Generation of Cell-laden Biopolymer Microgels with Tunable Mechanical Properties for Cancer Cell Studies.

By:

Alexander Kumachev

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Graduate Department of Chemistry
University of Toronto

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Abstract

This thesis describes the development of a high-throughput approach towards the encapsulation of cancer cells in biopolymer microgels with tunable mechanical properties. In particular, this thesis is focused on: i) the high-throughput generation of biopolymer microgels with tunable mechanical properties ii) the measurement of the mechanical properties of the microgels, and iii) the high-throughput encapsulation of a cancer cell line within biopolymer gels.

The microgels will be generated by (i) introducing in a microfluidic device two distinct streams of biopolymer solutions; (ii) mixing the streams; (iii) emulsifying the biopolymer and (iv) using thermosetting to transform the droplets in situ into microgels. By applying a compression force to the hydrogel microbead and measuring its deformation, the Young’s modulus and relaxation time of the microgel can be examined. The properties of cells were examined within the gels using various spectroscopic techniques such as absorption (UV-Vis) and fluorescence microscopy (fluorescent microscopy, confocal microscopy).
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1. Introduction

1.1. Effect of Cellular Environments on Cell Fate

In biological systems, cells are exposed to a multitude of extracellular environments which vary in size, structure, composition and distribution of extracellular macromolecules, and the density of cells.\(^1\) The extracellular surroundings provide important cues to guide cell fate, e.g., cell motility,\(^2\) phagocytosis,\(^3\) differentiation,\(^4\) and viability.\(^5\) Due to the importance of the properties of microenvironment adjacent to the cell (the cell 'niche'), a considerable effort has been applied over the last decade to determine the relationship between the properties of cellular microenvironments and cell behavior.

In particular, cells possess various mechanisms to sense the stiffness of the surrounding environment. Proteins on the membrane of the cell bind to extracellular molecules and subsequently, orchestrate an appropriate intracellular signaling cascade, thereby causing changes in the level of gene expression or rearrangement of the cytoskeleton.\(^6\) In turn, the level of protein expression affects the growth and the path of differentiation of the cell.\(^1\) Responses to physical stimuli occur through the formation and reinforcement of the extracellular matrix (ECM)-integrin–cytoskeleton complex.\(^6\) The binding by different amounts of integrins, as well as different integrin receptor subtypes, can modulate the strength of this extracellular-to-intracellular coupling.\(^7,8\) Furthermore, geometric confinement also impacts cellular process by regulating the cellular morphology, since the cell adapts its shape to satisfy its function within its particular environment.\(^9\) By examining the confinement of the cells, it has been established that cell fate can be switched from growth to apoptosis, regardless of the material surrounding the cell.\(^10\) Thus, by fine-tuning the composition and stiffness of artificial ECM, it is possible to control cytoskeletal contraction and promote a particular cellular response.
The microenvironment of a cell is characterized by its biochemical and mechanical properties. The biochemical – biological and chemical – properties of the cell niche are defined by the presence of specific molecules, such as metabolic gases, saccharides, lipids, proteins, cytokines, and extracellular fibres. These molecules can elicit a direct response from the cell by binding to a surface or cytoplasmic receptor, or may act to alter the cell’s niche, which would be subsequently sensed by the cell. Neighbouring cells also influence the biochemical nature of the cellular environment, due to either diffusion of the products of cellular secretion, or by forming direct cell-cell contacts within the microenvironment. The latter situation can result in the formation of cellular junctions, which would lead to the direct passage of molecules between cells, or can cause protein interactions located within the cellular membranes. Chemical or biological modification of the cell niche have been carried out in numerous works, in order to study cell cycles, migration, metabolism and survival.

On the other hand, studies of the role of the mechanical properties of cellular microenvironments are gaining the momentum. In the human body, non-mineralized tissues have elastic moduli ranging from approximately 0.01 to 100 kPa. For example, brain, muscle, and osteoid matrix have elastic moduli of 0.1-1, 8-17, and 25-40, respectively. The elastic modulus of mineralized bone is \( \sim 1.5 \times 10^7 \) kPa. Each biological environment is tailored to meet the particular functional and mechanical demands that are required from it. For example, fatty tissue is soft to provide cushioning for vital organs, while bones are more rigid, in order to protect organs and to withstand sizeable mechanical loads.

Currently, it is established that the stiffness of cellular microenvironments has many implications for cell fate. It has been established that microenvironments influence the development, remodeling, and growth of stem and progenitor cells in various types of tissues,
such as bone, muscle, brain, tendon, artery, and cartilage. Changes in the composition of the surrounding matrix have been closely related to the onset of various pathologies. For example, genetic defects in components of the connective tissue, which links similar types of cells together to form tissues, results in rheumatoid arthritis, scleroderma, poliomyositis, dermatomyositis and Ehlers-Danlos syndrome. It has been postulated that increased tissue rigidity may also heighten susceptibility to environmental perturbations, and the additive effects of the biochemical, and mechanical abnormalities may lead to the development of the disease.

Cells in tissues with abnormal stiffness are more vulnerable not only to biochemical disturbances, but also to the underlying genetic abnormalities. In patients with hereditary cancers, tumors develop in localized regions before spreading, despite the fact that every cell in the body carries the same mutation. This effect suggests that the local tissue environment is an important regulator in oncogene activation. Likewise, many oncogenes are maintained in the dormant state by environmental protective mechanisms - both mechanical and biochemical - until being triggered into an active state, a process most often observed in the older population and people with accumulated tissue damage.

Generally, in vivo studies of the changes in cellular properties in different microenvironments are correlative rather than causative, since cellular activities proceed through numerous intermediate steps that involve a wide range of biological responses. Thus, in vitro experiments using substrates with tunable rigidity have been used to explore the role of variations in tissue mechanics in disease development in the hepatic, cardiac, skeletal, muscular, and neuronal systems. In addition, embryogenesis, tumor development, and cancer metastasis have been studied in different environmental conditions.
As the cells of the embryo divide and differentiate, the surrounding matrix (niche) provides cues guiding cells along distinct differentiation lines. The effect of matrix elasticity on cell differentiation has been demonstrated in the breakthrough work of Disher et al. Stem cells seeded on substrates with identical chemical compositions but with varying elasticities have been guided by the substrate to transform into neurons, muscle cells and bone cells on soft, medium-stiffness and rigid substrates, respectively. Other groups have shown that substrate stiffness can also strongly influence cell adhesion, spreading, migration, and proliferation.

Similar cellular responses to the stiffness of niche were observed in partially and fully differentiated cells. It has been demonstrated that the sole tuning of the stiffness of the substrate enables control of the properties of human cardiomyocytes, neurons, osteoblasts, chondrocytes, endothelial cells, and epithelial cells. Human hepatic stellate cells and portal fibroblasts were both observed to differentiate to myofibroblasts on stiff polyacrylamide surfaces, mimicking the stiffness of diseased liver, but remain quiescent on soft matrices with elasticities close to that of healthy liver. Even anchorage-independent cells, such as circulating blood cells including monocytes and leukocytes, secreted different levels and types of cytokines, when plated on substrate with differing stiffness during adhesion and migration events. Therefore, the ability to promote proper force transmission by controlling the stiffness of cellular microenvironments is emerging as a new criterion for rational biomaterial design.
1.2. Substrate Regulation of Cancer Cell Growth and Metastasis

The ability of cells to sense their environment and elicit an appropriate response to it is particularly important in carcinogenesis and related signaling events.\textsuperscript{38, 39} For example, it has been established that cellular microenvironments that support proper tissue organization and function can serve as protective barriers against tumor formation.\textsuperscript{40} Abnormal extracellular cues can cause deregulation in the normal cell cycle by removing the naturally occurring cancer inhibition processes within the cell. The disruptions in these processes can result in abhorrent cell proliferation. Among the abnormal extracellular cues are elevated tissue stiffness’s, which can distort tissue structure, compromise its function and actively drive malignant transformation of premalignant cells.\textsuperscript{23} In addition, the variation in the mechanical properties of ECM may be one of the factors that control the ability of cancer cells to escape their regular environment and invade other tissues.\textsuperscript{40}

The change in mechanical properties of cellular microenvironments can magnify genetic instabilities in cells and activate dormant oncogenes (genes that can transform a cell into a tumor cell under certain circumstances). This effect can result in defective mechanotransduction, which desensitizes cells to their physical environment, thereby allowing internal oncogenic signals to overcome cellular constraints.\textsuperscript{6} The accumulation of these oncogenic signals can transform the normal cell into one a cancer cell. For example, premalignant mammary epithelial cells containing the pro-oncogene remained non-invasive when seeded on soft matrices, while on rigid matrices the cells transformed into cancer cells, and formed monolayers along the surface.\textsuperscript{41, 42}

Another area of research in cancer pathophysiology is focused on the growth and division of transformed cancer cells. These studies investigate the rate of the development of tumors, as
well as migration of individual cells out of the tumor (micrometastasis), and the generation of vascular microregions within the tumor after being cultured in and on a variety of substrates.\textsuperscript{43-45} It has been established that in 3D and 2D cultures, the cancer cells grow in the form of multicell spheroids and monolayers, respectively. As the tumor grew in 3D cultures, the number of cells that were proliferating decreased, while the proportion of quiescent cells increased. Generally, most of the proliferating cells in spheroids were located in the outer three to five cell layers of the tumor.\textsuperscript{46} These findings highlight the importance of the properties of the microenvironment that is in continuous contact with the outer proliferating layers of the tumor.

