked, exposed with a second shadow mask to pattern shell channel. The third layer for oil/separation channel was similarly patterned. All three exposures were aligned using an EVG620 automated mask aligner. In the end, the SU8 pattern on the substrate was developed in the SU-8 developer (MicroChem, MA), rinsed with isopropyl alcohol, and dried using nitrogen gas. Microfluidic devices were then made by replica molding of polydimethylsiloxane, PDMS (Sylgard 184, Dow Corning, MI). The mixture of cross-linker and PDMS pre-polymer (1:10) was poured on the patterned silicon wafer. PDMS was cured at 65 °C for at least 3 hr and then carefully peeled off the wafer. Two PDMS slabs with the same channel design were then plasma-treated for 30 s using a Harrick PDC-32G plasma cleaner at 18 W and 27 Pa, wetted with methanol, aligned and bonded together under a microscope to produce the final microfluidic device. Assembled device was kept on a hotplate at 80 °C for ~10 min to evaporate residual methanol and further kept at 65 °C for 2 days to make it sufficiently hydrophobic for experiments.

6.2.3. Preparation of nanoparticles

The nanoparticles were prepared using an improved double emulsion (water in oil in water or W/O/W) method. Briefly, 10 mg of PLGA and 20 mg Pluronic F127 (PF127) with or without the desired amount curcumin was dissolved in 2 ml of methylene chloride and the PLGA solution together with 0.4 ml of deionized (DI) water containing Rhodamine B or EGF protein was transferred into a centrifuge tube where the two immiscible solutions
were emulsified by sonication for 1 min using a Branson 450 sonifier. Afterward, this initial emulsion and 4 ml of 2% polyvinyl alcohol (PVA) solution (in DI water) were emulsified by sonication for 2 min to obtain the double emulsion that was further processed by slowly dropping into 6 ml of 0.6% PVA (in DI water) and stirred for 10 min at room temperature. After rotary evaporation of the double emulsion to remove the organic solvent, the PLGA nanoparticles were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice with DI water.

6.2.4. Characterization of nanoparticles
The morphology of nanoparticles was characterized using both transmission (TEM) and scanning (SEM) electron microscopy. For TEM, the nanoparticles were negatively stained with uranyl acetate solution (2%, w/w) as detailed elsewhere and examined using an FEI (Moorestown, NJ, USA) Tecnai G2 Spirit transmission electron microscope. SEM experiments were conducted by depositing 10 µl of an aqueous suspension of the nanoparticles (1 mg/ml) on a freshly cleaved mica grid and allowing them to dry for 2 hr in air. A thin film of Au was then sputtered onto the nanoparticles on the substrate. Samples were imaged with an FEI NOVA nano400 scanning electron microscope.

6.2.5. Encapsulation of nanoparticles in microcapsules
To generate bead microcapsules (300-400 µm), the fluids in the core and oil channels were 2% purified sodium alginate containing nanoparticles and mineral oil infused with calcium chloride solution, respectively. The shell channel was blocked with no flow. For the extraction channel (from I4 entrance), 1% sodium carboxymethyl cellulose solution was used which was necessary for establishing a stable interface between the oil and aqueous
phases. All the solutions were sterile and buffered with 10 mM HEPES to maintain pH 7.2 before use. Solutions were injected into the microfluidic device using syringe pump (Pump 11 Elite, Harvard Apparatus) at room temperature (RT) to generate bead microcapsules in oil and then extract them into the aqueous phase. To form smaller bead microcapsules (100-200 µm), a microfluidic device with smaller core channels (dimensions mentioned in Figure 6.2) was fabricated. Flow rates for the core, oil, and aqueous separation flows were regulated as mentioned in Figure 5.2 to generate bead microcapsules of different sizes. Outlets of the device were connected to a 50 ml centrifuge tube containing isotonic mannitol solution. To generate core-shell microcapsules, fluid in the core and shell channel were 1% cellulose containing nanoparticles and 2% purified sodium alginate, respectively. The same procedure as that aforementioned for generating the bead microcapsules was used to generate the core-shell microcapsules. Flow rates for generating core-shell microcapsules of different sizes are provided in Figure 6.2, as well.

6.2.6. Drug encapsulation efficiency and in vitro drug release

Encapsulation efficiency (EE) of drugs using the nanoparticles was calculated by the following equation:

\[
EE = \frac{A}{B} \times 100\%
\]

Where, A represents the amount of drug retained in nanoparticles and B is the initial amount of drug fed for encapsulation. The amount of Rhodamine B and curcumin in the nanoparticles was determined using a Beckman Coulter (Indianapolis, IN, USA) DU 800 UV-Vis spectrophotometer based on their absorbance at 550 and 450 nm, respectively. The amount of EGF was determined by the human EGF Elisa Kit (Abcam, MA, USA).

To determine the drug release of Rhodamine B and curcumin in vitro, the nano-in-
micro capsules (BM@NPs-RC and CSM@NPs-RC) were reconstituted in mannitol solution or medium with FBS (5 ml). The samples were placed at 37 °C and stirred at 110 rpm. At various times, 100 µl of the sample was collected and the remaining dialysate replenished with the same amount of fresh medium. The concentration of the released Rhodamine B and curcumin in the removed dialysate was determined using UV-Vis spectrophotometry based on absorbance at 550 and 450 nm.

