Development and evaluation of a novel approach to producing uniform 3-D tumor spheroid constructs

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Abstract

Producing three-dimensional (3-D) multicellular tumor spheroids (TSs) is valuable for characterizing anticancer drugs since they provide a stronger and more reliable model of the 3-D \textit{in vivo} tumor than a conventional two-dimensional (2-D) monolayer culture. The intercommunication of tumor cells with the extracellular matrix (ECM) in a 3-D culture environment is more similar to a tumor \textit{in vivo} than in a 2-D environment. Further, we can see that such cell-cell and cell-ECM interaction can influence cell behaviour, such as in response to drug treatment.

\textit{In vitro} tumor spheroid models have been developed using microfluidic systems to generate 3-D hydrogel beads containing components of alginate and ECM protein, such as collagen, with high uniformity and throughput. During bead gelation, alginate acts as a fast gelling component helping to maintain the spherical shape of beads and to prevent adjacent or underlying beads from coalescing when working with the slower gelling temperature and pH-sensitivity of collagen type I components. There are also well-known limitations in using microfluidic systems when working with temperature-sensitive components of collagen type I, and it is determined that to produce uniform hydrogel droplets through a microfluidic system, the mixtures must be homogeneous. However, the issue of collagen’s sensitivity to temperature causes concern for chunks of collagen gel inside of the mixture before bead encapsulation; therefore causing the mixture to become non-uniform and risking chip clogging. In order to overcome this limitation, previous approaches have used a cooling system during bead encapsulation while tumor cells were also present in the mixture, but this procedure assisted in postponing collagen gelation prior to bead production and potentially contributing to a delay in cell proliferation.
Here a novel yet simple method is developed to prepare homogeneous pre-bead-encapsulation-mixtures containing collagen type I through ultrasonication, while extending cell viability and proliferation. This method allows the cultivation of homogenous TS cultures with high uniformity and compact structure, and not only maintains cell viability but also stimulates the proliferation of cells in alginate/collagen hydrogel bead cultures. Depending on the sonication parameters, time and temperature, gelation of collagen is controlled by small sized fibrils to thick fibers. Here, the mixtures containing collagen are assessed for morphology of collagen fibers/fibrils, cell viability, and proliferation. Human source Michigan Cancer Foundation-7 (MCF-7) cells isolated from a breast cancer cell line are successfully incorporated into alginate/collagen mixtures, followed by sonication, and then bead production. After bead gelation, the encapsulated MCF-7 cells remained viable and proliferated to form uniform TSs when the beads contained alginate and collagen. Results indicate that ultrasound treatment (UST) provides a powerful technique to change the structure of collagen from fiber to fibril, and to disperse collagen fibers in the mixture homogeneously for an application to generate uniform hydrogel beads and spheroids while not disturbing cell proliferation.
Preface

Chapter two is based on work conducted at the BioMEMS lab by Solmaz Karamikamkar under the supervision of Dr. Karen C. Cheung. I was involved on all aspects of the project including the designing of the experiments, conducting experiments, data processing, image and data analysis, and writing of the results. A version of this chapter will be submitted for publication in a journal.

Chapter three is based on work conducted at the BioMEMS lab by Solmaz Karamikamkar under the supervision of Dr. Karen C. Cheung. I was involved on all aspects of the project including the designing of the experiments, conducting experiments, data processing, image and data analysis, and writing of the results. A version of this chapter will be submitted for publication in a journal.
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<th>Definition</th>
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<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7 Cell Line</td>
</tr>
<tr>
<td>TS</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>3-D</td>
<td>Three-Dimensional</td>
</tr>
<tr>
<td>2-D</td>
<td>Two-Dimensional</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>HMT-3522</td>
<td>Nonmalignant Human Breast Epithelial Cell Line</td>
</tr>
<tr>
<td>p-HEMA</td>
<td>Poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>UST</td>
<td>Ultrasound Treatment</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human Liver Carcinoma Cell Line</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>Alexander Hepatoma Cell Line</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly-(dimethylsiloxane)</td>
</tr>
<tr>
<td>hMSC</td>
<td>Human Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Buffered Salt Solution</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td>SHG</td>
<td>Second Harmonic Generation</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmitted Electron Microscopy</td>
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Dedication

To my dearest mom who acts as both father and mother physically and mentally all throughout my life when my father could not be with us physically, and my wonderful father who is always with me spiritually.

To my amazing husband who always supports me unconditionally and guides me academically no matter what, for his presence and great attention.

To my kindest sister who always makes my days fun and memorable ever from far away in back-home Iran.
1 Introduction and Overview

Throughout the past century, researchers have endeavoured to mimic tumor tissue in vitro for drug screening before clinical trial. Among in vitro screening tests, the tumor spheroid model has increasingly received attention for anti-cancer drug screening to eliminate poor drug candidates at preclinical trials and to acquire Food and Drug Administration (FDA) approval. In the tumor spheroid model, specific characteristics of spheroids are of great importance in determining their response to anti-cancer drugs. One of their most imperative characteristics is their size, which plays a substantial role in drug response due to chemical gradients in tumor spheroid cultures\(^1\). Spheroid cultures develop chemical gradients involving oxygen, catabolites, and nutrients at diameters between 200 and 500 µm, where necrosis typically occurs when spheroid sizes surpass 500 µm\(^2\). The small spheroids (<150 µm) exhibit a different expression profile as compared to the large spheroids (>400 µm) due to their different radial inward and outward gradients, as well as drug penetration and transport\(^2\). The variety of spheroid size in a batch influences the reproducibility of experimental results of drug dose response screening. Therefore, there is a strong need for an efficient and cost-effective model for uniform spheroid production for preclinical screening of anti-cancer drugs\(^3\).

The proposed study involves the development and evaluation of a novel approach to produce uniform spheroids without harming cells. The established procedure aims at rapid production of uniform tumor spheroids (TSs) as well as mimicking the cell culture micro-environment condition that is naturally occurring in vivo. The finished well-defined TS formation procedure along with micro-environment culture design could be used for regular production of
TSs for the testing of cancer treatments, allowing the correlation of treatment efficiency with micro-environment culture conditions.

1.1 Project Background

1.1.1 Three Dimensional (3-D) Micro-Tissue Model

Established cell-based series of *in vitro* functional high-throughput screening assays are an important topic for pharmaceutical companies\(^4\). Although two dimensional (2-D) monolayer cell-based screening assays are established in identifying potential drug candidates in the drug discovery process, their usefulness in predicting clinical response is limited due to the fact that such environments do not mimic the response of cells in the three dimensional (3-D) micro-environment present *in vivo* (in a tumor and tissue)\(^4\). The 2-D culture model suffers from the lack of physiological cell-ECM and cell-cell signalling. In contrast, these missing properties are present in 3-D multi-cellular matrix in vivo environments where signals are crucial to a range of cellular functions such as cell differentiation and proliferation\(^5\). The dissimilarities in cell polarity, morphology, interaction with ECM, mass transport, mechanical properties, and general cellular architecture have been recognized between 2-D cell cultures and the observed behaviour of cells *in vivo*\(^3\). Limitations of 2-D cell cultures (i.e. monolayer culture) suggest an alternative method in *in vitro* drug testing. It has been verified that cell behaviour in 3-D cultures is more representative of what naturally happens *in vivo* compared to 2-D monolayer cultures\(^6,7\). Recently, Imamura et al. have investigated the differential effects of chemotherapeutic drugs between 2-D and 3-D cell culture platform models, and have revealed that 3-D-cultured cells forming dense multicellular spheroids are exceptional candidates in simulating tumor characteristics *in vivo*, such as hypoxia, dormancy, and density\(^7\).
Apart from distinctions in the dimensional arrangement of cells when cultured in 2-D or 3-D, there are a number of biological differences that influence how the cells react to therapeutic agents. Highlighting these differences, there are well-documented studies on the substantial effects of microenvironment on the behaviour of cells in 2-D and 3-D cultures\textsuperscript{8,9}. Weaver et al. reported that culture in a 3-D membrane is required for complete expression of the reverted phenotype of non-malignant HMT-3522 cells. Pickl et al. also compared 2-D cultures to the effect of the spatial arrangement of cells when cultured as 3-D multicellular spheroids using poly(2-hydroxyethyl methacrylate) (p-HEMA)-coated plates\textsuperscript{10}. They stated that trastuzumab (anti-cancer drug) is less effective on cells cultured in 3-D as compared to those in 2-D. As a result, they concluded that the 3-D culture is a better representation of an in vivo signalling pathway than a 2-D monolayer culture\textsuperscript{10}. Hirschhaeuser et al. reported different cell responses to anti-cancer drugs in 3-D and 2-D cultures, where anti-cancer drugs were less effective in 3-D cultures\textsuperscript{1}. This confirms the observation by Torisawa et al. who investigated anti-cancer drug sensitivity by continuously evaluating 3-D multicellular spheroids based on their respiratory activity\textsuperscript{11}. In summation, 3-D culture models have been found to be a more reliable tool to evaluate drug efficiency and offer improved clinical efficacy predictions by restoring the micro-environmental and functional features of in vivo tumor tissue as compared to 2-D culture models.