Furthermore, certain cells in the center of the tumor became deprived of the molecules required for metabolic needs, such as oxygen and glucose, and when toxic waste products accumulated, metabolic gradients formed within the tumor. The gradients resulted in cell death and necrosis, in particular, within the centers of the spheroids, as these regions were most deprived of the environmental nutrients.\textsuperscript{46} A multitude of other factors influenced tumor cell growth in 3D environments, including the cell types, packing density, and the biochemical and mechanical properties of the encapsulating medium. It has been demonstrated that by modifying the properties of the tumor environment, it is possible to control tumor proliferation for histologically different cell types.\textsuperscript{43-51}

The response of cells to their environment is also important in understanding cancer metastasis. Tumor cell populations are heterogeneous in nature and only a subpopulation of cells possesses a capability to metastasize in late stages of tumorigensis.\textsuperscript{47,48} Although tumor tissues have an elevated rigidity, highly metastatic cells are extremely deformable.\textsuperscript{49,50} The reduced cytoskeletal stiffness is required to enable metastatic cell passage through small capillaries to invade distant tissues and form secondary tumors.
Selective homing and invasion of metastatic cells to distant organs seems to rely, in part, on the mechanical properties of cellular microenvironments in the target tissues, which provide motility and growth advantages for invading cells to take root and proliferate. While some cancer cells appear to grow equally well on substrates with different stiffnesses,\textsuperscript{41, 51} others show a sensitivity to matrix rigidity, which may be related to their preferential metastatic targets. For example, metastatic breast cancer cells with specific affinities for lung (soft) or bone (stiff) tissue exhibited preferential growth on the substrates with elasticity mimicking that of the target organ.\textsuperscript{42} An improved understanding of how the microenvironment couples with genetic factors that are implicated in tissue-specific metastasis may allow for prediction of potential sites of secondary tumor formation and facilitate preventive intervention.

The findings described above have sparked strong interest in the role of mechanical properties of cellular environments on cancer cell proliferation and metastasis. Thus, the ability to control cell division and migration by controlling cellular microenvironments is emerging as a new concept in studies of tumor formation, as well as the development of potential therapies for cancer treatment.

### 1.3. Comparison of the Effects of Two-Dimensional and Three-Dimensional Cellular Environments on Cancer Cell Fate

In early studies cell growth in different environments, the relationship between the behavior of cancer cells and the mechanical properties of the matrix has been studied for cells seeded on planar substrates. More recently, there has been an increased focus on studying growth of cancer cells in 3D environments, in which cells may experience mechanical signaling that is
distinct from that experienced on a two-dimensional (2D) matrix.\textsuperscript{4, 52} These 3D environments provide a physiological model that more accurately replicates microenvironments, compared to monolayer cell cultures in 2D,\textsuperscript{53-55} and can be functionalized to include key features of naturally derived ECMs, such as interactions \textit{via} integrin-binding sites. Consequently, 3D culture models are becoming increasingly crucial research tools, in particular, in cancer cell biology, as they bridge traditional cell culture on plastic substrates and \textit{in vivo} experiments that are done in a 3D format.\textsuperscript{56, 57}

Current, \textit{in vitro} 3D culture models include the growth of multicellular spheroids in suspension, as well as embedding cells within naturally derived extracellular matrices, e.g., collagen-laminin-heparansulphate gels and Matrigel\textsuperscript{46} (a gelatinous protein mixture, which resembles the complex extracellular environment found in many tissues).\textsuperscript{58, 59} Cell culture in 3D single-component synthetic and biopolymer gels may help to dismantle the complex cell-ECM interactions of carcinogenesis into a simpler and more defined \textit{in vitro} setting.\textsuperscript{60}

In a recent work, 2D and 3D platforms were used to culture, model and analyze interactions important for cancer progression (Fig. 1.1).\textsuperscript{60} Two cancer cell lines derived from human epithelial ovarian cancer were used in this work. The ability of both cancer cell lines to form multicellular spheroids from single cells, as well as their resistance to anti-cancer drugs, was compared to cell monolayers grown on conventional 2D plastic surfaces.\textsuperscript{61, 62}

The influence of the characteristics of extracellular microenvironment on the behavior of the cells was established by altering the physical, biological and biochemical properties of synthetic microenvironments.\textsuperscript{60} Similar to previous reports on cancer cells grown in 3D cultures, as well as \textit{in vivo} observations, the cancer cells grew as multi-cellular spheroids within the 3D matrices.\textsuperscript{46, 59, 63} The spheroids continued to grow for up to 28 days. It was also demonstrated that
upon increasing material stiffness, the spheroid formation and the size of spheroids were reduced. In addition, irregularly shaped and scattered spheroids formed in softer hydrogels, while compact and dense spheroids formed in stiffer hydrogels. In contrast, cells plated on 2D plastic surfaces formed monolayers of cells\(^6\) which reached confluence within 5 days and afterwards, their proliferation rates decreased.

**Figure 1.1** a) Schematic illustration of the differences in epithelial ovarian cancer (EOC) cell proliferation and organization on two dimensional plastic (left) and in physiologically applicable three dimensional hydrogels (right) cultures. B) Both EOC cell types OV-MZ-6, SKOV-3 e formed in 2D (left) typical monolayers shown by phase contrast (top panel) microscopy images. In 3D, both EOC cell types embedded within hydrogels grew as spheroids imaged by phase contrast microscopy. Scale bars are 75 µm.

The extra-dimensionality imposed by encapsulating cells in 3D hosts is influences the spatial organization of cell surface receptors engaged in interactions with surrounding cells, as well as rearrangement of cell cytoskeleton\(^6\). Furthermore, there is an additional physical constraint experienced by cells in 3D space\(^6\). Combined, these spatial physical aspects affect the signal transduction from the outside to the inside of cells, and ultimately, have a tremendous
impact on gene expression and cell behaviour. Previous 3D cancer studies have established that phenotypical differences (resulting from the changes in gene expression) between normal and malignant epithelial breast cells are observed exclusively in 3D cell culture.59, 67

Cells cultured in 3D and 2D environments also responded differently to chemicals that were introduced into the system, because the cells adapted differently to the environmental conditions.68 Ovarian cancer cell spheroids formed in 3D matrices had an enhanced resistance to chemotherapeutics, in comparison to 2D cultures, in particular to the anticancer drugs Paclitaxel and Cisplatin.62, 60, 69 Paclitaxel treatment of epithelial ovarian cancer cells in 2D cultures decreased cell survival and proliferation by 80%, while the same treatment led to only 40-60% reduction in cell viability in cell spheroids formed in 3D environments.60 In vivo experiments indicated that cells grown as spheroids developed multicellular-dependent chemoresistance.70, 71 The elevated chemoresistance of cell spheroids to anti-cancer reagents has been attributed to several mechanisms, including a decreased ability of the drugs to penetrate into the cell, an increased pro-survival signaling by the cell, and/or upregulation of genes conferring drug resistance.60 Therefore, evaluations of chemosensitivity to therapeutic agents can be done under circumstances, which reflect pathophysiological events seen in vivo.

Although 3D culture systems have profoundly improved our ability to understand cancer pathology, they have experimental limitations. Multicellular spheroids grown as independent cells in the absence of their physiological ECM have abnormal interactions with their extracellular environment, resulting in a cellular response not entirely representative of in vivo cell behaviour.72 Conversely, the standard ECM analogue Matrigel™ that has ECM-like biological properties offers limited design flexibility in fine-tuning the biological and physical matrix properties.33
Due to the complexity of *in vivo* extracellular environments and the complicated coupled interactions of their chemical and mechanical properties, many studies of the role of environments on cell fate have been conducted *in vitro* on model systems. Hydrogels of synthetic and biological polymers with various chemical compositions and mechanical properties have been utilized as substrates for cell seeding and as 3D matrixes for cell encapsulation. Typical synthetic polymers used for cell culture included collagen-coated polyacrylamide gels, poly(methyl methacrylates), and poly (ethylene glycol). Biological polymers included alginate, collagen, gelatin, agarose, and hyaluronic acid.

1.4. Microfluidic Generation of Hydrogel Microbeads (Microgels)

Polymer hydrogel microbeads with dimensions in the micrometer size range (microgels) have a variety of applications in the biomedical field. For example, microgels generated from biopolymers or biodegradable synthetic polymers have been used for the encapsulation of biologically active species such as enzymes, proteins, drugs, and cells. The encapsulation of cells within microgels with a controlled size, composition and structure paves the way for the generation of large libraries of cellular microenvironments. Microgels can be prepared by several methods, including spray drying, inverse emulsification techniques, coacervation, and shear break-up.