For EGF release, free EGF, NPs-EGF, and BM@NPs-EGF were placed in a medium with FBS (5 ml). At various times, 200 µl of the sample was collected and the remaining dialysate replenished with the same amount of fresh medium. The concentration of the released EGF in the removed dialysate was determined by the human EGF Elisa Kit (Abcam, MA, USA).

6.2.7. Characterization of nano-in-micro capsules

The morphology of nano-in-micro capsules was characterized using SEM. The samples were quickly frozen in liquid nitrogen and further freeze-dried for 24 h to obtain dry NP-RC@BM and NP-RC@CSM. A thin film of Au was then sputtered onto the samples on the substrate. Samples were imaged with an FEI NOVA nano400 scanning electron microscope. The distribution of nanoparticles within the capsules was studied using a Zeiss LSM 510 META confocal laser-scanning microscope, via visualizing the distribution of the fluorescence of Rhodamine B and curcumin inside the capsules.

6.2.8. Preparation of multiscale composite hydrogel system containing EGF and stem cells

After formation NP-E@BM, excess mannitol solution was removed by passing the sample
through 100 μm cell strainer. The neutralized collagen solution (formed by mixing stock collagen solution, 1N NaOH, 10X PBS, and DI water as per the manufacturer’s (BD Biosciences, San Jose, CA, US) instruction) was mixed with NP-E@BM or NP@BM + free EGF (0.5 μg ml⁻¹ EGF for all conditions), and ADSCs on ice to produce a final collagen concentration of 2.6 mg/ml excluding the 40% volume of bead microcapsules. The final density of ADSCs in collagen were 1.5 × 10⁶ cells/ml, respectively. The sample was carefully mixed for 1 min without introducing any air bubbles. Afterward, 20 µl of the sample were placed on a dish and allowed to gel at 37 °C in 5% CO₂ incubator to form the multiscale composite hydrogel system containing EGF and stem cells for in vitro studies. For the collagen hydrogel (CH) with ADSCs and NP-E, a similar method was used with the same final collagen concentration and cell density. For in vivo transplantation, all the samples were prepared as described above except that they were held at 4 °C on ice to prevent gelling before injection. 100 μl of the samples were injected around the cutting site.

6.2.9. Rheological characterization of CH (with or without NP-RC@BM), alginate (with or without NP-RC) and cellulose (with or without NP-RC)

Rheological measurements were carried out using a TA instrument AR-1000N rheometer with 40 mm cone or parallel plates. For alginate and alginate with nanoparticles, we prepared the gels on a PDMS mold and transferred them between parallel plates for the measurement. For solutions of sodium carboxymethylcellulose without or with nanoparticles, the rheological measurements were performed using cone plates. For CH and NP-RC@BM@CH, samples were prepared as aforementioned and placed between cone plates on the rheometer kept at 4 °C. The temperature was then raised to 37 °C for 30
min to crosslink collagen for further measurements. Stress sweeps at a constant frequency of 1 Hz were performed first to obtain the linear viscoelastic region. Frequency sweeps were then performed in the linear viscoelastic regime to determine values of the storage/elastic ($G'$) and loss/viscous ($G''$) moduli. The values at 1 Hz are reported in this study.

6.2.10. Cell culture and in vitro cell viability

Human ADSCs were cultured in ADSC™ Growth Medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO$_2$ incubator. For cell viability studies, ADSCs cultured in different composite hydrogels were transferred in 96-well plates. The cell viability was then evaluated using the CCK-8 cell proliferation reagent according to the instructions given by the manufacturer (Dojindo Molecular Technologies).

6.2.11. In vitro imaging

ADSCs cultured in different composite hydrogels were fixed with 4% paraformaldehyde first and then their actin stained with Alexa Fluor® 488 Phalloidin for 20 min. After incubated with DAPI for 10 min to stain the nuclei, the cells were moved on a cover slide for examination using an Olympus FluoView™ FV1000 confocal microscope. For cell uptake study, 2D cultured ADSCs were incubated with free Rhodamine B & curcumin, NP-RC, or NP-RC@BM at 37 °C for 3 hr. The cells were then fixed with 4% paraformaldehyde. The fixed cells were further treated with medium containing 75 nM LysoTracker Blue DND-99 to stain late endosomes/lysosomes for examination with an Olympus FluoView™ FV1000 confocal microscope.
6.2.12. Immunohistochemical staining

For staining of mouse CD31 (mCD31), human CD31 (hCD31), and human α-SMA (h-α-SMA), limbs were collected at 28 days after injection and put in frozen with the Tissue-Tek (Sakura Finetek, Torrance, CA, USA) O.C.T. Compound and Cryomold at -80 °C for 24 hr. The limbs were then cut into slices of 10 µm thick using a cryo-microtome and transferred onto microscope slides. The slides were incubated in 3% BSA in 1× PBST (1× PBS and 0.05% Tween 20) at room temperature for 1 hr to block potential non-specific binding, followed by overnight incubation at 4 °C with mCD31 (Abcam, Cambridge, MA, USA). The samples were then washed 3 times and incubated in dark at room temperature for 1 hr with Rhodamine B labeled secondary antibodies (Abcam, Cambridge, MA, USA) diluted in 1% BSA in 1× PBST (1:50 dilution). Afterward, the samples were incubated with hCD31 (R&D Systems, Minneapolis, MN, US) at 4 °C overnight. The samples were then washed 3 times and incubated in dark at room temperature for 1 hr with FITC labeled secondary antibodies (Abcam, Cambridge, MA, USA) diluted in 1% BSA in 1× PBST (1:50 dilution). Finally, the samples were washed and further stained for nuclei using Hoechst 33342 (5 µM) for further examination using an Olympus FV1000 confocal microscope.