Platforms for 3-D cell cultures include scaffolds and hydrogels. The most common and versatile 3-D cell culture approaches employed in applied tumor biology are briefly described in Table 1\textsuperscript{4}. 


Table 1. Brief summarization of 3-D cell culture approaches that are currently employed in tumor cell research

<table>
<thead>
<tr>
<th>3-D cell culture approach</th>
<th>Explanation</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multicellular spheroids</td>
<td>Micro-scaled spherical cell clusters</td>
<td>Tumor cell biology, nutrient gradient, drug efficiency and delivery, cell-cell interaction^{12,13}</td>
</tr>
<tr>
<td>Cellular multilayer</td>
<td>Layers of cells grown on a porous membrane</td>
<td>Drug transport and binding, drug efficiency^{14}</td>
</tr>
<tr>
<td>Matrix-embedded culture</td>
<td>Single cells or aggregates planted in a porous ECM</td>
<td>Cell-cell interaction, tumor cell biology, cell migration^{15}</td>
</tr>
</tbody>
</table>

Among the 3-D in vitro culture models, multicellular tumor spheroid cultures are a classical approach to achieve and retain the functional phenotype of in vivo tumor cells. Spheroids are one of the most versatile approaches to culturing cells in 3-D constructs^{16}, which exhibit many features of the natural tumor microenvironment and model the avascular region of natural tumors in vivo, dependent on diffusion^{17}. Spheroids are a reliable method for drug testing due to their similarity to in vivo tumor tissues in terms of their spatial multicellular arrangement and ECM deposition^{2}, comparable cell-cell interactions including tight junctions^{18}, inherent diffusional limits to nutrients, metabolites, oxygen, and mass transport of drugs^{19}, and the possibility they provide of using various cell types (Fig. 1)^{20}. The inherent gradient of spheroid metabolites, oxygen, and nutrients leads to a central necrotic region surrounded by quiescent viable cells and an outer shell of actively proliferating cells^{20}. This characteristic makes the use of spheroids a suitable, comparable model to those of natural tumor tissues.

Although the benefits of multicellular spheroids have been extensively recognized, tedious procedural challenges for spheroid culture production continue to restrain the biological
community from using spheroid tissue models for drug efficiency testing. A number of important reasons exist for why these 3-D culture systems have yet to be commercially introduced to contemporary drug screening procedures. These include the lack of simple, controlled techniques and protocols for rapid production and maintenance of uniform 3-D tumor spheroids⁴, a lack of cell connection and communication due to small numbers of cells, and the lack of straightforward protocols for a standardized assay of cellular responses or prediction of in vivo activities.

![Comparison of Tumor in Vivo and 3D Tumor Spheroid](image)

**Figure 1.** Schematic overview of a comparison of the components between an in vitro tumor spheroid model and in vivo tumor²⁰.
1.1.2 Previous Approaches and Limitations

A straightforward and reproducible method for spheroid formation is absolutely needed for spheroid-based studies. In the last two decades, numerous attempts have been made to bridge the gap between cell-based tests and animal studies by producing multicellular spheroids with different approaches. The proposed methodology for the production of multicellular spheroids will feature formation efficiency, spheroid size uniformity, and convenience for subsequent applications. Here, current spheroid production methods along with their pros and cons are briefly described.

1.1.2.1 Non-Adhesive Surface Method

Cultivation of cells on non-adherent surfaces is a traditional technique for generating multicellular spheroids\(^\text{21-23}\). In this technique, cells are essentially prevented from attaching to the culture flask surface by coating the culture ware with agarose thin films\(^\text{22}\) or hydrophobic polymers such as PHEMA\(^\text{21}\). Hence, this technique results in the aggregation of cells to form spheroids. This method is inexpensive and easy to scale up; however, it has a number of drawbacks, including variation in size (coefficient of variation [CV] of 40% to 60% \(^\text{24}\)), cell number, and shape. The application of non-adherent surfaces for spheroid formation frequently yields fragile aggregates with highly erratic geometry.

1.1.2.2 Rotary Bioreactor Method

Rotary bioreactors with appropriate surface treatment are also used to generate multicellular spheroids\(^\text{25,26}\). Cultures of cells in these systems produce spheroids via regular spinning of cell suspension to prevent the cells from settling down. This simple technique offers the potential of producing large numbers of spheroids with minimum effort. The rotary bioreactor method is suitable for co-cultures of different cell types in a dynamically controlled culture, but is
limited by requiring specialized equipment, variations in size and cell numbers, and high shear force\textsuperscript{16,27}.

1.1.2.3 **Hanging Drop Method**

In the hanging drop model, cell suspension drops (15-30 µl in volume) containing 300-3000 cells are deposited in a tissue culture dish on the underside of the lid. Drops are detained in place when the lid is flipped\textsuperscript{24,28} resulting in a single spheroid. The hanging drop technique is a simple, rapid and inexpensive method that enables co-culturing of different cell types to produce defined sizes of spheroids with a relatively narrow coefficient of variation: 10% to 15%, compared with 40% to 60% for non-adhesive surface methods. However, this method is labor-intensive and is not an ideal candidate for massive spheroid production.

1.1.2.4 **Microfluidic System**

One of the commonly used techniques in forming hydrogel beads is the use of microfluidic systems, which produce beads with high uniformity\textsuperscript{29}. In this technique hydrogel beads are generated on microfluidic chips through flow focusing\textsuperscript{30}, incorporating elements of ECM such as collagen type I\textsuperscript{31}.

In general, the gelation of natural polymers is reported to be less controllable, although ECM protein hydrogels are more compatible for cell-encapsulation compared to synthetic polymers such as polylactic acid (PLA) and polyglycolic acid (PGA)\textsuperscript{32}. In vitro, collagen type I solutions undergo self-assembly into hydrogels induced by pH, temperature or ionic strength, and the modulus and compressive strength of collagen hydrogels are reported to increase with a rise in collagen concentration and temperature\textsuperscript{33,34}. We also know that collagen type I is a triple helical molecule in which three α chains are bonded to each other by hydrogen bonding, where individual molecules are self-assembled into fibrils (diameter of 20-200 nm) that further aggregate to form
fibers of diameters in the range of 0.25-100 µm\(^3\). A collagen solution undergoes gelation at room temperature when pH is equal or higher than neutral pH levels. Therefore, there are two requirements for fast collagen gelation: 1) temperature higher than 4ºC, and 2) pH ≥ 7.4 at the same time. On the other hand, it is important to keep the solution at this physiological pH in order to maintain cell viability while processing hydrogel beads in a microfluidic system. In addition, collagen gelation must be prohibited during bead production to prevent chip clogging and to maintain bead size uniformity. Therefore, this is performed by using a cooling system to maintain collagen at a low temperature (0-4ºC), below its gelling point\(^3\). Keeping cells chilled during the bead encapsulation period is imperative for this process, as cells heating up to physiological temperature during beads collection could potentially affect cell viability and alter cell function, thus reducing proliferation efficiency.

1.1.2.5 Using External Forces

Generally, any system that concentrates hanging cells in a high-density suspension can potentially promote multicellular spheroid formation. Examples of these systems include magnetic fields\(^3\), low speed centrifugations\(^3\), and ultrasound waves\(^3\), which are all reported to enhance cell aggregation and spheroid production. These methods allow rapid formation of cell aggregates; however, they are restricted due to their demand for special instruments.

1.1.3 Spheroid Formation Mechanism

The multicellular spheroid model was first demonstrated by Holtfreter\(^4\) using self-assembly of cell suspension. Applying this self-assembly procedure from suspended cells, Moscona et al.\(^4\) have stated that cells are able to resume \textit{in vitro} a tissue-like configuration and re-establish their distinctive histogenetic development. Multicellular spheroid self-assembly
Involves three steps for full completion, as reported by Lin et al.\textsuperscript{28}. These three steps include cell aggregation, cell accumulations, and compact spheroid formation, respectively (\textbf{Fig. 2})\textsuperscript{19,28}.

\textbf{Figure 2}. Spheroid formation process that is divided into three steps (1) binding of integrin to ECM fibers with multiple RGD motifs for rapid aggregation of dispersed cells, (2) delay phase for regulated cadherin expression and accumulation, and (3) homophilic cadherin-cadherin binding between cells forming adherens junction for final compact spheroid formation. (Figure is reproduced from Lin et al. paper\textsuperscript{19})

The resulting compact multicellular tumor spheroids model the 3-D architecture of real tissues, including ECM deposition and cellular arrangement as well as some of the mass transport limitations through cellular barriers\textsuperscript{1}.