These methods produce hydrogel polydisperse particles with a relatively high polydispersity. Polydispersity of microgels can be a disadvantage in the encapsulation of the controlled number of cells in the microgel interior and subsequent cell studies. In the past decade, microfluidic synthesis and assembly of microgels have offered a facile method to the
continuous production of micrometer-size hydrogel polymer particles with unprecedented control over their sizes, shapes, and morphologies.\textsuperscript{80-82}

Microfluidic generation of microgels includes two steps: microfluidic emulsification of an aqueous solution of a monomer, an oligomer, or a polymer in an immiscible non-polar liquid, followed by \textit{on-chip} or \textit{off-chip} gelation of the precursor droplets. The dimensions, shapes and morphologies of microgels are conveniently tuned by changing the flow rates of the droplet and continuous liquids and by varying the properties of the liquids.\textsuperscript{83} \textit{On-chip} polymer gelation (that is, gelation occurring in the precursor droplets during their residence in microchannels) paves the way for the continuous generation of microgel beads with a narrow size distribution.

Emulsification can be carried out in microfluidic devices with different geometries. Figure 1.2 shows two designs of microfluidic droplet generators that are most frequently used for emulsification: a flow-focusing device and a T-junction device.\textsuperscript{79}

In a flow-focusing device (Figure 1.2a) an aqueous phase and a nonpolar liquid (labeled as A and B, respectively) are introduced to the central and side channels, respectively. A stream of the aqueous droplet phase is focused in the orifice due to the shear imposed by the stream of the continuous phase. A highly periodic break-up of the thread of the aqueous phase yields droplets with a narrow size distribution.\textsuperscript{84} The size of droplets is controlled by varying the ratio of flow rates of the continuous-to-droplet phases: with increasing flow rate ratio, the diameter of droplets (and the resulting microgels) decreases.

In a T-junction droplet generator (Figure 1.2b), as the droplet phase flows into the junction, the continuous phase forms a thin film layer between the disperse phase and the walls of the device, causing an increase in pressure which “squeezes” the disperse phase to form a
droplet. The size of droplets typically decreases with increasing ratio of flow rates of the continuous-to-droplet phases.

**Figure 1.2.** Schematic drawings of various types of (a) flow-focusing, (b) T-junction microfluidic droplet generators.\(^7^9\)

Following the formation of the precursor droplets, the gelation can be achieved by chemical or physical mechanisms. Chemically crosslinked microgels are produced by emulsifying aqueous solutions of monomers, and subsequently, polymerizing or crosslinking these monomers in the droplets. This method was used for the preparation of microgels such as \(N\)-isopropylacrylamide,\(^8^5\) and dextran-hydroxymethyl methacrylate.\(^8^6\) The advantage of covalently crosslinked microgels is that they are mechanically strong, however, these particles cannot be easily enzymatically cleaved and metabolized.

Physically gelled microgels are generally prepared from biological polymers, such as agarose, alginate, carrageenan, chitosan, gelatin, or pectin.\(^1^1, 8^7-8^9\) Crosslinking of polymers is achieved by hydrophobic interactions, hydrogen bonding, or ionic interactions.\(^9^0\) These forces are induced by changing the temperature of the system (e.g., by thermosetting agarose or gelatin)\(^9^1\), or introducing crosslinking ions inside the droplets (e.g., for \(\text{Ca}^{2+}\)-crosslinked alginate).\(^9^2\) The physical methods lead to the preparation of gels with low toxicity and are
beneficial for cell encapsulation. For example, cell encapsulated in alginate and agarose microgels had a high viability of ca. 70-75%, which supported future applications of these microgels in cell studies. The challenge is the microfluidic emulsification of precursor polymer solutions: even at moderately high polymer concentration, the solution may have a high viscosity, which makes the emulsification difficult.

In contrast with microfluidic encapsulation of cells in droplets, encapsulation within microgels offers the ability to transfer the cell-laden microgels into an aqueous culture medium for prolonged cell culture and subsequent studies.\textsuperscript{79} The encapsulation of cells in micrometer-size three-dimensional hydrogels offers the ability to control the shear forces imposed on cells, the ease of cell visualization, the reconfigurability of the cell-laden hydrogel modules, and the transport of oxygen, nutrients, growth factors, and waste.\textsuperscript{93} These advantageous characteristics have led to a large number of biomedical applications of microscopic cell-laden hydrogels, including clinical diagnostics, pharmaceutical research, and regenerative medicine.\textsuperscript{94}

Currently, most of the applications of MF cell encapsulation have been reported for cell-laden droplets, e.g., to create cell arrays for single-cell bioassays, including measurements of single-cell respiration rates,\textsuperscript{95} for drug screening on the scale of a single cell,\textsuperscript{96} in cell viability studies within different microenvironments,\textsuperscript{97,98} and for monitoring cellular gene expression\textsuperscript{99} and intercellular interactions.\textsuperscript{100,101} In addition, it offers the ability to transfer cell-laden microgels into an aqueous culture medium for prolonged cell culture.

Importantly, the formation of libraries of cellular microenvironments often relies on the simultaneous change in several properties of microgels. For example, increase in microgel stiffness by increasing polymer concentration will concurrently change microgel pore size, fiber structure, and local polymer deformability, which will influence cell motility. Decoupling of the
different properties is a significant challenge, which should be addressed through collaborations of groups specializing in microfluidics, cell biology, and polymer and materials science.

Currently, analysis of encapsulated cells is primarily performed using optical methods such as optical microscopy, however more efficient cell analysis can be performed by flow cytometry, using microgel degradation and the release of the cells. Alternatively, the generation of smaller microgels (with dimensions below 40 µm) would enable direct injection of the cell-laden microgels into standard laboratory flow cytometers and allow for the analysis of the encapsulated cells without the need to remove them from the microgels. Finally, innovation and improvements in microfluidic platforms will enable cell encapsulation, culture, and characterization using integrated probes, all conducted on a miniaturized lab-on-chip.

The ability to change in a controllable, high-throughput manner the physical and chemical properties of the hydrogel, by changing the relative amounts of gel components, paves the way for studies of the role of the properties of 3D microenvironments on cell fate.

1.5. Properties of Agarose Hydrogels

Agarose is a neutral polysaccharide extracted from agar, a gelatinous substance that accumulates in the cell walls of agarophyte red algae. The molecule of agarose is composed from alternating units of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose (Figure 1.3)
Agarose dissolves in water at temperatures above 60-95 °C and forms a hydrogel as the solution is cooled to 25-40 °C. The mechanism of the formation of agarose gels is illustrated in Figure 1.2. At elevated temperatures individual agarose molecules exist in solution as flexible random coils. Gelation begins from the creation of double helices, due to the formation of intermolecular hydrogen bonds between agarose coils. The presence of the (1,4)-linked sugar units in the agarose chain introduces kinks into the double helices, which leads to the termination of helix formation. The truncated helices associate with individual agarose coils or other helixes to form larger agarose aggregates, which associate with each other and form a macroscopic gel (Figure 1.4).

The exact temperatures of agarose dissolution and gelation depends on the molecular weight and concentration of agarose in solution, as well as the type of agarose used. The
temperature of the formation of agarose gels reduces with an increasing molecular weight of the polymer and the concentration of agarose in the solution. Agarose exhibits hysteresis with respect to the temperature of the solution-gel transition: it gels at temperatures that are lower than the temperatures of gel melting. For example, when a solution of agarose is cooled, it remains liquid at 37 °C, but once the agarose is gelled, it exists in a gel state upon heating to 37 °C, the temperature used for cell culture. The gelation temperature range of agarose solutions may be tuned by changing the degree of hydroxyethylation per agarobi unit.

Agarose gels are extensively used for cell encapsulation and culture. Agarose is non-adsorptive to proteins and non-adhesive to cells, however, it can be readily functionalized with adhesion or signaling molecules, such as collagen or growth factors, respectively. In general, the mechanical properties of agarose gels can be tuned by varying agarose concentration in the solution prior to its gelation: the strength of a gel increases with an increasing agarose concentration. Agarose can form hydrogels at concentrations as low as 0.45 wt% and as high as 30 wt%. The preparation of gels with a higher polymer concentration is limited by the solubility of agarose in aqueous solutions.

The preparation of gels with a higher polymer concentration is limited by the solubility of agarose in aqueous solutions.

1.6. Characterization of the Mechanical Properties of Polymer Hydrogels

Techniques such as scanning transmission electron microscopy and surface plasmon resonance have improved the capacity to probe, visualize, and quantify structures and properties on both the surface and inside the microstructures. The general methods used for studies of the mechanical properties of biological samples, such as gels, include pipette aspiration, atomic
force microscopy (AFM), tensile tests using laser tweezers, and strain-controlled rheometry, e.g., including parallel plate, cone and plate, Coutte, and conical cylinder compression tests.

The mechanical properties of gels are typically characterized by their Young's and shear moduli. The Young's modulus is measured by the indentation method using the stress-strain equation

$$E = \frac{\text{tensile stress}}{\text{tensile strain}} = \frac{\sigma}{\varepsilon} = \frac{F/A_0}{\Delta L/L_0} = \frac{FL_0}{A_0\Delta L}$$ (eq. 1.1),

where $E$ is the Young's modulus, $F$ is the force applied to the gel; $A_0$ is the original cross-sectional area of the gel, to which the force is applied; $L_0$ is the original length of the gel, and $\Delta L$ is the amount by which the length of the gel changes.