6.2.13. FluoroSpot assays for IL-2 and INF-γ

A dual color Fluorospot assay (R&D Systems, Minneapolis, MN, US) was used to detect the secretion of IL-2 and INF-γ by ADSCs cultured in A@CH, NP-E&A@CH, NP@BM&E&A@CH and NP-E@BM&A@CH in the specialized FluoroSpot assay plate, according to the manufacturer's instructions. Briefly, 100 µl of 35% alcohol was added into each well of the specialized plate to activate the Polyvinylidene difluoride (PVDF)
membrane. Then, 100 µl of diluted (1:50) capture antibodies was added to each well and incubated overnight at 4 °C. After washing with PBS, 200 µl of blocking buffer was added to each well and incubated for 90 min at room temperature. @CH, NP-E&A@CH, NP@BM&E&A@CH and NP-E@BM&A@CH cultured for different times were transferred into each well and incubated at 37 °C in a humidified 5% CO2 incubator for 24 hr. Afterward, we added IL-2 detection antibody into each well overnight at 4 °C, and then added INF-γ detection antibody and incubated at room temperature for 2 hr. Finally, the northernLights fluorescence enhancer solution was added to each well and incubated for 15 min at room temperature. The fluorescence images were taken using a Zeiss LSM 510 META confocal laser-scanning microscope to examine the number of fluorescence spots.

6.2.14. ELISA analysis of VEGF and TGF-β
To detect VEGF and TGF-β by ELISA (Novex™, Frederick, MD, US for VEGF and Abcam, Cambridge, MA, US for TGF-β), the experiment was conducted according to the respective manufacturer's instructions. Briefly, for VEGF, 50 µl of the incubation buffer was added to all wells except that for chromogen blanks. For samples and controls, 50 µl of standard diluent buffer was mixed with 50 µl of sample or controls and added to the appropriate wells. Then, the plate was incubated for 2 hr at room temperature. After adding 100 µl of human VEGF-biotin conjugate solution into each well for 1 hr at room temperature, 100 µl of streptavidin-HRP was added to each well except the chromogen blanks. The plate (with plate cover) was incubated for 30 min at room temperature. Afterward, 100 µl of stabilized chromogen was added into each well. Finally, 100 µl of stop solution was added to each well to stop the reaction. The plate was then measured for absorbance at 450 nm using a BioTek Synergy HT multi-detection microplate reader.
For TGF-β, 100 µl of each standard and samples were added to appropriate wells. After incubating overnight at 4 °C with gentle shaking, 100 µl of 1 × biotinylated TGF-β detection antibody was added to each well and incubated for 1 hr at room temperature with gentle shaking. Then, 100 µl of 1 × HRP-streptavidin solution was added to each well and incubated for 45 min at room temperature with gentle shaking. Afterward, 100 µl of TMB one-step substrate reagent was added to each well and incubated for 30 min at room temperature in the dark with gentle shaking. Finally, 50 µl of stop solution was added to each well to stop the reaction and absorbance at 450 nm was measured immediately as aforementioned.

6.2.15. Statistical analysis

All data are reported as mean ± standard deviation (SD) from at least three independent runs. The Kruskal-Wallis H-test and the Mann-Whitney U-test were used to assess the overall among-group and two-group differences, respectively. Statistical analyses were carried out with the IBM SPSS 22 software. In all cases, a p value less 0.05 was considered to be statistically significant.

6.3. Results and discussion

6.3.1. Generation of nano-in-micro capsules and formation of the multiscale delivery system

To generate the nano-in-micro capsules, we first prepared nanoparticles with Pluronic F127 (PF127) and poly (lactic-co-glycolic acid) (PLGA) using an improved double emulsion
method for encapsulating both the hydrophobic and hydrophilic drugs[232-234]. For visualization and characterization of the nanoparticles, we encapsulated two fluorescent dyes, Rhodamine B (R, hydrophilic) and curcumin (C, hydrophobic) in the nanoparticles. The amount of Rhodamine B and curcumin in nanoparticles was controlled to be the same.