\subsection{1.1.4 Effect of ECM on Spheroid Formation}

The ECM provides physical support in which cells can adhere, signal, and interact with each other, but ECM is more than a matrix that maintains tissue structure. Indeed, it regulates cell proliferation, growth, migration, and other aspects of cell behaviour. Successful cell-ECM signalling through cell surface receptors (integrin) is essential for cell migration, proliferation, differentiation, apoptosis and a range of vital cellular functions\textsuperscript{8}. Therefore, providing an environment with the same properties found in naturally occurring ECM \textit{in vivo} is a great consideration with widespread benefits.
Lin et al. showed the correlation between the amount of ECM and spheroid-forming activity\textsuperscript{28}. They reported enhanced cell aggregation as a result of adding ECM proteins such as collagen to a culture. Their result strongly suggests that the key reason for the significantly high aggregation of the Alexander hepatoma cell line, PLC/PRF/5, is the presence of collagen in the culture medium\textsuperscript{28}. Collectively, their results suggest that optimal concentrations of ECM proteins are required for the promotion of spheroid production.

In addition, Kelm et al. observed tightly packed spheroid formation of HepG2 and MCF-7 with extensive ECM secretion visible at the surface\textsuperscript{24}. Bissel et al. have also demonstrated the direct effect of ECM proteins on the differentiation process by the observation of clonal growth of myoblasts to myotubes in response to the presence of collagen\textsuperscript{42}.

In order to overcome slow cell proliferation due to the lack of cell-cell and cell-ECM interactions, the addition of ECM proteins to such collagen cultures has been reported to enhance spheroid formation by providing cell-ECM support\textsuperscript{17,43–46}.

1.1.5 Hydrogels

Hydrogels are a network of hydrophilic polymer chains, in which water is the dispersion medium. These hydrophilic gels are cross-linked polymers, absorbing large amounts of water without dissolving. Some specific polymers that have an attached hydrophilic group to their backbone can make hydrogels, in which the cross-links between network chains resist the dissolution. The gel is a state that is neither completely solid nor completely liquid, as O. Okay stated in 2009\textsuperscript{47}. These half-liquid half-solid properties cause many interesting behaviours that allow various applications in areas such as wound care\textsuperscript{48}, drug delivery\textsuperscript{49}, dental materials\textsuperscript{50}, injectable polymeric systems\textsuperscript{51}, and tissue engineering and implants\textsuperscript{52,53}. Hydrogels may also
exhibit dramatic volume changes in response to a variety of external stimuli, such as the solvent, pH levels, electric field, and temperature, among others\textsuperscript{54}.

### 1.1.5.1 Hydrogels from Natural Polymers

There are many different reviews that highlight various natural polymers for hydrogel production, which are recommended for further reading\textsuperscript{53,55}. Here, two of the important polymers, alginate and collagen, and their material-dependent characteristics are discussed.

**Alginate**

Alginate is a hydrophilic and negatively charged polysaccharide that is obtained from brown algae and can form hydrogels. Alginate is a well-known biomaterial due to its simple gelation with divalent cations such as Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Mg\textsuperscript{2+}, and Ba\textsuperscript{2+}. Alginate structure consists of two basic building blocks, \(\alpha\)-L-guluronic acid (G) and \(\beta\)-D-mannuronic acid (M) residues, linearly linked together by 1-4 linkages (Fig. 3)\textsuperscript{56,57}. The distribution of these building blocks depends on the type of algae from which the alginate is isolated, as well as on the age and region of the plant. For example, alginate from the stem may have a different sequence and block composition than alginate isolated from the leaves\textsuperscript{57}. The formation of gels, by addition of calcium ions, involves G blocks where a higher proportion of G blocks results in a greater gel strength.

![Figure 3. Components of alginate: \(\alpha\)-L-guluronic acid (G) and \(\beta\)-D-mannuronic acid (M)\textsuperscript{57}.](image-url)
Alginate is widely used in tissue engineering, biomaterials, wound dressing, drug delivery, and cancer research due to its beneficial characteristics of biocompatibility, low toxicity, low cost, and a simple gelation method. However, alginate alone may not be an ideal material due to its fast degradation into the surrounding medium via a process involving the loss of divalent ions\textsuperscript{58}. Another limitation in using alginate gels alone is the lack of cellular interaction\textsuperscript{58}. Alginate tends to discourage protein adsorption due to its hydrophilic character resulting in an inability to interact with cells\textsuperscript{59}. Therefore, for the purpose of studying biomaterials, alginate needs to be either modified or used in a mixture combined with other materials to help with its limitations.

\textbf{Collagen}

Collagen is a triple-helical protein that forms fibrils of great tensile strength (\textbf{Fig. 4})\textsuperscript{60}. Collagen is a major insoluble fibrous protein component of extracellular matrices of mammalian tissues. There are at least 16 types of collagen, but 80 – 90 percent of collagen in the body consists of types I, II, and III\textsuperscript{61}. Collagen molecules are stable in acidic solutions at low temperatures and are capable of forming nanofibrils\textsuperscript{62}. Collagen forms gels via two different paths. One path is through temperature change that forms physical cross-links (hydrogen bonding) between chains\textsuperscript{62}, These physically formed collagen gels are thermally reversible. Collagen contains hydrophilic and lipophilic amino acids that exhibit changes in solubility that are temperature dependent, which is why it is able to form thermo-reversible gels\textsuperscript{55}. A second way to produce collagen gels is forming chemical cross-links between chains by using glutaraldehyde\textsuperscript{63} or diphenylphosphoryl azide\textsuperscript{64}. This cross-linking method is recognized as being impractical in a clinical setting because of cytotoxicity due to the direct exposure of chemical cross-linkers to the living cells (e.g., glutaraldehyde treatment)\textsuperscript{62} and prolonged treatment times (e.g., glyceraldehydes)\textsuperscript{65}. 
Figure 4. The individual triple helices or tropocollagen molecules are arranged to form fibrils, which are of high tensile strength and flexibility and can be further assembled and cross-linked\textsuperscript{60}.

Collagen has many advantages, including biocompatibility, bioabsorbability, and cell adhesiveness\textsuperscript{62}. Collagen is composed of a combination of amino acid sequences that are
recognized by cells, which makes it a great candidate in biological application. This specific amino acid sequence design helps collagen degradation by enzymes secreted from cells, named collagenase\textsuperscript{58}. However, there are common concerns with collagen regarding immunogenicity and contamination with viruses due to the use of animal sources to obtain it\textsuperscript{55}. Collagen gels are still short of physical strength, potentially immunogenic, and expensive\textsuperscript{58}.

1.1.5.2 Hydrogels in General

This section will review the mechanism of network formation in hydrogels, as well as the different methods producing them, including physical, chemical, and radiation cross-linking. We will also review hydrogels made from natural polymers such as alginate and collagen.

1.1.5.2.1 Mechanism of Network Formation

The linking together of polymer chains is referred to as “sol”, and the continuous linking process results in a solution of larger branched polymers with decreasing solubility. The resulting branched state is called a “gel” or a “network”. Accordingly, the sol state is referred to as a finite branched polymer system, whilst the gel state is called an infinite branched polymer system, in which the transition of the system from finite to infinite is called a “sol-gel transition”. This is also known as “gelation”\textsuperscript{66}.

Gelation can take place by two different types of mechanisms including physical linking and chemical linking. Physical linking can be either strong or weak. Strong physical gels are effectively permanent gels, strengthened by physical bonding between polymer chains. Weak physical gels are formed from temporary chain associations, such as hydrogen bonds, with a limited lifetime, which makes them reversible as they continuously break and reform. In general, the chemical linking process involves covalent bond formation that results in strong gel.
1.1.5.2.2 Classification of Hydrogels and Their Characteristics

Hydrogels are classified into two types: chemical gels (permanent) and physical gels (reversible). Chemical gels refer to networks held together by covalent bonding. On the other hand, physical gels are networks that held together by hydrophobic interaction, hydrogen bonding, ionic bonding, and molecular interaction, which are all reversible with any changes in physical conditions. Some of the most important characteristics of hydrogels are their permeability, water holding capacity, and biocompatibility.

1.1.5.2.3 Gelation Mechanism

Many different polymer modification methods are reported in biomedical fields, such as radiation crosslinking, grafting polymerization, chemical crosslinking, and physical crosslinking. In this section, general methods used to create hydrogels are described.