The method selected for the measurements of the elastic modulus of a gel depends on sample preparation. Generally, measurements of elastic or shear moduli are carried out on macroscopic gel samples by using conventional rheometers, whereas micropipette aspiration and atomic force microscopy (AFM) methods are used to characterize micrometer-size samples.

Micropipette aspiration imposes a deformation onto the surface of the micrometer-size object (e.g., a cell, a microgel, or a vesicle), by the complete or partial suction of the sample into the orifice of a glass micropipette using a small negative pressure. The shear modulus of the aspirated sample is determined from the relationship between the suction pressure, the diameter of the pipette orifice, the diameter of the undeformed object, and in the case of partial suction, the protrusion length of the object in the pipette. Several challenges in the usage of this technique including the large effect of mechanical vibration, temperature and humidity
fluctuations, as well as difficulties in aligning the pipette tip with the focal plane of the imaging system and the surface of the target object. In addition, pipette aspiration experiments are typically performed in air, resulting in a continuous loss of the sample liquid during the course of experiment. Therefore, for polymer hydrogels, a constant loss of liquid would result in a change in the mechanical properties with time.

An alternate technique to studying the mechanical properties of micrometer-size soft objects such as hydrogels is the AFM method, in which a microgel is indented with a cantilever or tip probe. The surface of the tip may be conjugated with biological molecules such as lipids or proteins. A laser is aligned with the edge of the cantilever directly above the point of indentation on the microgel. The laser is reflected from the back surface of the cantilever onto a photodiode that acts as a position-sensitive detector.

![AFM Diagram](image)

**Figure 1.5.** Schematic of the AFM setup which probes the mechanical and structural properties of a sample.

When the measurements are carried out using a tipless cantilever, the diameter of the microgel has to be equal to- or smaller- than the width of the cantilever to ensure an equal
compression across the entire sphere. It is highly desirable to conduct these measurements in water, so that the fast evaporation of water within the microgel does not change its properties.

For studies of the mechanical properties of microgels as cellular environments, the use of atomic force microscopy is particularly useful, because it allows the examination of the local microenvironment surrounding the encapsulated cell during its culture leading to e.g., growth or differentiation. It also enables a study of the feedback between the cell and the adjacent surroundings: as the cell secretes proteins, ions and byproducts of cell metabolism, the environment surrounding the cell adapts to the changes.
2. Materials and Methods

2.1. Materials

Ultra-low gelling temperature agarose with a gelling temperature in the range of 8-17 °C was purchased from SeaPrep (Lonza, Switzerland). Phosphate Buffer Saline (PBS) and Hank’s Buffer Saline Solution was purchased from Gibco-BRL (Rockville, MD, U.S.A). A non-ionic surfactant Span-80, mineral oil and glycerol were supplied by Aldridge-Sigma Canada. Mineral Oil with a viscosity of 30 cPs, and an oil soluble surfactant Sorbitan monooleate 80 (Span 80) were purchased from Sigma-Aldrich (Canada). All biopolymer solutions were filtered and sterilized using syringe filters (Millex GP, 0.22 µm pore size, Millipore, Canada).

2.2. Methods

2.2.1 Preparation and Characterization of the Agarose gels

2.2.1.1 Preparation of Agarose Solutions

A 5 wt.% stock agarose solution was prepared by dissolving 2.5 g of agarose in 47.5 g of a 1 wt.% PBS (phosphate buffer solution) under heating to 70 °C, and subsequent cooling of this solution to room temperature. The stock solution was used to prepare the 0.75, 1, 2, 3 and 4 wt % agarose solutions used in the experiments. The solutions were kept in a gelled state at r. t., but before the microfluidic experiments they were heated to 70 °C and then cooled to 37 °C.
2.2.1.2 Preparation of Planar Agarose Gels

Two glass slides were separated by a rubber spacer with a thickness of 2.3 mm and were clamped together to ensure a constant height between the glass slides. Agarose solutions with varying concentration were poured into the rectangular space between the glass slides. The sample was placed into a 4°C fridge for 45 min to allow the solution to gel. The agarose gel was removed from the fridge, peeled off the slide and placed into a solution of 1 wt% PBS for 24 hrs prior to measurements of its mechanical properties. The thickness of the different gels was measured using a camera (CAM101, KSV Instruments LTD, Finland), using a glass slide as a scale.

2.2.1.3 Viscosity and Surface Tension Measurements

The measurements of viscosity of the agarose solutions were carried out at 32 °C in a Brookfield DV-III rheometer (MA, USA) at a constant shear rate of 10 s⁻¹. After each measurement, the sample was replaced with a fresh agarose solution, in order to avoid the effect of evaporation during the measurement.

The value of interfacial tension, $\gamma_{12}$, between the agarose solution and the oil phase was determined by the pendant droplet method using a tensiometer (DSA100, Kruss). A droplet of the agarose solution with a particular concentration was immersed into the 3 wt. % solution of SPAN 80 and light mineral oil.
2.2.2 Microfluidic Preparation of Agarose Microgels

2.2.2.1 Fabrication of Microfluidic Devices by Soft Lithography

Microfluidic (MF) devices were fabricated in poly(dimethyl siloxane) (PDMS) elastomer (Sylgard 184, Dow Corning) using a standard soft-lithographic procedure\(^3\). Masters with microchannels were prepared with features of SU-8 25 photoresist (Micro-Chem) in bas-relief on silicon wafers with a diameter of 3 inches by a photo-lithography technique.\(^{126}\) PDMS elastomer was prepared by mixing a prepolymer with the cross-linking agent in a weight ratio of 10:1, respectively, and placing the mixture was placed under vacuum for 25 min to remove air bubbles. The prepolymer mixture was then poured onto the master and cured in an oven at 75°C for 24 hours. The replica was peeled from the master and cleaned with tape. Inlet and outlet holes were punched into the designated positions in the device using a needle. The replica and a PDMS substrate without any bas-relief features were oxidized in a plasma cleaner chamber (PDC-3XG, Harrick, USA) for 90 sec. The plasma-treated replica and substrate were brought into contact and sealed.

For cell encapsulation experiments, the sealed MF device was hydrophobized by maintaining it for 12 h in an oven at 140 °C and subsequently exposing it to the vapor of 1,1,1,3,3,3- hexamethyldisilazane (99.9% pure, Sigma, U.S.A.). During silanization, the MF devices were placed on a hot plate at 80 °C for 1 h. The silanizing agent was placed into a 25 mL vial sealed with a sleeve style rubber stopper (Wheaton Science Products, U.S.A.). An N\(_2\) gas was bubbled through the silanizing agent. The vapor of 1,1,1,3,3,3- hexamethyldisilazane was supplied to the MF device via polyethylene tubing (Small Parts, U.S.A.). The 17 gauge polyethylene tubes (Small Parts, U.S.A) were inserted into the inlet holes
and sealed with epoxy glue. Prior to experiments, the MF devices were stored in a desiccator with CaCl$_2$ to avoid exposure to moisture).

### 2.2.2.2 Microfluidic Generation of Agarose Microgels

The generation of agarose microgels with precisely controlled dimensions and tunable elasticity was achieved by MF emulsification of solutions with varying agarose concentration and subsequent rapid gelation of the precursor droplets. The high-throughput variation in agarose concentration was achieved by introducing into a MF droplet generator two streams of agarose solutions, one with a high concentration of agarose and the other one with a low concentration of agarose, at varying relative volumetric flow rate ratios of the two streams. The microgels were imaged using an optical microscope (Olympus BX51) equipped with a digital camera (CoolSnap ES, Photometrics, Roper Scientific). The diameters of at least 50 microgels were measured in each sample using ImagePro Plus software (Media Cybernetics Inc.). The distributions of microgel diameters were fitted to the Gaussian distribution. The polydispersity of microgels was measured as the standard deviation in their dimensions divided by the mean diameter. A more detailed description may be found in section 3.1.

### 2.2.3 Generation and Characterization of Cell-laden Agarose Gels

#### 2.2.3.1 Generation of Cell-laden Microgels

The human leukemia cell line AML2 was used in the present work. Cells were removed from cell culture media and stained with a fluorescent CMTMR cell surface marker in a ratio of 1.5 µL per 1 mL of cell solution. Cell suspensions were prepared to a concentration of $1 \times 10^6$
cells/mL in the agarose solutions described above. Two suspensions containing an equal concentration of cells but different concentrations of agarose were supplied into the two inlet channels of the MF device. The total flow rate of the droplet phase was 0.12 mL/hr. The cell-laden agarose microgels were collected in a conical tube placed in an ice bath for 20 min. The tube was maintained in the ice bath for an additional 20 min. The microgels were then centrifuged at 1000 rpm at 4 °C for 5 min, washed once with PBS buffer, and placed in new PBS buffer.