**Figure 6.2. Fabrication of nano-in-micro system.** (A) A schematic illustration of the nanoparticles for encapsulating both hydrophilic and hydrophobic drugs (NP-RC, Rhodamine B: R or Rho B; curcumin: C or Cur). Typical transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images showing the uniform size, core-shell morphology. (B) A schematic view of the microfluidic systems used for producing NP-RC encapsulated beads microcapsules (NP-RC@BM) and NP-RC encapsulated core-shell microcapsules (NP-RC@CSM). (C) Fluorescence images of both Rho B and Cur shows its successful encapsulation into the microcapsules. NP-RC were distributed in the whole BM while only the core of the CSM.
for characterization. As shown in **Figure 6.2A**, Rhodamine B and curcumin were encapsulated in the hydrophilic core and hydrophobic shell of the nanoparticles (NP-RC), respectively. Typical transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images showing a uniform size, spherical morphology, and core-shell structure of the NP-RC are given in **Figure 6.2A**. The nano-in-micro capsules were prepared by using microfluidic flow-focusing devices (**Figure 6.2B**), similarly to what we did before[46, 198]. The NP-RC can be distributed in the whole bead microcapsule (NP-RC@BM) or only in the core of core-shell microcapsules (NP-RC@CSM) by using different devices. Alginate was used to fabricate the bead microcapsules (BMs) or the shell of the core-shell microcapsules (CSMs) because of its biocompatibility and reversible gelation with divalent cations such as Ca$^{2+}$ under mild conditions that are not harmful to living cells[4, 27, 28, 235, 236]. Cellulose was used as the core solution of the NP-RC@CSM[46]. Under the microscope, both the red fluorescence of Rhodamine B and green fluorescence of curcumin were co-located and distributed homogeneously in the BMs or the core of CSMs (**Figure 6.2C**). However, when we used microcapsules to encapsulate the free Rhodamine B directly (curcumin is hydrophobic that cannot be encapsulated directly in the hydrogel), all the Rhodamine B was released from microcapsules into mannitol solution as soon as we collected microcapsules. This is not surprising as the pore size of the microcapsules is much bigger than the size of Rhodamine B[200, 237].

Furthermore, we could create nano-in-micro capsules of various sizes (100-500 µm in diameter) with low polydispersity (**Figure 6.3 A and B**), by tuning the flow rates of oil and aqueous core and/or shell flows, and the geometry of the microfluidic channel. We
could also accurately control the drug concentration in the nano-in-micro capsules (Figure 6.3 C and D). During the formation of nano-in-micro capsules, isotonic mannitol solution was used for collection and purification of the capsules. We found that the amount of Rhodamine B or curcumin in the mannitol solution was negligible. Therefore, independent of the concentration of drug in the capsules, the encapsulation efficiency (EE) of the nanoparticles in the microcapsules was 100%. The morphology of the nano-in-micro capsules was determined by using SEM. For NP-RC@BM, the size was ~150 µm after drying (Figure 6.4A). Inside the capsules, we could see the alginate matrix, as well as the
nanoparticles. The core-shell structure of NP-RC@CSM could be readily identified in the SEM image and nanoparticles could be seen inside the core of the capsules (Figure 6.4B).

We next investigated the stability and drug release behavior of the nano-in-micro capsules in cell culture medium. Both NP-RC@BM and NP-RC@CSM were stable in the medium. The fluorescence intensities of Rhodamine B and curcumin in NP-RC@BM and NP-RC@CSM gradually decreased in the capsules and increased in the medium with time, showing apparent and gradual drug release from the nano-in-micro capsules in cell culture medium. Overall, the drug release kinetics of NP-RC@BM was slower than NP-RC@CSM.
in both mannitol solution and medium. Since our goal was to achieve sustained release of protein for therapy of the ischemic tissue, we utilized NP-RC@BM of ~ 300 µm for both in vitro and in vivo studies. It is worth noting that the nano-in-micro capsules could be freeze-dried for long-term storage without altering its morphology after rehydration.

We further integrated the nano-in-micro capsules and stem cells into collagen hydrogel to form a composite system for stem cell delivery. The structures of both the pure collagen hydrogel (CH) and NP-RC@BM assembled in CH (NP-RC@BM@CH) are shown in Figure 6.5A. Interestingly, the NP-RC@BM@CH had a more heterogeneous microstructure that pure CH, which should make the CH stronger and more stable. Indeed, both the storage ($G'$) and loss modulus ($G''$) moduli of NP-RC@BM@CH were much higher than that of CH (Figure 6.5B). However, the nanoparticles have no significant impact on the mechanical properties of alginate hydrogel or cellulose solution in the nano-in-micro capsules.

### 6.3.2. Sustained EGF release to promote stem cell proliferation in the multiscale system in vitro

To assess the ability of the nano-in-micro capsules and CH in supporting cell growth in vitro, EGF was encapsulated (similarly to Rhodamine B) in the nano-in-micro capsules
with an EE of ~74%. The EGF-laden nano-in-micro capsules and adipose-derived stem cells (ADSC) were then assembled together in CH by simply mixing. We hypothesize that the EGF could release slowly from the nano-in-micro capsules and bind to the EGFR on the plasma membrane of ADSCs (Figure 6.6A). Meanwhile, the CH and the alginate shell of the microcapsules could provide a 3D scaffold for cell adhesion and proliferation (Figure 6.6A). To test our hypothesis, we first encapsulated both Rhodamine B and EGF in the nano-in-micro capsules for visualization. After 12 hr of culture, cells were stained with green fluorescence (Alexa Fluor® 488 Phalloidin) to show that they have spread and grown within the spaces between the capsules, while all the nanoparticles (containing Rhodamine B with red fluorescence) were located inside the microcapsules (Figure 6.6B). Importantly, we could see some red fluorescence of Rhodamine B in the ADSCs, indicating the release of Rhodamine B from the nano-in-micro capsules.