**Physical Cross-linking**

As various articles discuss, physical gels occur in response to different stimuli. Some examples of physically-crosslinked hydrogels include: heating and cooling of a polymer solution to form a junction zone, helix and association of helices, addition of di- or tri-valent counterions to ionic polymers (Fig. 5), changing the pH of an aqueous solution of polymers carrying carboxyl groups to form H-bonded hyrogels (Fig. 6), and heat induced aggregation through maturation. In general, physical cross-linked gels have the benefit of being reversible. Physical gels are also relatively easy to produce, and do not use cross-linking agents, which negatively affect the integrity of entrapped substances and need to be removed before application.
Figure 5. Gelation of homopolymeric blocks of α-L-guluronic acid junction with calcium ions. Binding of divalent cations by alginate: the “Egg-box” model.

Figure 6. Hydrogel network formation due to intermolecular H-bonding in carboxymethyl cellulose at low pH.
**Chemical Cross-linking**

This section briefly discusses a number of reported methods for producing permanent chemically cross-linked hydrogels. One chemical cross-linking method involves using cross-linkers such as glutaraldehyde to induce bonding between polymer chains (Fig. 7)^68^.

![Chemical Cross-linking Diagram](image)

**Figure 7.** Schematic illustration of using a chemical cross-linker to obtain a cross linked hydrogel network^68^.

Another versatile method for obtaining chemically cross-linked hydrogels is grafting, which involves the polymerisation of a monomer on the backbone of a polymer. This method is carried out by using either chemical reagents (chemical grafting)^78^ or high-energy radiation (radiation grafting)^70^ to activate the polymer chain, leading to branching and cross-linking.
1.2 Project Overview

In response to the foremost need to address the lack of a suitable model for drug efficiency tests, our study aims to generate a uniform-sized multicellular tumor spheroid model similar to natural tumor tissues, with applications in drug screening through an innovative, straightforward method. Our technique is based on the simultaneous in situ ultrasound-treatment of cells and ECM proteins prior to hydrogel bead production using microfluidic systems, which enables rapid production of large-scale spheroids for drug screening applications. The following elaborates on hydrogels in general, hydrogel bead design, ultrasonic treatment (UST), and the effect of collagen morphology on modeling 3-D tumors.

1.2.1 Microfluidic Hydrogel Bead Design

The microfluidic encapsulation of tumor cells within hydrogel beads has been extensively studied\textsuperscript{79-82}. The great interest in hydrogel beads is due to their similar properties to ECM, such as high water content, biodegradability, biocompatibility, and tuneable chemical and physical properties\textsuperscript{83}. One of the challenges with an alginate bead culture is that cells tend to migrate out of beads to proliferate on the culture flask, resulting in monolayer proliferation on the culture flask surface\textsuperscript{84}. This incident results in the mixture of a 2-D monolayer and 3-D spheroid in a culture flask, unless the culture flask is changed frequently. Changing a culture flask and dispersing beads into a new culture flask only prevents the mixing of a 2-D monolayer at the bottom of the flask with cells staying inside the beads. This does not provide a tool to prevent future cell migration in the new culture flask, and does not promote cells to stay inside the bead. Horning et al\textsuperscript{85} used non-tissue culture petri dishes to prevent monolayer attachment of cells. However, the need remains for a method to promote cells to stay inside the beads. One possible way to convince cells to stay inside of the hydrogel beads is providing them with a more analogous environment to ECM by
introducing an ECM polymer such as collagen inside of the hydrogel bead. This section will elaborate on microfluidic hydrogel beads and collagen’s morphologic effect on modeling 3-D tumor tissue.

A common practice in forming hydrogel beads is the use of microfluidic systems, which produce beads with high uniformity. Hydrogel beads are generated on microfluidic chips through flow focusing, incorporating elements of ECM such as collagen type I. Briefly, droplets of alginate are generated in the poly-(dimethylsiloxane) (PDMS) microfluidic chips through the breakup of a stream of the mixture of alginate and collagen containing cells (dispersed phase). The breakup is facilitated by shear from the cross flow of an immiscible, acidified oil (continuous phase) (Fig. 8). As alginate droplets flow through the continuous oil phase, acid diffuses into the alginate, causing Ca\(^{2+}\) release and alginate gelation. Therefore, the presence of alginate polymer in the mixture acts as a fast gelling component to maintain the spherical shape of the beads once gelled in the chip during their flow in the acidified oil. Moreover, collagen in the mixture is used as an ECM component to mimic the natural tumor in vivo.

In order to use microfluidic chips (as shown in Fig. 8) to produce hydrogel beads, each channel must be filled with a fluid that can flow inside the chip. For much of the TS production process in microfluidic systems, collagen gelation must be prohibited during bead production since collagen is a temperature-sensitive polymer and starts to form gel at room temperature. Using a cooling system provides a tool to keep collagen at a temperature below its gelling point during its flow inside the microfluidic chip, and prevents chip clogging due to in-site gelation of collagen before droplet breakup inside the chip. This results in bead size uniformity. However, keeping cells chilled during the bead encapsulation period could potentially affect cell viability.
and alter cell function, reducing the cell’s proliferation efficiency. Another drawback of using a cooling system set-up is its time-consuming and labor-intensive nature.

**Figure 8.** The presence of an acidic environment persisting within the flow, allowing CaCO₃ to release free Ca²⁺ ions capable of binding with the available ionic blocks of the alginate chains, resulting in hydrogel beads containing cells yet inside the chip. This figure is a schematic of flows in the chip. As the blue color of the dispersed phase gets darker, the gelation of alginate becomes greater.

1.2.2 Ultrasound Treatment (UST)

Ultrasound has been used for making homogeneous polymer mixtures⁸⁸–⁹¹, and UST is commonly employed to uniformly disperse nanoparticles in suspensions as a preparation step⁹². The main purpose of using UST in our project was to disperse particles and fibers in the mixture uniformly. Besides the application of UST in particle/fiber dispersion, UST has been recently developed to form multicellular aggregates in suspensions⁹³,⁹⁴.

It is reported⁵⁷,⁹³–⁹⁶ that low-intensity ultrasound can be used to stimulate the activity and viability of different cell types such as human articular chondrocytes in a 3-D alginate bead culture⁹⁵. Wong et al. used ultrasonication to accelerate the gelation of silk fibroin for human bone
marrow derived from mesenchymal stem cell (hMSC) encapsulation, and they showed that cells retained viability and proliferation in a culture for several weeks. Additionally, Liu et al. have applied an ultrasound-based technique to rapidly produce HepG2 cell aggregates followed by culturing on a P-HEMA-coated surface. Bazou et al. have also applied ultrasonication to show the generation of 2-D cell aggregates from chondrocytes and neural cells in suspensions. Despite the effectiveness of these ultrasound-based techniques, an application for the formation of 3-D multicellular tumor spheroids and long-term cultures has not yet been investigated.

In this study, a new ultrasonication-based technique is developed and then used to induce an in situ structural transition of collagen type I in a controllable manner. This technique enables the researcher to eliminate the cooling of ECM mixture during bead formation by initially changing the structure of collagen from temperature-sensitive to partially-temperature-sensitive. This technique permits post-sonication bead production, followed by rapid incubation. The technique is then assessed in terms of cells proliferation, mixture and bead homogeneity, and spheroid uniformity.

1.3 Summary of Project Objectives

The objectives of this work can be described as the following:

1) To incorporate collagen into tumor spheroid model.

2) Address the challenge of inhomogeneity in mixtures of alginate and collagen that hinders the ability to generate uniform hydrogel droplets/beads

3) Address the poor controlled procedure to form hydrogel droplets/beads that reduce the viability of cells entrapped in the hydrogel beads.

4) Address the improvement of spheroid size uniformity to produce more reliable 3-D cell culture model for anti-cancer drug screening.
2 Characterization of UST Effect on Cell Proliferation and Bead/Spheroid Size Uniformity

Here a novel yet simple method is developed to prepare homogeneous pre-bead-encapsulation-mixtures containing collagen type I through ultrasonication, while extending cell viability and proliferation. This method allows the cultivation of homogenous TS cultures with high uniformity and compact structure, and not only maintains cell viability but also stimulates the proliferation of cells in alginate/collagen hydrogel bead cultures.

2.1 Materials and Methods

2.1.1 Mixture Preparation

MCF-7 breast tumor cells were cultured in an RPMI medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (10,000 U/ml penicillin, 25 μg/ml fungizone, and 10 mg/ml streptomycin, Gibco BRL, NY) for 24 hours under 5% CO₂ at 37°C. After washing twice with PBS, the cells were detached with 0.025 mM trypsin + EDTA and then resuspended in 1 mL of RPMI media. Cells were then centrifuged and washed in Hank’s Buffered Salt Solution (HBSS). Briefly, a mixture containing 2% w/v sodium alginate and 1.5 mg/ml collagen solution, with 80 mM calcium carbonate and MCF-7 cells (5×10⁶ cells/ml) was prepared. The pH was set to 7.4 by the drop-wise addition of calcium bicarbonate (sample 1: S1: no sonication) (Table 2). Additional mixtures were likewise prepared and ultrasonicated for 30 minutes at temperatures of 32°C, 37°C, and 42°C, resulting in S2, S3, and S4 respectively.