2.2.3.2 Cell Culture

Acute Myeloid Leukemia (AML2) cells were acquired from consenting donors according to the procedures accepted by the ethics board of Mt. Sinai hospital (Toronto, ON, Canada). Cells were cultured under sterile conditions in a media composed of Dulbecco’s Modified Eagle Media (DMEM, Gibco-BRL) supplemented with 15% (v/v) Fetal bovine serum (FBS, Gibco-BRL). Cells were passaged every four days (1:6 split ratio). Cells were maintained under sterile conditions and maintained in a 5% CO₂ humidified incubator at 37°C. Agarose bulk and microgels laden with AML2 cells were cultured under identical conditions. Fluorescence microscopy images of encapsulated cells were captured using a Zeiss Microscope (Axio Observer D1, U.S.A.) coupled with a digital camera (Axio Cam HRm, Zeiss, U.S.A.).

2.2.3.4 Analysis of Cell Growth in Bulk Agarose Gels

Cell-laden macroscopic gels with different concentrations of agarose were prepared in 96-well plate. 100 µL of cell media was added to each well and the samples were incubated for 7 days under standard conditions for cell culture described above. On the 7th day, 10 µL of CCK-8
dye (Dojindo Molecular Technologies Inc., Japan) was added to each sample and was incubated in 5% CO₂ at 37 °C for 3 hours. The 96-well plate was then placed in a UV/Vis plate reader (SpectraMAX GeminiXS, SOFTmax Pro). Absorbance measurements were acquired at 450 nm (the λmax for CCK-8 dye) for the samples.

2.2.4 Characterization of the Mechanical properties of Agarose Gels

2.2.4.1 Measurements of the Young’s Modulus of Bulk Agarose Gels

The Young’s modulus of macroscopic agarose gels was determined using an Instron 5848 Microtester with a 50N load cell. The agarose gel slab stored in a PBS buffer was cut into cylinders with a diameter of 18 mm and a height of 2.3 mm. The sample was loaded into the microtester and compressed at a rate of 0.23 mm/min. The compression of the gel was conducted at 22 ºC (room temperature).

2.2.4.2 Modification of Gold-Coated Platinum AFM Cantilevers

Gold-coated platinum cantilevers were conjugated with thiol-terminated methoxy poly (ethylene glycol) (CH₃O-PEG-SH) (RAPP Polymere- GMBH, Germany) to reduce the adhesion of the agarose hydrogels to the cantilever during AFM measurements. The cantilevers were first irradiated with UV light for 15 min., and subsequently, rinsed with acetone and deionized water, to remove dirt from the surface. The cantilevers were placed in a 1.0 mM solution of (PEG-SH) in deionized water for 5 hrs and then rinsed with deionized water. The successful modification of the cantilever was verified by conducting a similar modification of the macroscopic gold
substrate with the area of 10 mm$^2$ and measuring the contact angle of the modified surface (KSV Instruments LTD, Finland).

2.2.4.3 Measurements of the Young`s Modulus of Bulk Agarose Gels using AFM

All AFM measurements on the macroscopic agarose gel sheets were performed using an atomic force microscope (MFP-3D, Asylum Research, Santa Barbara, CA) with conducting probe module—a modified cantilever holder with a trans-impedance amplifier, and related software for operating mode-control (ORCA kit, Asylum Research, Santa Barbara). Pyramidal-tipped silicon-nitride triangular cantilevers (Bruker, U.S.A) with a spring constant of 0.15 N/m were used to compress the gels. The dimensions of the tip were measured by analyzing scanning electron microscopy images. The force indentation results were fitted to the modified Hertz law equation using IgorPro software as in:

\[
F = \frac{2 \tan \alpha}{\pi (1 - \nu^2)} E \delta^2 \tag{eq. 2.1}
\]

where $F$ is the force (pN), $E$ is the Young’s modulus (Pa), $\alpha$ is the half-angle of the tip, $\nu$ is the Poisson’s ratio (assumed to be 0.5), and $\delta$ is the indentation distance.
2.2.4.4 Measurements of the Young`s Modulus and Relaxation Time of Agarose Microgels

AFM measurements on the agarose microgels were performed using the method described above for the macroscopic gels, however tipless silicon-nitride or gold coated silicon-nitride cantilevers were used with a spring constant of 0.03 N/m and a width of 100 µm were used (Nanoworld, Switzerland). Gold-coated silicon tipless cantilevers (Applied NanoStructures, U.S.A) with a spring constant of 0.1 N/m and a width of 43 µm were also used for microgel compression experiments. In all AFM experiments, the spring constant of the cantilever was experimentally determined during a calibration step using the thermal noise method, and was often different from the manufacturer’s given spring constant.

AFM measurements were conducted by placing an agarose microgel in PBS buffer on a 10 mm² gold substrate (Ssens Bv, Netherlands). The tipless cantilever was lowered and aligned with the center of the microgel. A loading force was applied to compress a microgel at a frequency of 0.2 Hz, until a set force of 30 nN or 10 nN was reached for gels measured at room temperature, and 37 °C, respectively. For each microgel composition, a minimum of five microgels were examined by collecting six force-distance curves for each of them. The force indentation results were fitted to the following modified Hertz law equation using IgorPro software:¹²⁸ ¹²⁴

\[
\alpha = (3\pi)^{2/3} P^{2/3} \left( \frac{1 - \sigma^2}{E} \right)^{2/3} \left( \frac{1}{D} \right)^{1/3}
\]  

(eq. 2.2),

where \(\alpha\) is the indentation distance, \(P\) is the force (pN), \(\sigma\) is the Poisson’s ratio (which was assumed to be 0.5), \(E\) is the Young’s modulus (Pa), and \(D\) is the microgel diameter.
The relaxation of the microgels after compression was studied in stress relaxation measurements. After the initial loading force was applied at a frequency of 0.2 Hz, the cantilever position was maintained for 1 s, that is, the cantilever dwelled. After the dwelling, the cantilever was retracted from the microgel to reach the original position above the point of contact with the microgel. The dwell portion of the force vs. time curves was fitted to the Kohlrausch-Williams-Watts function (stretched exponential function) using IgorPro software:

$$F(t) = F_r \exp \left[ -\left(\frac{t}{\tau}\right)^\beta \right] + F_\infty$$

(eq. 2.3),

where $F_r$ is the amplitude of the relaxation force (nN), $F_\infty$ is the force after a length of time much longer than the mean relaxation time $\tau$, and $\beta$ is the stretching exponent. The stretching exponent provided a measure of the distribution of the relaxation times in cases when the system could not be fitted to a single exponential decay.

Following the dwell phase, the cantilever was retracted from the surface of the microgel, thus completing the compression-dwell-retraction cycle (Figure 2.2). Alternatively, the dwelling phase may be removed so that the cycle consists of only a compression and retraction.

**Figure 2.2.** Standard force-time acquired for a microgel showing the approach, dwell, and retract segments, respectively.
The Young’s modulus and relaxation time of microgels at 37 °C were measured by placing the gold substrate coated with the microgels into a heated fluid cell. A thermocouple was coupled to the fluid cell. The microgels were placed on the substrate, and the cell was sealed using a rubber spacer. The temperature was increased at a rate of 3 °C/min and upon reaching 37 ± 0.1 °C, the microgels were incubated for 1 hr prior to the measurement.
3. Microfluidic Generation of Cell-laden Microgels with Different Agarose Concentrations

The motivation behind studying the microfluidic emulsification of agarose solutions was to develop a process of the rapid, continuous generation of microgels with controllable mechanical properties. First, we determined the effect of the concentration of agarose solution on the size of precursor droplets. The viscosities of the agarose solutions were measured to relate the viscosity to the oil flow rate needed to emulsify the solution. Secondly, the effect of the flow rates of liquids was studied to achieve control over droplet (and microgel) size. Finally, the variation in the sizes of precursor droplets and agarose microgels was studied to ensure that the generated microgels had a narrow size distribution.

3.1. Microfluidic Preparation of Agarose Microgels

Agarose microgels with varying mechanical properties were produced by the MF emulsification of solutions with varying agarose concentration and subsequently to that, gelling the precursor droplets by thermosetting. The MF device contained a T-junction droplet generator, which was followed by a straight downstream channel for partial gelation of droplets of the agarose solution (Figure 3.1).
Two agarose solutions with concentrations of the polymer of 3.0 and 0.5 wt.% were supplied to the two inlets of the MF device using two independently controlled syringe pumps (Harvard Apparatus 33 Dual Syringe Pump, U.S.A.). Later in the text we refer to the solutions with agarose concentration of 3.0 and 0.5 wt.% as to Streams 1 and 2, respectively. Both solutions were heated to 37 °C by placing the syringe pumps in an oven. The tubing connecting the syringe pumps to the MF device was enclosed in a larger tube that was also heated to 37 °C. At the point of emulsification the temperature of the solutions was reduced to 32 +/- 1.0 °C (verified by measuring the temperature with a thermocouple). In the MF device (Figure 2.1), the solutions were mixed in a serpentine channel with the length of 250 mm. Thorough mixing of the solution in the wavy channel was verified by introducing a blue food dye in Stream 1 and monitoring the distribution of the dye in the mixed solution along the mixing channel.

The concentration of agarose in the droplets was varied by tuning the ratio between the volumetric flow rates of the Streams 1 and 2, while maintaining the total flow rate of the streams
at $Q_d = 0.12 \text{ mL/hr}$. To generate 4 or 5 wt% microgels, two syringes that contained agarose solutions with identical concentrations supplied the solutions into the inlets of the device.