To understand the composite hydrogel of EGF-laden nano-in-micro capsules and ADSCs in CH (NP-E@BM&A@CH) on cell proliferation, it was compared with ADSCs in CH (A@CH), EGF-laden nanoparticles and ADSCs in CH (NP-E&A@CH), empty nano-in-micro capsules mixed with free EGF and ADSCs in CH (NP@BM&E&A@CH).
As shown in Figure 6.7A, phalloidin (green) stained ADSCs in A@CH and NP-E&A@CH groups were mainly located on the surface of the scaffolds after one-day culture. The CH shrank more than 8 times after one-day culture, which may limit the diffusion of nutrients into the interior of the CH. However, the CH with nano-in-micro capsules did not shrink much and the cells were observed throughout the CH (Figure 6.7A). Overall, more cells were observed inside the NP-E@BM&A@CH than the CH of the other three conditions at days 6 and 9 (Figure 6.7A-B). Figure 6.7C shows the sustained release of EGF from the
NP-E@BM, while free EGF degraded quickly in the medium. Although a sustained release of EGF could also be achieved by using the nanoparticles alone, the ADSCs could take up the nanoparticles directly to minimize the binding of EGF to the EGFR on the cell surface. Indeed, when we cultured the ADSCs with NP-RC and NP-RC@BM, most of the NP-RC were observed to co-localize with the lysoTracker that stains lysosomes/endosomes, indicating that the nanoparticles were taken up by the cells via endocytosis (Figure 6.7D). In contrast, the fluorescence stains of cells treated with NP-RC@BM were similar to that of free Rho B & Cur, indicating the Rhodamine B and curcumin were released from the nano-in-micro capsules before their interactions with the cells. These data indicate both maintaining the initial porosity of the CH by the nano-in-micro capsules and the sustained release of growth factor (EGF) from the capsules promote cell adhesion and proliferation inside the CH.

6.3.3. Augmented therapy of ischemic limb with the multiscale system in vivo

The murine hindlimb ischemia model was used to evaluate the capability of the multiscale system for co-delivery of EGF and ADSCs as a therapy for ischemic diseases. The ischemia was induced by unilateral femoral artery ligation as shown in Figure 6.8A. A laser doppler perfusion imaging (LDPI) system was used to non-invasively quantify blood perfusion in the ischemic limbs. The blood perfusion in both limbs was normal before surgery, and it was reduced dramatically in the right limb immediately after the induction of ischemia on day 0 (Figure 6.8B). After confirmation of successful surgery, a total of 100 µl of saline, simple mixture of nanoparticles/microcapsules in CH (BM&NP@CH), NP-E@BM in CH (NP-E@BM@CH), NP@BM with free EGF and ADSCs in CH (NP@BM&E&A@CH),
NP-E with ADSCs in CH (NP-E&A@CH), and NP-E@BM with ADSCs in CH (NP-E@BM&A@CH) treated mice show better recovery of blood perfusion. (C) Quantification data from the LDPI images showing the blood perfusion in NP-E@BM&A@CH treated mice are significantly higher than other groups after day 7. Error bars represent ± s.d. (n = 5). **p < 0.01, *p < 0.05 (D), H&E staining and average muscle fiber area (E) of the mice with different treatments showing the restoration of blood perfusion after 4 weeks without compromising the host muscle fibers. Error bars represent mean ± s.d. (n = 50). **p < 0.01, *p < 0.05 (Kruskal-Wallis H-test). (F) Confocal images of actin and fibronectin of mice with different treatments showing the NP-E@BM&A@CH treated mice have longer and larger muscle fiber. (G) H&E staining of the injected area of different treatments showing the newly formed blood vessels only can be observed in the NP-E@BM&A@CH treated mice.
E@BM&A@CH), were evenly injected into the ligation area at five different locations immediately. The LDPI images were taken at different days for all the groups, which shows that the restoration of blood perfusion in the hind limb was faster in mice with the NP-E@BM&A@CH treatment than all the other treatment (Figure 6.8B). This difference was further found to be statistically significant after day 7 (Figure 6.8C).