2.1.2 Ultrasound-Based Treatment (UST)

The ultrasonic stimulation was performed using an ultrasonic processor model from the Branson ultrasonic corporation, 8510R-DTH, with transducer placement at the tank bottom. The
equipment was used to apply ultrasound energy (in Joules per ml of mixture) to previously made mixtures in order to disperse mixture contents homogeneously. Ultrasound energy at a power of 2000 J/ml in a continuous wave fashion was applied on prepared mixtures for 30 minutes in a water bath at temperatures of 32°C, 37°C, and 42°C (S2, S3, and S4) (Table 2). The temperature was increased from 32°C to 42°C to observe the effect of temperature on possible structural changes and the distribution of collagen fibers. The entire chamber was sealed during sonication to maintain the temperature at the same fixed value. The water bath was used to transmit ultrasound energy indirectly and to also maintain temperature at the desired fixed value.

Table 2. Sonication treatment condition and mixture composition for all samples

<table>
<thead>
<tr>
<th>Sonicated (2000 J/mL mixture)</th>
<th>Mixture Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous wave fashion</td>
<td></td>
</tr>
<tr>
<td>S1 No sonication-ambient temperature</td>
<td>2% (w/v) sodium alginate + 80 mM CaCO₃ + 1.5 mg/ml collagen + 5*10⁵ cells/ml of MCF-7 cells</td>
</tr>
<tr>
<td>S2 30 min continuous pulse at 32°C</td>
<td>2% (w/v) sodium alginate + 80 mM CaCO₃ + 1.5 mg/ml collagen + 5*10⁵ cells/ml of MCF-7 cells</td>
</tr>
<tr>
<td>S3 30 min continuous pulse at 37°C</td>
<td>2% (w/v) sodium alginate + 80 mM CaCO₃ + 1.5 mg/ml collagen + 5*10⁵ cells/ml of MCF-7 cells</td>
</tr>
<tr>
<td>S4 30 min continuous pulse at 42°C</td>
<td>2% (w/v) sodium alginate + 80 mM CaCO₃ + 1.5 mg/ml collagen + 5*10⁵ cells/ml of MCF-7 cells</td>
</tr>
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</table>

2.1.3 Microfluidic-Based Bead Production

The bead generation procedure relies on the method of internal alginate gelation, where Alginate gels are formed through calcium-mediated crosslinking. Calcium, bound in the insoluble form of CaCO₃, is mixed into an alginate solution of pH 7.4. When pH is lowered, the acid reacts with the calcium carbonate to release water, CO₂ and Ca²⁺, initiating alginate gelation (Fig. 9).
The oil phase was prepared by mixing 50 mL mineral oil (Sigma Aldrich) with 0.5 g Span80 (sorbitane monooleate nonionic surfactant, Sigma Aldrich) and 1 μL/mL acetic acid. The Span80 surfactant was used to stabilize the hydrogel droplets and prevent their coalescence prior to gelation.

![Diagram](image)

\[ \text{CaCO}_3 + 2H^+ \rightarrow \text{Ca}^{2+} + \text{H}_2\text{O} + \text{CO}_2 \]

**Figure 9.** The presence of an acidic environment persisting within the flow, allowing CaCO₃ to release free Ca²⁺ ions capable of binding with the available ionic blocks of the alginate chains.

The droplets of the alginate hydrogel precursor were generated in the poly-(dimethylsiloxane) (PDMS) microfluidic chips through flow-focusing with an immiscible, acidified oil continuous phase. As alginate precursor droplets flow through the continuous oil phase, acid diffuses into the alginate, causing Ca²⁺ release and alginate gelation. Once gelled, the alginate beads were collected in a tube of culture media, and washed via centrifugation. At 15-minute intervals, the contents of the collection tube (containing oil, beads and media) were centrifuged to bring the rest of the beads from the oil into the media. Beads were then washed twice with media, and incubated overnight in a flask of culture media at 37°C and 5% CO₂. Sample mixtures were introduced into the chip in the following order: oil solutions were introduced at the same time, then the cell-alginate-collagen mixture tube was added (Fig. 10). Pressures were
adjusted as required to obtain droplet bead production. It is possible to make a range of droplet bead sizes (from 50 µm to 600 µm in diameter), due to changes in the applied pressure. After droplet formation and gelation into beads, beads remained in the tubing for ~30 seconds until they reached the collection tube.

**Figure 10.** Schematic of a microfluidic chip for hydrogel core bead encapsulation. Inlets to chip are supplied by reservoirs with pressure flow control. The chip was originally designed for making core/shell structure beads; therefore, the channel 2 must be blocked in order to produce core beads.

### 2.1.4 MCF-7 Cells Viability and Proliferation Test

The viability of MCF-7 cells was first monitored by applying the Trypan Blue (Gibco BRL, NY) dye exclusion staining method to mixtures before and immediately after UST. This technique was used to determine the number of viable cells present in the mixtures. Mixtures containing MCF-7 cells were immediately mixed with Trypan Blue 50/50 for 10 minutes at 37°C. The cells
were stained with 0.4% Trypan Blue, and the numbers of viable and non-viable cells were counted. If cells take up Trypan Blue, they are considered non-viable\textsuperscript{97,98}.

The cell proliferation was determined using a standard MTS assay (Sigma Aldrich) in which the cell-laden beads and MTS solution were mixed with a ratio of 5:1, and cultured in 96-well plates followed by culturing in an incubator at 37°C and 5% CO\textsubscript{2} for 4 hours\textsuperscript{99}. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product, and in this method the quantity of formazan product measured at a 490 nm absorbance indicates the number of viable cells in the sample; such proliferation assays can be used with cells embedded in 3-D matrices\textsuperscript{99} or with multicellular spheroids\textsuperscript{100}.

2.1.5 Evaluation of Size Uniformity of Beads and Spheroids

To assess the bead size consistency for sonicated and non-sonicated mixtures, hydrogel beads were dispersed in media and the diameters were measured by averaging in bright field images utilizing NIS-Elements BR software (Nikon Apparatus). Hydrogel bead size consistency was characterized by the CV value. Beads with different diameters ranging from 210 to 310 µm in different batches were obtained in this study using different oil jet pressures in the microfluidic chip and applying different sonication conditions. The size distribution of the beads is represented by the coefficient of variation (CV=\(\frac{\text{Standard deviation}}{\text{mean}}\)).

2.1.6 Second Harmonic Generation (SHG) Imaging

Second harmonic generation (SHG) backward propagation is a powerful tool to determine the distribution of micro-sized collagen gel in the matrix\textsuperscript{101–105}. Collagen type I is a triple helical
structure that is abundant in the human body (particularly in ECM) and consisting of tropocollagen molecules. The individual tropocollagen molecules are covalently self-assembled into collagen fibrils in a diameter of 25 to 150 nm, which are capable of aggregating and forming collagen fibers in diameter of 0.25-100 µm via lateral hydrogen bonding\textsuperscript{35} (Fig. 11).

![Schematic view of hierarchy of fibrous collagen](image)

**Figure 11.** Schematic view of hierarchy of fibrous collagen\textsuperscript{106}.

The generation of SHG signals is highly dependent on the non-centrosymmetric structure of the material (no inversion symmetry). In SHG microscopy, the regular arrangement of scatterers in the material is an essential factor for generating a significant amount of SHG signals. Another requirement for generating SHG signals from a specimen is matching fiber thickness with the coherence length of SHG\textsuperscript{101}. The material should have enough non-centrosymmetric structure density to be able to produce an efficient SHG signal. This clarifies why not all non-centrosymmetric materials emit SHG signals (the collagen molecule tropocollagen is a non-centrosymmetric molecule).
Here, SHG backward propagation imaging was used to determine the distribution of collagen type I in the matrix of cell-laden beads, both before and after the sonication of cell-laden beads and the pre-bead-production mixture. The focusing objective of a 2p 25X water immersion with a 1.05 numerical aperture (NA) water immersion objective lens (Olympus objectives) was used. The SHG signal was excited at 810 nm, and acquired with a HQ 405/30M-2P emission filter removing any back-propagated signal not originating from collagen fibers. Backward-propagating SHG is examined at microscopic scales to examine the aggregation of fibers.