At the T-junction, the stream of the mixed agarose solution broke up and released droplets of the mixed agarose solution. The droplets traveled 250 mm in the downstream channel towards the outlet of the MF device and then exited into an outlet tubing. The outlet tubing was enclosed by a hose connected to a water circulator cooled to 2 °C by a 1:4 vol. mixture glycerol and water. The droplets moved through the cooled tubing for approximately 5 min and were then collected in a 15 mL conical tube filled with HBSS solution. The tube was placed in an ice bath for 45 min to ensure complete gelation of the microgels. The resulting suspension was centrifuged at 1000 rpm at 4 °C for 5 min, and washed with twice with an HBSS buffer.

We set the following requirements to the system and the process of the generation of cell-laden microgels.

(i) The concentrations of agarose in the concentrated and dilute solutions (stream 1 and 2, respectively), as shown in Figure 3.1, had to be such that sufficient mixing between the solutions occurred in the serpentine channel and controlled emulsification took place in the MF device;

(ii) The lowest concentration of agarose, $C_{ag}$, in the precursor droplets was limited by the integrity of agarose microgels in PBS solution at 37 °C under cell culture conditions;

(iii) Microgels derived from solutions with varying $C_{ag}$ had to have uniform, well-defined volumes;
(iv) To avoid the broadening in the polydispersity of microgels due to their coalescence in the outlet tubing, it was important to achieve sufficient gelation of droplets in the downstream channel of the MF device (complete droplet gelation occurred off-chip);

(v) A 100% cell encapsulation efficiency was achieved by using a Poisson distribution equation:\textsuperscript{130}

\[
P(x, \lambda) = \frac{e^{-\lambda} \lambda^x}{x!}
\]  

(3.2),

where P(x, \lambda) is the fraction of microgels containing x cells, and \lambda is an average number of cells per microgel. For the exemplary concentration of cells in the droplet phase of 8 x 10\textsuperscript{6} cell/mL, we estimated that a 100% encapsulation efficiency would be achieved by generating droplets with a diameter of 110 \mu m.

In MF experiments, we denoted \alpha as the ratio of the flow rate of the agarose solution with a concentration of 3 wt.% to the flow rate of the agarose solution with a concentration 0.5 wt. % (Stream 1-to-Stream 2). The value of \alpha was tuned from 0.11 to 9.0 and the total volumetric flow rate of agarose solutions, Q_d, was 0.12 mL/hr.

Figure 3.3 (a-d) shows typical optical microscopy images of the droplets of agarose solution that were generated at various values of \alpha. The droplets formed in the pinching regime without obstruction of the downstream channel, due to the shear stress imposed by the oil phase.\textsuperscript{131} The diameters of droplets were tuned to be in the range from 125 to 100 \mu m by varying the flow rate of the continuous oil phase from 0.3 to 1.5 mL/hr. The frequency of droplet generation was \sim 40 s\textsuperscript{-1}.
Figure 3.3. Optical microscopy images of agarose droplets forming at a T-junction of the MF device. The images of droplets in (a) and (b) were taken at the flow rate of the continuous phase of $Q_o$ of 0.4 and 0.6 mL/h, respectively, at $\alpha = 0.11$. The images of droplets (c) and (d) were taken at $Q_o = 0.9$ mL/h at $\alpha$ of 0.25 and 1.50, respectively. $Q_d = 0.12$ mL/h. The scale bar is 100 $\mu$m.

The effect of the flow rate of the continuous phase, $Q_o$, on the droplet dimensions is shown in Figure 3.3 a and b. At an $\alpha = 0.11$, a larger value of $Q_o$ resulted in smaller droplet dimensions. For a particular value of $Q_o$, the size of droplets became smaller with decreasing values of $\alpha$. For example, at $Q_o = 0.9$ mL/h the average diameters of droplets were 104 and 117 mm for $\alpha = 0.25$ and $\alpha = 1.50$ (Figure 3.2c and d, respectively).

These two effects are presented in Figure 3.4a, where the variation in the mean diameter of droplets, $D$, is plotted as a function of $Q_o$ for the different values of $\alpha$ (an exemplary targeted diameter of the droplets was 110 $\mu$m). The decrease in $D$ with increasing value of $Q_o$ occurred due to the increasing shear stress imposed by the oil phase on the stream of the mixed agarose
solution. Similarly, with increasing $a$ (resulting in the increase in viscosity of the agarose solution) the shear stress imposed on the stream of the droplet phase by the continuous phase reduced, which resulted in the generation of larger droplets. Figure 3.4 provides guidance for the generation of droplets with a particular diameter from agarose solutions with the varying values of $a$. In order to produce droplets with $D = 110 \, \mu m$ (shown with a horizontal dashed line), the value of $Q_o$ had to be tuned from 0.6 to 1.4 mL/hr for the concentration of agarose in the droplet phase in the range from 0.75 to 2.75 wt. %.

![Figure 3.4](image)

**Figure 3.4.** (a) Variation in droplet diameter, $D$, plotted as a function of the flow rate of the continuous phase, $Q_o$, for $a$ of 0.11 (♦), 0.25 (+), 0.67 (●), 1.50 (▲), and 9.00 (■). For each experimental point we analyzed 100 droplets generated in three independent experiments. (b) Conditions for the generation of 110 mm-diameter droplets. The combined total flow rate of the agarose solutions was 0.12 mL/hr.

The concentration of agarose in the mixed stream (Stream 1 plus Steam 2) was estimated as calculated as

$$C'_{ag} = \frac{[(C_{ag,1} \cdot Q_{ag,1}) + (C_{ag,2} \cdot Q_{ag,2})]}{(Q_{ag,1} + Q_{ag,2})}$$  \hspace{1cm} (3.5)
where $C_{ag,1}$ and $C_{ag,2}$ are the concentrations of agarose in Streams 1 and 2, respectively, and $Q_{ag,1}$ and $Q_{ag,2}$ are the flow rates of Streams 1 and 2, respectively.

To generalize the conditions for the generation of droplets of agarose solution with varying concentration, we characterized the properties of the agarose solution for $0.5 < C_{ag} < 3.0$ wt.% at the temperature of 32 °C (the temperature measured with a thermocouple at the point of MF emulsification). The value of interfacial tension, $\gamma$, between the 3 wt.% solution of Span-80 in mineral oil and agarose solutions at $0.5 < C_{ag} < 3.0$ wt.% was ca. 2.2 mN/m for the entire range of concentrations of agarose solutions. The variation of viscosity of agarose solution with increasing $C_{ag}$ is presented in Figure 3.3a. In the designated range of $C_{ag}$, the viscosity of the solution increased from $0.5 \times 10^{-2}$ to $2.4 \times 10^{-1}$ Pa s.

Figure 3.3c shows the variation in the mean diameter of droplets, $D$, plotted as a function of the product $Ca_d x b$ where $Ca_d$ is the Capillary number of the droplet phase and $b= Q_d/(Q_d + Q_o)$. The value of the $Ca_d$ was calculated as $Ca_d = m_d U_d/g$ where $m_d$ and $U_d$ are the dynamic viscosity and the velocity of the aqueous agarose solutions, respectively. While for $C_{ag} < 2$ wt.% ($m_d < 0.08$ Pa s) the relationship $D$ vs. $Ca_d x b$ followed the master curve and provided a practical guidance for the generation of droplets with a particular diameter, for higher values of $C_{ag}$ concentrations it was difficult to find a single model. It is important to stress that in the range of $C_{ag}$ used in the present work, the solutions had properties of a Newtonian liquid.132
Figure 3.3. (a) Variation in the viscosity of the droplet phase measured at 32 °C, plotted as a function of $C_{ag}$. (b) Variation in droplet diameter plotted as a function of the product of the Capillary number of the droplet phase and the ratio $Q_\alpha/(Q_d + Q_o)$ for $\alpha$ of 0.11 (◆), 0.25 (■), 0.67 (▲), 1.50 (●) and 9.00 (➕).

Figure 3.4a shows typical optical microscopy image of agarose microgels derived from precursor droplets and transferred to the PBS buffer. The microgels had a round shape and the average diameter that was close to the mean diameter of the corresponding precursor droplets. The microgels had a narrow size distribution (Figure 3.4b). The polydispersity of microgels did not exceed 6%.
Figure 3.4. Optical microscopy images (a) and size distribution (b) of agarose microgels transferred in the PBS buffer. $C_{ag} = 2$ wt.%. The scale bar is 100 mm.

The variation in mechanical properties of the microgels with varying concentrations of agarose is described in Chapter 4.

3.2. Discussion

Our results show that agarose microgels with a narrow size distribution can be readily generated by MF emulsification of agarose solutions, followed by the thermosetting of agarose compartmentalized within the droplets. The diameter of microgels is determined by the shear stress imposed by the continuous oil phase on the agarose droplet phase. Since with increasing agarose concentration the solution’s viscosity increases, a larger shear stress (controlled by the flow rate of the oil phase) was needed to generate droplets with a target diameter of 100 µm (Figure 3.2.b).