Since the induction of ischemia in the hind limb can lead to remarkable muscle degeneration[238], we next checked the hematoxylin and eosin (H&E) staining of the limb muscle. The result shows that the muscle fibers became largely disconnected in all the groups except the NP-E@BM&A@CH group (Figure 6.8D). The mean muscle fiber area (Figure 6.8E) determined from the H&E images and the staining of actin and fibronectin (Figure 6.8F) also show that the NP-E@BM&A@CH treatment resulted in the longest and largest muscle fibers. Interestingly, we also found that blood vessels were newly formed at the injection area for the NP-E@BM&A@CH treated group (Figure 6.8G). More importantly, red blood cells could be seen in the newly formed vessels, indicating the blood perfusion in the vessels (Figure 6.8G). Equally important, we did not notice any apparent side effect of the NP-E@BM&A@CH treatment. No mice died during the entire experiment. Major organs from mice in the NP-E@BM&A@CH group were collected on day 28 for histology analysis. No obvious damage to the critical organs was observable in the H&E stained tissue slices. These results indicate the excellent safety of the NP-E@BM&A@CH treatment in vivo.
6.3.4. Mechanisms of the multiscale system for augmented therapy of ischemic limb *in vivo*

Having shown the newly formed blood vessel in the H&E staining, we next sought to investigate the origin of the new blood vessels. As the ADSCs used in this study is isolated from normal (non-diabetic) human adult lipoaspirates collected during elective surgical liposuction procedures, we labeled both human CD31 (hCD31) and mouse CD31 (mCD31) in the sections to identify whether the newly formed blood vessels were formed due to differentiation of the implanted human ADSCs[239, 240]. The fluorescence images show that the NP-E@BM&A@CH treated mice indeed have blood vessels that could be stained with hCD31, which was not observed for the other groups (*Figure 6.9A*). In order to further confirm this result, we labeled the ADSCs with CellTracker™ CM-DiI Dye for implantation. In saline, BM&NP@CH, NP-E@BM@CH, NP@BM&E&A@CH, and NP-E&A@CH treated groups, we did not observe any red fluorescence of the DiI dye at 28 days after implantation. In contrast, we saw some DiI-labeled ADSCs around blood vessels in the NP-E@BM&A@CH treated mice (*Figure 6.9B*). Furthermore, these blood vessels could be labeled with hCD31 and human α-SMA (h-α-SMA), further indicating that the blood vessels were newly formed and matured from the implanted ADSCs (*Figure 6.9B*)[241]. As a control, we also saw some blood vessels in NP-E@BM&A@CH treated mice that were not stained by hCD31 and h-α-SMA, which should be the native blood vessels in mouse (*Figure 6.9B*).

The H&E staining of the limb tissue injected with DiI-labeled ADSCs also shows that only NP-E@BM&A@CH treated group has strong fluorescence of DIL tracking dye (*Figure 6.9C*). Interestingly, we could see that the cells with fluorescence were in two
different stages. First, the red fluorescence stained in few blood vessels suggesting that the
implanted ADSCs differentiated into epithelial cells and contributed to the blood vessel formation. Second, the red fluorescence was observed in the hydrogel liked area suggesting some of the implanted ADSCs stayed within the hydrogel (Figure 6.9C). As stem cells can repair/regenerate damaged tissue/organ by either direct differentiation or indirectly by delivering regenerative signals, we further checked the secretion of cytokines from the ADSCs[242, 243]. Dual cytokine fluorospot analysis of human IL-2 and INF-γ was used to determine the immune regulation of ADSCs. As shown in Figure 6.9D-E, the NP-E@BM&A@CH has significantly higher production of the IL-2 and INF-γ than the other groups starting from day 14. These cytokines are reported to be involved in the regeneration process[244, 245]. Besides immune response, the stem cells also can secrete cytokines to promote the repairmen of the injured tissue [242, 246]. Indeed, the ELISA results of both vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β) indicated their elevated levels in NP-E@BM&A@CH, which are important for blood vessel formation (Figure 6.9F). Based on these results, we could conclude that the implanted ADSCs in NP-E@BM&A@CH can participate in the formation of new blood vessels by direct differentiation and modulate the microenvironment in the ischemic tissue by secreting cytokines and growth factors to promote blood vessel formation. The main challenge of using stem cells is to keep them alive and functional after implantation, which can be resolved by our multiscale composite delivery system.

6.4. Conclusions
In summary, we have created a multiscale composite system consisting of ADSCs, nano-in-micro capsules, and collagen hydrogel. The biodegradable collagen hydrogel served as the carrier of both cells and nano-in-micro capsules to maintain them at the injecting area.
The nano-in-micro capsules were used to achieve sustained release of the EGF in the extracellular space, which enables its interaction with the receptors on cell membrane for proper function. This system can be used to overcome the drawback of nanoparticles based system that is usually for intracellular delivery. In addition, the nano-in-micro capsules can minimize the shrinkage of the collagen hydrogel, allowing for cellular growth in the hydrogel. The ADSCs delivered in the composite system were found to contribute to the formation of new blood vessels \textit{in vivo} via direction differentiation to endothelial cells. Moreover, the ADSCs secreted cytokines that further promote regeneration and healing of injury in the ischemic hind limb. Ultimately, the co-delivery of EGF and ADSCs with the multiscale composite system leads to complete restoration of blood perfusion after 4 weeks without compromising the host muscle fibers. Collectively, the present work provides a new strategy for stem cell delivery with great potential for treating ischemic diseases.
Chapter 7: Conclusions and prospective

The main objective of tissue engineering is to construct living tissues that can be used to replace or repair damaged tissue or organs. This is to address the critical gap between the growing number of patients and the limited availability of tissues and organs for transplantation. However, since the last decade or so, only a few tissue engineered products have been approved by US Food and Drug Administration (FDA). As discussed in Chapter 1, scalable/high-throughput 3D biomimetic culture systems, ability to control cellular proliferation and differentiation, sustainable delivery of growth factors and cells after transplantation could facilitate the wide application of tissue engineering.