2.2 Results and Discussion

2.2.1 Cell Viability and Proliferation

The number of viable cells in the non-sonicated (91% of 348 counted cells) and sonicated samples (89% of 457 counted cells) was almost the same. This implies that the applied sonication energy does not induce cell death, confirming the results presented by Choi et al.\textsuperscript{95}. Choi et al. found that an alginate culture combined with sonication treatment could better support the cell’s viability, rather than using alginate culture alone. As shown in Fig. 12, UST can stimulate proliferation of MCF-7 cells in alginate hydrogel bead compared to non-UST mixture. The enhanced MCF-7 cells proliferation by UST in the long time culture may have a beneficial effect on the construction of uniform engineered 3-D TSs. We can see that UST clearly enhanced the MCF-7 cells proliferation in alginate culture compared to the non-UST sample. This was also observed when collagen was added to the mixture. As shown in Fig. 12, the proliferation of MCF-7 cells was enhanced when sonicated along with the rest of mixture. When MCF-7 cells where present in the mixture while being sonicated they showed a greater proliferation after day four compared to the proliferation result from the mixture that where alginate and collagen were first
sonicated and MCF-7 cells were subsequently mixed into solution. This shows that UST enhances the cells proliferation. Our finding is in a good agreement with the findings of Choi et al. with respect to the enhancement of cell proliferation after ultrasound treatment.

The observations of our study showed that MCF-7 cells proliferation is significant in the samples sonicated at 37°C. As illustrated in Fig. 13, sonication at 37°C (S3) enhances the proliferation of MCF-7 dramatically compared to the lower temperature sonication.
treatment. However, there is a significant drop in MCF-7 proliferation in the samples sonicated at 42°C (S4). This shows that the optimal temperature for sonication treatment of MCF-7 is a culture temperature of 37°C. The data indicate that MCF-7 cells survive and proliferate longer within the sonicated collagen/alginate bead culture than in the non-sonicated sample, S1. The non-sonicated sample provides improved MCF-7 cell proliferation during the first five days, but then levels off after the proceeding two days, while the sonicated samples stay viable and proliferate twice as long as the non-sonicated sample (Fig. 13).

![Graph showing proliferation of MCF-7 cells in 3-D Hydrogel Bead Culture](image)

**Figure 13.** Comparison of MCF-7 cell proliferation in non-sonicated and sonicated samples at 32°C, 37°C, and 42°C. Proliferation was measured using standard MTS assay. Error bars were calculated based on the results obtained from experiments at the trial numbers of the same condition meaning three replicates within the same trial. The same condition is tried three times for UST at 32°C, three times for 37°C, two times for 42°C, and one time at room temperature. The UST temperature and the number of each trial is mentioned in the title box.
2.2.2 Size Uniformity Evaluation

As noted above, bead size was determined by measuring bead diameter in bright field images using an NIS Element (Nikon Instrument). **Table 3** shows the size distribution (CV) and mean diameter (AV) of the produced beads with no sonication (S1) and sonicated at temperatures of 32°C, 37°C, and 42°C (S2, S3, S4 respectively). We see that the size distribution drops with an increase of temperature from 32°C to 42°C compared to the non-sonicated sample (S1). Beads had the highest uniformity compared to S1, S2, and S3. S1 showed the least bead uniformity compared to the sonicated samples. When the temperature of UST was 42°C (S4), the bead size presented the highest order of uniformity, where the CV value of the beads was 0.04 compared to the CV value of sonication at 32°C, which was 0.11 (**Table 3**).

The average diameter of the hydrogel beads prepared from S1, S2, S3, and S4 are presented in **Table 3**. The size distribution of the higher sonication temperature sample (42°C) (S4) is much narrower than that those with lower sonication temperatures (32°C, 37°C) (S2, S3) and the non-sonicated sample (S1).

The result demonstrates that S4 (sonicated for 30 minutes at 42°C) is the best condition for making uniform-sized hydrogel beads containing collagen. It can be concluded that the sonication temperature plays an important role in the production of uniform hydrogel beads in terms of their size. As the temperature goes higher the distribution of collagen fibril becomes more uniform, therefore the production of uniform sized hydrogel beads becomes more possible.
Table 3. Size distribution of beads and spheroids. Bead size was measured on the first day of production. Spheroid size was measured on day 7. The average diameter (AV), standard deviation (SD), and coefficient of variation (CV) (CV=standard deviation/mean) for beads and spheroids are given. (Number of beads; N≥80)

<table>
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<tr>
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<th>Beads</th>
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<th>Spheroids</th>
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<td></td>
<td>AV (μm)</td>
<td>SD (μm)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>S1: control; non-treated</td>
<td>293</td>
<td>116.4</td>
<td>39</td>
</tr>
<tr>
<td>S2: UST at 32°C</td>
<td>210</td>
<td>23.4</td>
<td>11</td>
</tr>
<tr>
<td>S3: UST at 37°C</td>
<td>264</td>
<td>22.9</td>
<td>8</td>
</tr>
<tr>
<td>S4: UST at 42°C</td>
<td>303</td>
<td>13.2</td>
<td>4</td>
</tr>
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</table>

2.2.3 Mixture Uniformity Evaluation

Representative SHG images from dispersed collagen fibers inside the mixture are illustrated in Fig. 14. These images qualitatively track the changes related to the collagen fiber remodelling in the UST samples at 32°C (S2), 37°C (S3), and 42°C (S4), as well as the non-sonicated (S1) sample. Significant reduction in collagen fiber aggregation in the UST sample at 42°C (S4) is clearly observed, while a lack of well-distributed collagen fibers is evident in the sonicated sample at 32°C (S2) and the non-sonicated sample (S1) (Fig. 14). This observation is an indication that higher UST temperature corresponds to better collagen distribution within the mixture.
Figure 14. SHG polarization and bright field images of mixtures and hydrogel beads. Scale bar represents 100 μm. Dark particles in the bright field images are calcium carbonate particles dispersed in a mixture before gelation of the mixture inside of the microfluidic chip. S1: non-treated, S2: UST at 32°C, S3: UST at 37°C, and S4: UST at 42°C.

Collagen type I fibers in the non-sonicated sample (S1) exhibit a strong photo-stable image, while they do not produce many detectable backward SHG signals in the sonicated samples (S2, S3 and S4) with the same concentrations (1.5 mg/ml). Since the generation of SHG signals is
highly dependent on the non-centrosymmetric structure of the material, as well as the matching of fiber thickness with coherence length\textsuperscript{102,101}, the stated observations may be due to changes in the fiber diameter.

To further study the effect of the UST on collagen fiber structure and aggregation, bond dissociation energies of the Carbon-Carbon (C-C), Carbon-Hydrogen (C-H) covalent bond, and Hydrogen-Oxygen (H—O) hydrogen bond are compared with emitted energy from the UST. The energy needed to dissociate the C-C and C-H bonds in the collagen fiber backbone is 348 kJ/mole and 413 kJ/mole, respectively\textsuperscript{107,108}, while the emitted energy from the applied UST is less than 154 kJ/mole of collagen (2 kJ/ml of mixture). This indicates that collagen fibers do not break from the backbone during UST, which inserts a much lower energy into the mixture. There is a possibility that collagen fibers are dissociated during sonication, since the energy needed to dissociate H—O hydrogen bonding is only 1-3 kJ/mol.

As shown in Fig. 15, there is a noticeable difference in the size of individual collagen fibers in the sonicated samples from their aggregation in the non-sonicated sample. A similar discrepancy is observable in all samples sonicated at different temperatures, such as at 32°C and 42°C. We can see that sonication treatment eases the uniform dispersion of the collagen fibers among the mixture by preventing lateral hydrogen bonding of fibers. The observations show that collagen fibers (micro scale size) are mostly restructured into collagen fibrils (nano scale size) through the breakage of hydrogen bonding between fibrils after the sonication treatment. Thus, the fiber dispersion can be tuned by the sonication temperature.

These results suggest that the UST process may either reduce the density of the non-centrosymmetric structure defined by collagen type I, or change the order of non-centrosymmetric structure at the molecular level, since less SHG signal was detected after the UST process. To rule
out the effect of collagen concentration on the findings, the same amount of collagen (1.5 mg/ml) was used in all the samples.

![Non-sonicated sample (S1) vs UST at 37°C for 30 min (S3)](image)

**Figure 15.** Left: Backward-propagating SHG polarization from non-sonicated (control) mixture (sample S1). Right: Backward-propagating SHG polarization from sample S3. The scale represents 50 µm.