We assumed that up to the agarose concentration of 3 wt.%, the solutions behave as Newtonian liquids, however we were unable to find a single relationship between the diameter of droplets and the product of the Capillary number and the ratio $Q_d/(Q_d + Q_o)$, as shown in Figure 3.3b. We speculate that a higher contribution of extensional flow for agarose solutions
with a higher polymer concentration led to the transition from a dripping regime of droplet formation at \((11<\alpha<0.67)\) to a jetting regime at \((\alpha >0.67)\).

In addition to the uniform distribution of microgel dimensions, MFs enabled a high through-put variation in the concentration of agarose within the microgels. In the present work we used agarose concentrations of 3 and 0.75 wt.% in streams 1 and 2, respectively (Figure 2.1). Further increase in agarose concentration above 3 wt.% would lead to more “asymmetric” conditions in mixing of the two streams and droplet formation, as well as a non-Newtonian behavior in the flow of the solution. These features may require the modification in the design of the MF device and an optimization in flow parameters of the liquids.
4. Studies of the Mechanical Properties of Agarose Gels

The motivation behind the measurements of the Young’s moduli of agarose gels was to correlate the mechanical properties of the gels with the cellular responses of the encapsulated cells. Compression tests were initially conducted on macroscopic agarose gels to determine the values of the Young’s moduli using a standard, well-established method. The moduli of agarose microgels were then measured by AFM using silicon-nitride tipless cantilevers. To establish a protocol, we carried out AFM experiments using non-modified cantilevers and cantilevers modified with thiol-terminated methoxy polyethylene glycol (CH$_3$O-PEG-SH). Furthermore, by carrying AFM experiments with cantilevers with various spring constants, we selected the cantilever with the appropriate rigidity to measure the Young’s modulus and the relaxation time of the agarose microgels. Finally, the Young’s modulus of the microgels was measured at 37 °C to provide a better understanding of the mechanical properties of the gels at physiological temperatures.

4.1. Compression Measurements of Macroscopic Agarose Gels

The Young’s modulus of macroscopic agarose gels was measured using a plate-plate compression method, with a 50 N load cell. The measurements were conducted at 21 ± 1 °C on agarose gels with a concentration of agarose, $C_{ag}$, of 1.0, 2.0, 3.0, 4.0 and 5.0 wt%. The samples were prepared as described in the Materials and Methods section. For each $C_{ag}$, we examined three samples. The Young’s moduli of the bulk agarose gels increased by a factor of 50, when the value of $C_{ag}$ increased from 1.0 to 5.0 wt% (Figure 4.1).
Figure 4.1. Variation in the Young’s moduli ($E$) of macroscopic agarose gels with agarose concentration, $C_{ag}$. For each $C_{ag}$ the average value of Young’s modulus was obtained by examining 3 gel samples.

We used the values of the $E$ determined for macroscopic agarose gels to narrow the range of moduli that would be expected from AFM compression experiments conducted for agarose microgels with identical values of $C_{ag}$.

4.2 Optimization of AFM measurements

4.2.1 Experiments Conducted Using Silicon-Nitride Tipless Cantilevers

The Young’s modulus of agarose microgels was measured using a silicon-nitride tipless cantilever with a spring constant of $0.028 \pm 0.005$ N/m. Experiments were conducted at $21 \pm 1^\circ$C in PBS solution. A minimum of five microgels were studied, with five compression-retraction experiments performed on each microgel particle. The measurements were not conducted
continuously, that is, after the first compression-retraction cycle, the cantilever was lifted up from the microgel for 5 sec, after which the microgel was compressed again.

For microgels with a $C_{ag}$ of 0.75 and 2.0 wt% we determined the values of $E$ of 131 and 306 Pa, respectively. The modulus for agarose microgels with $C_{ag} = 2.0$ wt% gels was almost two and a half times greater than for microgels with $C_{ag}=0.75$ wt%. For both values of $C_{ag}$, the AFM measurements led to a broad variation in the values of $E$, especially for the softer microgels, with $C_{ag} = 0.75$ wt% (Table 4.2). In particular, the standard deviations in the values $E$ at $C_{ag}$ of 0.75 and 2.0 wt% were 58 and 47 Pa, respectively. The standard deviation was similar for measurements on the same microgel, as well as between microgels, for both concentrations.

<table>
<thead>
<tr>
<th>$C_{ag}$, wt%</th>
<th>$E$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>131 ± 58</td>
</tr>
<tr>
<td>2.0</td>
<td>306 ± 47</td>
</tr>
</tbody>
</table>

During the AFM measurements, we found out that following the compression phase of the measurements, the microgel moved unilaterally towards the cantilever platform. Such unidirectional movement was indicative of adhesion of the microgel to the surface of the cantilever. Due to slipping, the movement prevented the conduction of continuous repetitive compression measurements on the same microgel. To decrease the adhesion of the microgels to the cantilever, in the next series of experiments, we modified the surface of the cantilever by coating it with gold and attaching thiol-terminated methoxy poly (ethylene glycol) (CH$_3$O-PEG-SH) to the gold surface, as described in Chapter 2.
4.2.2. Experiments Conducted Using Gold-Coated Silicon Cantilevers Modified with Thiol–Terminated Methoxy Poly(ethylene glycol)

We assumed that, as previously described,\textsuperscript{\textcolor{red}{133}} coating cantilevers with CH\textsubscript{3}O-PEG-SH described previously will reduce the adhesion of the microgels to the gold-coated silicon-nitride cantilever. The SH-group was used to achieve a strong binding of the polymer to the surface of gold.\textsuperscript{\textcolor{red}{134}} Successful modification of the cantilevers with CH\textsubscript{3}O-PEG-SH was verified by measuring the contact angle of deionized water on the planar gold substrate before and after the surface modification. Following adsorption of CH\textsubscript{3}O-PEG-SH, the contact angle of water reduced from 95 to 22 ± 2°.

For each $C_{\text{ag}}$, we studied a minimum of 5 microgels using 5 compression-retraction cycles for each microgel. The measurements were conducted in a continuous fashion in the following way: after microgel compression, the cantilever retracted for 5 sec, and another compression of the microgel was initiated.

The measurements of the Young’s moduli were performed for agarose microgels with 0.75, 2.0, and 3.0 wt\%. Figure 4.3 shows that the value of $E$ increased with an increasing agarose concentration in the microgels. The Young’s moduli of agarose microgels were 127 ± 27, 231 ± 26, 342 ± 27 Pa for $C_{\text{ag}}$ of 0.75, 2.0, and 3.0 wt\%, respectively. These values of the Young’s moduli obtained in these measurements are similar to values obtained when using non-modified cantilevers. The differences between successive measurements conducted on the same microgel, and the differences between the measurements carried out for different microgels with identical $C_{\text{ag}}$ were significantly smaller that those obtained in the measurements with non-modified silicon nitride cantilevers.
Figure 4.3. Effect of agarose concentration, $C_{ag}$, on the microgel Young’s modulus, $E$. The average value of $E$ was obtained by examining 5 individual microgels, with 5 compression-retraction cycles carried out for each microgel. The AFM experiments were conducted using gold-coated silicon cantilevers modified with thiol–terminated methoxy poly(ethylene glycol), with an spring constant of 0.03 N/m.

The force-separation curves contained two regions: (1.) a slow-rising curve that spanned roughly 1 µm of separation, and (2.) a sharply rising curve (Fig. 4.4). The acquired force curves did not fit to the hypothetical force curve obtained by the modified Hertz model (eq. 2.2).
Figure 4.4. Force separation curve obtained from the indentation of an agarose microgel with C_{ag} = 2 wt.%. The force curve consists of (1.) a slow rising curve between a separation of 3-4 µm, and (2.) a sharply rising curve between 4-5 µm.

The values of the $E$ determined by fitting only region 1 of the force curve to the Hertz model (eq. 2.2) were significantly smaller than the expected values of $E$ (measured for the bulk agarose gels), whereas the values of $E$ extracted by fitting only region 2 of the force curves to the Hertz model were closer to those acquired from the compression tests carried out for bulk agarose gels. Thus we concluded that to probe region 2 of the curve more accurately, a more rigid cantilever was required, which could compress the microgel without strong deflection. To determine an appropriate stiffness of the cantilever, that is, to correlate the results of macroscopic compression tests with AFM measurements, we conducted AFM experiments on planar bulk agarose gels.

4.2.3. Determining an Appropriate Spring Constant for the Cantilever

The value of $E$ of a planar 2 wt % agarose gel was measured using a silicon-nitride pyramidal tipped cantilever with a spring constant of 0.15 ± 0.02 N/m. The spring constant for this cantilever was selected based on previous studies$^{135, 136}$, in which the values of $E$ were similar to ones that we measured macroscopically for different agarose gels. A film of agarose gel was prepared in a similar fashion as the sample used for bulk plate-plate compression measurements. The gel was probed using the pyramidal cantilever at three different points by doing five measurements for each point. For the 2 wt% gel, the value of $E$ was measured to be 4.0 ± 0.4 kPa. This value of $E$ was approximately two-fold greater than 2.2 ± 0.2 kPa, which was
measured by macroscopic measurements. Importantly, the force curve obtained from these measurements did not contain the slow rising fragment (region 1 in Figure 4.4), which was observed in the AFM experiments conducted using a cantilever with a spring constant of 0.3 N/m. Therefore, in later experiments with agarose microgels, tipless cantilevers with a spring constant of 0.1 N/m were used.