Employing stem cells as a cell source is an important aspect in tissue engineering/regenerative medicine since they can be programmed to differentiate into any cell type. However, large quantities of cells with high pluripotency is essential for any tissue engineering applications. In Chapter 2, development of a novel non-planar microfluidic device that enables high-throughput encapsulation of pluripotent stem cells in 3D biomimetic core-shell microcapsules is discussed. It was shown that the miniaturized biomimetic culture maintains high pluripotency of pluripotent stem cells, which resulted in efficient differentiation towards cardiac lineage when guided with a small molecule (Cardiogenol C). This demonstrated the importance of our miniaturized 3D culture platform. However, the reasons for high pluripotency and differentiation potential of the stem cells cultured in the 3D biomimetic microcapsules warrant more investigation. It is
possible that cytokines and growth factors secreted by the encapsulated cells are present in increased concentration within the microcapsules which otherwise dilutes very fast in the bulk medium as in the case of traditional 2D cultured cells. Presumably, this increased autocrine and paracrine signaling may affect the “stemness” of the encapsulated cells. For example, a recent study where the mESCs were cultured in a microfluidic chamber with minimal perfusion demonstrated that the endogenous leukemia inhibitory factor (LIF) and other growth factors produced by the cells alone can help maintain stem cell properties [247]. The microfluidic system developed in this study has the ability to address limited availability of highly pluripotent stem cells for tissue engineering purposes. However, future work would entail encapsulation and characterization of embryonic stem cells from human sources. In Chapter 3, the application of core-shell microcapsules for assisted reproduction were explored. By employing the microfluidic platform, the ovarian microtissue that recapitulates the 3D mechanical, physiological, and anatomical milieu in the ovary was engineered. With these biomimetic ovarian microtissues, the important role of mechanical heterogeneity in the ovary in regulating follicle development and ovulation was revealed. It was demonstrated that not only the biomechanical properties of the core of the microcapsules but the shell is equally important for the development of preantral follicles. The potential future application of such a system could be to study the role of the microenvironment in diseases such as premature ovarian failure (POF) and polycystic ovary syndrome (PCOS). This could be done by designing the shell of the microcapsules that correlate/mimic the mechanical properties of the cortex in POF and PCOS patients. Then, in Chapter 4, development of a more efficient method to study the role of the microenvironment in cellular/tissue proliferation and development is discussed. It was
shown that by using microfluidic platform, core-shell microcapsules with different core extracellular matrix (ECM) with varying adhesion ligands and mechanical stiffness to investigate its role in proliferation, differentiation, and development of stem cells/mouse preantral follicles. It was shown that the embryonic stem cells maintain high pluripotency in soft/liquid core ECM compared to stiff core ECM. Optimization of the ECM for culture and development of preantral follicles to the antral stage was also performed. Developed antral follicles could be used to obtain MII oocytes after \textit{in vitro} maturation. Furthermore, MII oocytes can be fertilized to form 2 cell stage embryos. The results are significant in restoring/preserving the fertility of women. However, further research is required to optimize protocols to increase the efficiency of obtaining MII oocytes and ultimately transplantable blastocysts before it can be used in humans.