### 2.3 Conclusion

This study has introduced a novel sonication treatment of a mixture to enhance the proliferation and viability of human source MCF-7 cells. Only a few reports have previously discussed the effects of sonication on the activity of non-human source cells in vitro in a monolayer\(^95\)\(^{,109}\), and the present study is distinguished from these through its use of MCF-7 from a human source in a 3-D bead culture, supported by the understanding that working with human source cells provides more applicable information for human-related research.
The findings above show an increased cell proliferation after day 5 in the sonicated sample at 37°C compared to the non-sonicated sample. The enhanced MCF-7 viability through sonication treatment seen in the longest culture timeframe provides an advantage for constructing an engineered tumor spheroid in vitro. Beads made of the sonicated mixture stay in the spherical shape roughly for 20 days, whereas beads made of the non-sonicated mixture degraded in only 10 days. The presence of alginate component in the mixture is firstly to maintain the 3-D shape of culture beads during the formation of multicellular spheroids and secondly to discourage cell proliferation out of beads. Experimental observation showed that alginate hydrogel beads produced from non-UST mixture was degraded to the culture medium more quickly than those made of UST mixture. The presence of this pro-longed lasting alginate hydrogel beads made of UST-mixture could potentially be one of the reason to help producing uniform-sized spheroids. A uniform sized spheroid was obtained from the beads made from the sonicated sample at 37°C (CV=0.08).

Sonication treatment is thus found to be of great significance in dissociating collagen fibers to fibrils, and in allowing a uniform distribution of collagen fibrils throughout the mixture, to better serve the mixture’s homogeneity and provide greater surface area for cells to attach during culturing. Uniformly-positioned collagen fibrils made by sonication treatment in the pre-bead-production mixture allow the forming of more uniform beads than those made from the non-sonicated mixture. Since sonication treatment breaks the lateral hydrogen bonding in collagen fibers, it thus turns them into nano-sized fibrils.
3 Characterization of the Effect of UST on Collagen Morphology

Ultrasound has been used for making homogeneous polymer mixtures\cite{88-91}, and UST is commonly employed to uniformly disperse nanoparticles in suspensions as a preparation step\cite{92}. The main purpose of using UST in our project was to disperse particles and fibers in the mixture uniformly.

3.1 Materials and Methods

3.1.1 Mixture Preparation

To investigate the effect of UST on collagen fiber/fibril micro/nanostructure, aqueous solutions of collagen type I, both UST and non-UST, were prepared, as briefed in Table 4. First, 820 µl of collagen type I at an 11 mg/ml concentration was transferred into a conical tube. Then 180 µl of phosphate-buffered saline (1X-PBS) was added, along with a few droplets of sodium bicarbonate until the desired pH (7.4) was reached and sufficiently vortexed. The mixture was either imaged with no treatment or imaged after being sonicated for 30 minutes at 37°C.

Table 4. UST condition and mixture preparation

<table>
<thead>
<tr>
<th></th>
<th>UST</th>
<th>Mixture Composition</th>
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<tbody>
<tr>
<td>P-Col-UST</td>
<td>30 min continuous pulse at 37°C</td>
<td>9 mg/ml collagen type I solution, pH=7.4</td>
</tr>
<tr>
<td>P-Col-N-UST</td>
<td>No UST-ambient temperature</td>
<td>9 mg/ml collagen type I solution, pH=7.4</td>
</tr>
</tbody>
</table>

3.1.2 Transmitted Electron Microscopy (TEM)

Transmitted Electron Microscopy (TEM) was used to determine the collagen fiber/fibril thickness in both UST and non-UST Samples. To distinguish fibrils and fibers, TEM was used to determine the possible structural differences between UST samples and non-UST samples. To
visualize type I collagen fibers and fibrils a Hitach H-7600 TEM at an accelerating voltage of 80 kv was used. The samples were prepared for TEM by a negative staining technique\textsuperscript{110}. In short, this technique was administered by placing the aqueous suspension that contains the specimen on the formver-carbon TEM grids, after which excess solution was removed and the grids were air-dried. These grids were then negatively stained with a 2\% uranyl acetate. No fixation or dehydration methods were used other than air-drying after staining (this is standard for negative staining). Negative staining is a common method for TEM imaging, in which the background is stained, leaving the actual specimen untouched and thus visible.

3.1.3 Second Harmonic Generation (SHG) Imaging

To investigate the effect of UST on collagen fiber/fibril length, aqueous solutions of collagen type I, both UST and non-UST, were prepared (Table 4) and observed by using SHG. Since these samples contained a higher collagen concentration (9 mg/ml) compared to S1, S2, S3, and S4 (in chapter 2) (1.5 mg/ml), SHG imaging was expected to be able to observe collagen fibrils’ or thinner fibers’ structures since there were more non-centrosymmetric features to produce a strong detectable SHG signal. Images were processed and interpreted using ImageJ/Fiji software.

The samples were prepared for SHG by depositing a few droplets (400\(\mu\)l) of the specimen-containing solution on the glass slide and sealing it with a top cover slip, producing a very thin film.

SHG backward propagation imaging was used to determine the distribution and size of the collagen type I in the matrix, both before and after mixture sonication. The focusing objective of a 2p 25X water immersion with a 1.05 numerical aperture (NA) water immersion objective lens (Olympus Objectives) was used. The SHG signal was excited at 810 nm, and fundamental blocking
was carried out with a channel one HQ 405/30M-2P emission filter to avoid receiving a back-propagated signal from anything except the collagen fibers. Backward-propagating SHG is examined at both macroscopic and microscopic scales to examine the length and dispersion of fibers and fibrils.

### 3.1.4 Image Processing

Image Processing is a technique that utilizes computation algorithms to enhance digital images, and in this project, we used image processing to estimate fiber dispersion and size distribution. Some images were not easy to interpret; therefore, we performed image processing to extract the size of fibers from the noisy background before further analysis. Quantification and image processing were carried out using the ImageJ/Fiji software. The types of obtained images were first changed to 8-bit, followed by threshold adjustment using the [Image → Adjust → Threshold] command in ImageJ/Fiji by dragging the two sliders until the desired area (the collected signal) was covered by the black and white background mode. We maintained the threshold value at the same level between images from different specimens and defined particle sizes as larger than background noise dots to omit the effect of background noise pixels. This allowed us to compare all specimens together and to identify the fibers. After readying the images ready for analysis, they were analyzed using the freehand tool to determine the length of fibers.

The threshold level has been chosen on the basis of discrimination of signal from collagen fiber/fibril against the background noise. This has been calibrated for the field of view, as ImageJ/Fiji has an internal pixel size calibration. **Fig. 16** shows an ImageJ/Fiji screenshot of the procedure for a single SHG section of a collagen fiber/fibril, wherein the left and center images are the raw and threshold images, respectively, and the output of fiber/fibril length is given on the right. This procedure was based on the method developed by Chen *et al.*\textsuperscript{104}. 

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Figure 16. Using ImageJ/Fiji to measure collagen fiber/fibril length. Left, raw SHG image; center, thresholded image used to identify fibers. One fiber (in the red circle) is selected and a freehand line (yellow) is overlaid to measure its length using the Analyze.measure function. Right, measurement result showing the fiber length in the relative unit of the image (1 pixel=0.1µm for a field size of 102.4 × 102.4 µm).

3.2 Results and Discussions

3.2.1 Collagen Fiber/Fibril Length Analysis

We assessed collagen fiber length distribution using SHG microscopy imaging. Non-treated solutions were characterized by long collagen fibers (Fig. 17a). As the ultrasound exposure pressure was introduced to the solutions, collagen fibers became visibly shorter fibrils (Fig. 17b). Changes in collagen type I fiber morphology were quantified through measuring fiber length using ImageJ/Fiji software (Fig. 18). As shown in Fig. 18, the average size of fibers in non-UST samples were measured at 10µm with CV of 0.8; however, the average size of fibrils in UST-collagen samples were measured at 5µm with CV of 0.4. These results suggest that UST treatment changed the fiber structure to a shorter-length fibril and with a more uniform size distribution. The results also indicate a large decrease in fiber length compared to non-treated samples.
Figure 17. Representative back-propagated SHG image of the collagen fibers/fibrils of non-sonicated (a) and sonicated (b) samples. Each scale bar represents 10 µm.
Collagen fiber length from sonicated (P-Col-UST) and non-sonicated (P-Col-N-UST) samples as determined by second harmonic generation images. The number of fibers in sample P-Col-UST is 856, and the number of fibers in sample P-Col-N-UST is 717. (N_{P-Col-UST}=856, N_{P-Col-N-UST}=717).

### 3.2.2 Collagen Fiber/Fibril Diameter Analysis

Collagen type I fibrils can range from 20 to 200 nm in diameter\textsuperscript{111}, while fibers are larger in diameter (0.25-100 μm) depending on the tissue and its location\textsuperscript{103}. Given that the size of each pixel in SHG imaging is 0.14 μm, it is thus impossible to distinguish fibrils and fibers of collagen type I\textsuperscript{112}. In light of this, we use the term “fiber” to include both fibers and fibrils when discussing observed structures in SHG. Further, to ultimately distinguish fibers and fibrils there is a need for a stronger tool, such as TEM, to determine the possible structural differences between UST samples and non-UST samples.
TEM examinations of untreated collagen type I (P-Col-N-UST) showed collagen fiber diameters in the range of 0.25 µm to 200 µm in our experiments which is similar to other published TEMs of collagen fibers\textsuperscript{113–115}. For all UST samples (P-Col-UST), collagen dissociation into thinner fibrils was observed (Fig. 19), while P-Col-N-UST samples were not subject to fiber dissociations.