In Chapter 5, encapsulation of human breast cancer cells in core-shell microcapsules to form microtumors is discussed. The interactions of breast cancer cells and stromal cells with surrounding extracellular matrix have been shown to play a crucial role in angiogenesis and thus, responsible for tumor growth and metastasis. The extracellular matrix was also reported to support the unique patterns of vascular morphogenesis of endothelial cells \cite{248}. Thus, the tumor microenvironment of breast tumor is essential for the breast cancer pathogenesis. One essential component of this microenvironment is the extracellular matrix, which includes a complex network of macromolecules with diverse physical, chemical, and mechanical characteristics \cite{249}. The composition and physio-chemical properties of extracellular matrix is precisely regulated during embryonic development, but it gets deregulated during the process of carcinogenesis \cite{249}. Therefore, in this study the effects of different stiffness and adhesion
molecules on cancer cell proliferation and gene expression were tested. Next, these microtumors were used as building blocks to generate a 3D vascular architecture. A 3D vascularized tumor was fabricated by assembling microcapsules with stromal cells in a microfluidic perfusion device. The ability of this system is also to study direct or indirect cell-cell interactions between cancer cells and stromal cells and their role in vascular formation. The ability of breast cancer cells to induce vascularization was found to be increased substantially when alginate was removed. Thus, this study suggests that there must be some direct cell-cell interactions occur between cancer and stromal cells beside the indirect communications that enhances vascular formation which can subsequently facilitate tumor growth and metastasis. Recent experimental evidences indicate a critical role of not only stromal cells but also parenchymal cells from tissue where benign tumors form [250]. The tumor microenvironment was also reported to play an essential role in regulating neighboring cells behaviors [250]. The accumulating experimental evidences also suggested that angiogenesis is an important characteristic of tumor growth and metastasis. The process of angiogenesis is initiated by cancer cells through a wide range of cell-cell communications with nearby stromal cells [251]. It has been reported that cancer cells can induce the changes in behavior of endothelial cells by directly targeting them via diverse signaling pathways that include soluble factors, gap junctions, vesicles and adhesion receptors [251]. The endothelial signaling pathways can also be activated in an indirect way by different means, e.g., secreting proteases into the extracellular space, activating stromal cells, or even modulating the temperature, pH and availability of nutrients and oxygen [251]. Further studies are warranted to find out the importance of each of these factors in facilitating vascularization and tumor growth and metastasis.
Lastly, this study reported that the human breast cancer cells cultured in 3D core-shell microcapsules are more resistant to anticancer drug. Moreover, this drug resistance is further increased in the case of 3D vascularized tumor. It has been reported that tumor angiogenesis appears to be a significant factor in defining the therapeutic efficiency and also patient prognosis. The already published results on tumor angiogenesis evidently shows that most solid tumors are heterogeneously vascularized/oxygenated to contain hypoxic regions [252]. These hypoxic areas were also shown to affect the response of cancer patients to a variety of anti-cancer drugs. Thus, the measurement of hypoxic regions and oxygen tensions will also need to be done in order to evaluate the efficiency of different anti-cancer drugs in highly vascularized tumors. The work also reported that encapsulated cells in 3D microcapsules are composed of increased population breast cancer stem cells, which may contribute their enhanced drug resistance. However, further research is warranted to study the exact and detailed molecular mechanisms responsible for the increased drug resistivity. For example, it is possible that improved secretion of ECM proteins from the cancer/stromal cells and increased autocrine and paracrine signaling between the cells lead to enhanced drug resistance. Therefore, the 3D vascularized tumor can be utilized as a much better platform for drug screening than the conventionally used 2D cells. Furthermore, the developed system has the potential to be utilized to fabricate various 3D vascularized tumor tissues (solid tumor: breast, liver, lung, pancreatic) using cells obtained directly from cancer patients for personalized drug screening. Based on the findings here, microfluidic systems can also be made for anticancer drug testing even in non-solid tumor, such as leukemia by encapsulating leukemic cells in core-shell microcapsules with an aqueous core, since leukemic cells are naturally grown in
suspension. Lastly, the inherent cost effectiveness (PDMS-based microfluidic devices can be mass-produced in pennies with low consumption of medium and drugs) and high throughput nature of microfluidics can drive to significantly reduce the cost of healthcare.

In addition to using the core-shell microcapsules in regenerative medicine, ART, and cancer drug screening, in Chapter 6, an attempt to solve two major problems that currently persists in regenerative medicine was done: low retention/survival of transplanted cells and rapid degradation of proteins/small molecules. To overcome these problems, a novel nano-in-micro system to encapsulate epidermal growth factor (EGF) was developed. The encapsulation of EGF laden nanoparticles in alginate microcapsules enable its sustained release. Further, it prevents direct cellular uptake of the nanoparticles which is important since EGF and many other proteins interacts with its receptor on the cell surface to perform normal function. Furthermore, a novel multiscale composite hydrogel system was developed that was composed of nano-in-micro capsules and collagen ECM for the co-delivery of EGF and stem cells to treat ischemic limb in mice. The delivery system enables high proliferation and viability of cells in vitro as well as in vivo. The system developed here can be used to deliver a wide variety of therapeutic proteins/drugs/cells to treat many life-threatening diseases.

To conclude, in this dissertation novel biomaterial systems involving microencapsulation of cells were developed. Various applications of microencapsulation were shown in stem cell-based tissue regeneration, assisted reproduction, and cancer drug screening with promising results. Without doubt, the studies conducted here are only a small part in the field of tissue engineering. Building on these findings, hopefully the
biomaterial systems can be further developed for therapy and/or diagnosis of diseases in the clinic in the near future.
References


**Appendix 4**: p. Appendix 4I.


Appendix: Fabrication of the non-planar microfluidic device

Schematic of the fabrication process to make patterned silicon (Si) wafer. First, a positive photoresist is spin coated on the Si wafer. After the UV exposure and development, Si wafer is etched using CF$_4$ and O$_2$ plasma. After the etching, S1813 is dissolved by a methanol wash and dried. Next, SU8-2025 is spin coated on the wafer followed by baking. The shadow mask having core channel is aligned on top of etched marks and then exposed to the UV light. After the post-exposure bake, a new layer of SU8-2025 is coated on the Si wafer. Then, a second shadow mask with shell channel is aligned and exposed again to the UV light. After the post-exposure bake, the third layer of SU8-2025 is coated and exposed.

Figure A.1 Schematic of fabrication process to make complete microfluidic device. Alignment marks are first etched on the silicon wafer. Multi-layer photolithography is performed to fabricate silicon master mold. Soft-lithography followed by device alignment is done to get final device in PDMS.
to UV light through the shadow mask having oil channel. After the final bake, the Si wafer is developed. Then using soft lithography technique, a negative of Si wafer is imprinted upon the PDMS. Finally, the channels on two identical PDMS slabs are cleaned, O₂ plasma treated, and aligned as shown in above figure to fabricate a non-planar microfluidic device for cell encapsulation.