**Figure 19.** TEM image of a collagen type I solution at 60000x magnification. (a) Pure collagen type I UST at 37°C, (b) pure collagen type I at room temperature with no treatment. Yellow bars indicate the thickness of the collagen fibril/fiber. White arrows show the random spot chosen to measure the diameter of the collagen type I fiber/fibril. Scale bar represents 500 nm.

TEM observation showed collagen fibers in P-Col-N-UST as irregular networks composed of a wide range of fiber diameters (from 490 to 983 nm) that were randomly bundled together, whereas collagen fibrils in P-Col-UST formed narrower diameters (ranging from 30 to 40 nm).
The TEM images verified a decrease in collagen fiber diameter (P-Col-N-UST) to diameters in the range of reported collagen fibrils in the UST sample (P-Col-UST).

**Fiber/fibril diameter analysis**

<table>
<thead>
<tr>
<th></th>
<th>AV (nm)</th>
<th>SD (nm)</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Col-N-UST</td>
<td>708.9</td>
<td>210.26</td>
<td>0.3</td>
</tr>
<tr>
<td>P-Col-UST</td>
<td>36.2</td>
<td>3.8</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**Figure 20.** Diameter distribution of collagen type I observed in TEM images for non-sonicated (P-Col-N-UST) and sonicated (P-Col-UST) samples. (N=14).

### 3.2.3 Collagen Fiber/Fibril Dispersion Analysis

Uniform dispersion expression is very useful for descriptive purposes due to its prevalence in the literature. To analyze the uniformity of fiber dispersion here, a statistical simulation method has been adapted from a paper by H.S. Khare and D.L. Burris. Briefly, this method finds a domain that has no fibers and is defined as $L_f$. Fiber size, concentration, and dispersion are variables that can be measured directly by this parameter. As dispersion becomes more uniform for constant concentration, $L_f$ becomes smaller. Moreover, as the concentration is increased, $L_f$ is reduced. As fiber size is reduced for a given concentration and distribution, $L_f$ is reduced. Here the
concentration remains the same and the UST only changes the dispersion and size of fibers. Therefore, this parameter directly measures the effect of variables that are fiber length and dispersion. The method for obtaining $L_f$ begins with presenting an SHG image of the dispersed fiber and fibrils. Then the image is converted to a black and white bitmap where black distinguishes the fiber from the white background. Luo and Koo$^{117-119}$ defined the free-space length ($L_f$) as the width of the largest placed square in which the highest possible number of crossing black pixel is zero. In this method first, a square of length, $L$, is placed randomly on the SHG image. The number of crossing black pixels is counted and stored. The code used here is adopted from H.S. Khare and D. L. Burris$^{116}$. This method has been applied on three images of each sample and has been repeated 1,000 times for each sample (representative image is shown in Fig. 21). This code finds the largest square for which the most probable number of crossing black pixel in a randomly placed square is zero. The results of the analysis for each sample are compared.

The dispersion in Fig. 21a is a relatively uniform dispersion of ±5μm fibrils at 9% black pixel, while the field of view is 102×102 μm, compared to Fig. 21b which is a non-uniform dispersion of ±10μm fibers with the same loading and field of view. The value of the free-space length in the P-Col-UST sample and P-Col-N-UST sample is 3 μm and 16 μm respectively. This calculation showed that fibers are dispersed more uniformly in the UST samples compared to the non-UST samples.

Given that the size of each pixel in SHG imaging in our set-up is 0.14 μm, fibrils smaller than that are either not visible or removed during the noise filtering step in the image processing procedure. Therefore, the reported values are for the purpose of comparison between the non-UST fibers and UST fibrils and cannot be reported as the absolute value for the treated or non-treated fibers measurements.
Figure 2. Illustration of the method used to measure free-space length. The free-space length ($L_f$) is the size of the largest placed square in which the highest possible number of crossing black pixel in the placed square is zero. a) a SHG image of collagen fibrils from the UST sample, b) a SHG image of collagen fibers from the non-treated sample. $L_{AV}$ represent the average value of collagen fibrils/fibers, $L_f$ represents the free-space length.

3.3 Conclusion

Based on both qualitative and quantitative assessment of the TEM and SHG images, it was found that the collagen fibers became fibrillary after UST. It was observed that UST collagen became thinner, shorter, and more highly dispersed than non-UST collagen fibers.
4 Conclusion and Future Work

This study introduced the novel ultrasound-based treatment of mixture of collagen and cells to enhance the proliferation of human source MCF-7 cells while not disturbing the viability of them. Although a few studies have discussed the effect of sonication on the activity of some non-human source cells in vitro in a monolayer\textsuperscript{95,109}, the present study distinguishes itself with human source MCF-7 being used in a 3-D bead culture, implementing UST for the first time in this context. Indeed, as noted previously, it is clear that human source cells provide a more applicable base of information for human-related researches, adding to the value and relevance of our current study.

The preliminary findings of our study shows an increase in cell proliferation after day 5 in the sonicated samples compared to the non-sonicated sample. As noted in our observations, the enhanced MCF-7 proliferation by sonication treatment in the lengthiest culture has been shown to be advantageous for the construction of engineered tumor spheroids in vitro. Beads made of the sonicated mixture remain in spherical shapes for 20 days whereas beads made of the non-sonicated mixture degrade in only 10 days. Uniform sized spheroid is obtained from the beads made of the sonicated sample at 37°C (CV=8%).

Sonication treatment is found to be helpful in the uniform distribution of collagen fibrils throughout a mixture, to better serve the mixture’s homogeneity. Uniformly positioned collagen fibrils created by sonication treatment in the pre-bead-production mixture, along with induced cell aggregates through UST, allows for the formation of more uniform beads than those made from the non-sonicated mixture. Since sonication treatment breaks lateral hydrogen bonding in collagen fibers, it turns them into nano-sized fibrils.
The body of work presented in this thesis was aimed at producing uniform-sized spheroids in order to provide the same necrotic and quiescent layer at all the spheroids in a same batch to build a reliable *in vitro* model for anti-cancer drug efficiency test before clinical trials. This was achieved through the development of a novel treatment prior to bead encapsulation, UST. The presented work would contribute to achieving reliable treatment of ECM mixture containing human cells prior to bead encapsulation through microfluidic chip. Through the careful design of UST set-up, it can lead to the development of a reliable and efficient procedure to make uniform mixture for hydrogel bead encapsulation. This method helped in producing uniform spheroids.

To obtain more insight on the collagen concentration effect and core-shell structure being incorporated with UST, future work could investigate the effect of a shell with UST in promoting MCF-7 cells to stay inside of beads. There are few studies indicating the effect of collagen concentration on conducting fluid movement in the tumor interstitial matrix which is necessary in delivery of sufficient amounts of therapeutic agents to tumors in order to optimal therapy\textsuperscript{120,121}. Ramanujan et al. reported the importance of high collagen concentration in molecular diffusion in tumors\textsuperscript{121}. Their results revealed the major dependency of diffusive hindrance in tumors *in vivo* to the collagen concentration. Netti et al. reported the correlation between diffusion of large molecules in tumors and collagen organization and concentration\textsuperscript{122}. Future work could also investigate the effect of high collagen concentration\textsuperscript{120–122} in mimicking the natural 3-D tumor tissue since mimicking the collagen concentration similar to those found in tumor tissues is necessary to be able to produce representative models for anti-cancer drug screening.

It is also worthwhile to compare the response of TSs and micro-dissected tumor tissue to anti-cancer drugs. To validate the reliability of 3-D TSs model, anti-cancer drug efficiency test can
be carried out comparing with *in vivo* test results. This study helps in investigating the effect of UST on ultimate TSs as a model for anti-cancer drug screening.

A number of improvements can be implemented in future studies to expand upon the knowledge that has been generated through this study. For instance, exploring other UST conditions, particularly treatment duration and ultrasound power, could be beneficial. Conducting an anti-cancer drug efficiency platform would be highly informative in terms of validating the similarity of such a 3-D model to *in vivo* tumor tissue. Future studies should involve larger sample sizes to achieve higher statistical significance.

In conclusion, this study is highly beneficial to the pharmaceutical industry, since it will address the challenges in producing and using a 3-D *in vitro* model before clinical trials for drug screening. Further, producing uniform 3-D TSs with the aid of ultrasound treatment of ECM will benefit the pharmaceutical industry that is currently struggling with a lack of a straightforward procedure to produce 3-D *in vitro* models for drug screening.
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