4.3 Measurements of Mechanical Properties Using SH-PEG-Modified Gold-Coated Tipless Rigid Cantilevers

After optimizing the conditions of AFM experiments, the Young’s modulus of agarose microgels was measured using a gold coated silicon-nitride tipless cantilever with a spring constant of 0.1 N/m at 20 ºC. The cantilevers were modified with CH₃O-PEG-SH as described previously. After modification with CH₃O-PEG-SH, the spring constant of the cantilevers increased to 0.14 ± 0.03 N/m. This value was used to determine the value of Young’s modulus of agarose microgels.

For each agarose concentration, Cₐg, we studied a minimum of 5 microgels with 5 compression-decompression cycles conducted for each microgel. The microgels prepared for these measurements had an average diameter of 40 +/- 3 µm, to ensure diameter a smaller than the width of the cantilever (43µm). The measurements were conducted in a continuous fashion: the microgel was compressed, the compressive force was maintained for 1 sec, the cantilever was retracted, and a new compression of the microgel was initiated after 5 sec.

The measurements of the Young’s modulus were performed for agarose microgels with 1.0, 2.0, 3.0, 4.0 and 5.0 wt%. Figure 4.4 shows that the value of the Young’s modulus increased
with an increasing $C_{ag}$ in the microgels. The value of $E$ at $C_{ag}=1.0$ wt.% was 34 times greater at $C_{ag}=5.0$ wt.%.

**Figure 4.4.** Effect of $C_{ag}$ on the microgel $E$. The average value of $E$ was obtained by examining 5 individual microgels, with 5 compression-dwell-retraction cycles carried out for each microgel.

The values of the Young’s modulus were comparable with values determined from bulk compression tests (Figure 4.5). The Young’s modulus increased with increasing agarose concentrations in an exponential manner. The average error in the modulus between the two methods was 12 %, primarily due to the larger error obtained for 3 wt% agarose microgels.
Figure 4.5. Comparison between bulk and microgel $E$, with increasing agarose concentration, $C_{ag}$. Bulk measurements were conducted using a 50N load cell, and a compression rate of 0.23 mm/min, at $22 \pm 1 \, ^\circ C$. Microgel measurements were conducted using a CH$_3$O-PEG-SH modified gold coated silicon-nitride tipless cantilever with an average spring constant of 0.14 N/m, at $21 \pm 1 \, ^\circ C$.

The microgel relaxation time, $\tau$, was also measured by determining the time of the decay of the force $F$, due to the relaxation of the stress in the microgel particle. The relaxation time of the microgels was measured for $C_{ag}$ of 1.0, 2.0, 3.0, 4.0 and 5.0 wt.%. The measurements were taken in a continuous manner, as described previously. To analyze the relaxation times, we used compression-dwell-retraction force curves that were used in the analysis of the Young’s modulus, and profiles that showed a spontaneous decay - characteristic of the microgel slipping during the dwelling period - were not included.

Figure 4.6 shows that the value of $\tau$ decreased with increasing agarose concentration. The decrease in the relaxation time followed an exponential decay trend.
Figure 4.6. Effect of $C_{ag}$ on the microgel relaxation time, $\tau$. The average value of $\tau$ was obtained by examining 5 individual microgels, with 5 compression-dwell-retraction cycles carried out for each microgel. Microgel measurements were conducted using a CH$_3$O-PEG-SH modified gold coated silicon-nitride tipless cantilever with an average spring constant of 0.14 N/m, at 21 ± 1 $^\circ$C. The dwelling period was 1 sec.

4.4 Measurements of Mechanical Properties Using Modified Gold-Coated Tipless Rigid Cantilevers at Physiological Temperatures

To determine the mechanical properties of agarose gels under physiological conditions, the value of $E$ of agarose microgels was measured at 37 $^\circ$C. We used the same protocol as was used in AFM experiments for microgels gels at room temperature. The change in the value of $E$ followed a similar trend at 37 $^\circ$C and room temperature, increasing exponentially with increasing $C_{ag}$ (Figure 4.7). The value of $E$ at 37 $^\circ$C was roughly 57 times greater at a concentration of 5.0 wt.% than at 1.0 wt.%, compared to the 34 fold increase at room temperature. The measured
Young’s modulus reduced dramatically upon incubation of the gels when compared to the value at room temperature. For gels with a C\textsubscript{ag} of 1.0, 2.0, 3.0, 4.0 and 5.0 wt.%, the value of $E$ decreased by a factor of 8, 27, 14, 8, and 4, respectively.

![Figure 4.7](image)

**Figure 4.7.** Effect of C\textsubscript{ag}, on the microgel $E$ at 37 °C. The average value of $E$ was obtained by examining 5 individual microgels, with 5 compression-dwell-retraction cycles carried out for each microgel. Microgel measurements were conducted using a CH\textsubscript{3}O-PEG-SH modified gold coated silicon-nitride tipless cantilever with an average spring constant of 0.14 N/m, at 37 ± 0.1 °C.

To determine the real concentration of agarose in the microgel at an elevated temperature, we prepared bulk agarose gels with an initial concentration C\textsubscript{ag} of 1.0, 2.0, 3.0, 4.0 and 5.0 wt.% at room temperature, incubated them in PBS solution at 37 °C for 1 hr and measured concentration of agarose, C\textsubscript{ag, 37} after incubation. We note that the incubation time may not be sufficient to get an equilibrium value of C\textsubscript{ag, 37}, however prolonged incubation of the microgels in the fluid cell resulted in solvent evaporation, thus confounding our measurements. Figure 4.8 shows the variation in C\textsubscript{ag}, plotted as a function of the original agarose concentration C\textsubscript{ag}. The
values of $\text{C}_{\text{ag}}$ were consistently $(18 \pm 4 \text{ wt.\%})$ lower than that at room temperature. The variation in Young’s modulus at 37 °C was then replotted as a function of $\text{C}_{\text{ag}, 37}$ (Figure 4.9).

**Figure 4.8.** Change in the $\text{C}_{\text{ag}}$ of macroscopic agarose gels between 21 °C and upon incubation for 1 hr at 37 °C in PBS solution.

**Figure 4.9.** Variation in Young’s modulus, $E$, of agarose microgels with the $\text{C}_{\text{ag}, 37}$. 

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4.5. Discussion

In the present work, we developed a method for the measurements of the mechanical properties of agarose microgels by using AFM. We identified two important conditions that influenced the measurements. First, we observed a large scatter in the values of Young’s moduli measured using silicon-nitride tipless cantilevers without prior to modification with a CH$_3$O-PEG-SH layer (Figure 4.6). We ascribe this effect to the strong adhesion between the microgel and the cantilever surface. The modification of the cantilever with CH$_3$O-PEG-SH reduced adhesion, and resulted in a smaller deviation in the Young’s modulus for microgels with the same concentration of agarose.

The second factor was the rigidity of the cantilever. A comparison between the values of the Young’s modulus of bulk agarose gels and microgels measured using cantilevers with a low spring constant (0.03 N/m) suggested that these cantilevers did not probe the elasticity of the microgels. The values of $E$ were approximately two orders of magnitude smaller than the values obtained in the macroscopic compression test. Therefore, a more rigid cantilever with an average spring constant of 0.14 N/m was used to probe the microgel elasticity.

In the optimized AFM experiments, the experimental data showed that the Young’s moduli of microgels at $1.0 \leq C_{ag} \leq 5.0$ wt% were close to the values of $E$ measured for macroscopic gels. The value $E$ increased with increasing $C_{ag}$, which was ascribed to the increasing crosslinking density of the polymer network in the microgels. We note that the values of Young’s moduli measured for microgel particles were similar to those measured under compression for macroscopic agarose hydrogels with similar $C_{ag}$ and prepared using similar protocols (i.e., gelation time and temperature). In one such study, the value of $E$ of a 2 wt.% agarose gel prepared by gelation at 21 ºC for 20 min was measured to be 1.5 kPa, whereas in
our work the average value of $E = 2.5$ kPa was obtained. We stress that this variable is particularly important: it has been established that it influences significantly the values of $E$ of agarose gels with identical concentrations.$^{138,139}$

A strong decrease in the modulus of the microgels at 37 °C, in comparison with that measured at room temperature, was caused by the weakening of hydrogen bonds between individual agarose molecules and agarose double helices, thereby increasing the strain exhibited by the microgel under compression. The reduction in attraction forces between led to the uptake of water upon incubation at 37 °C, resulting in decrease in the concentration of agarose microgels.$^{140}$ By comparing the values of $E$ of 2.5 and 1.5 kPa measured for, e.g., 3.0 wt.% agarose microgels at room temperature and 37 °C (Fig. 4.4 and 4.7 respectively), we conclude that the decrease in $E$ was caused by the combined effect of reduced concentration and a weaker polymer network formed at 37 °C.

The decrease in the relaxation time $\tau$ with increasing $C_{ag}$ (Fig. 4.6) also originated from the increasing crosslinking density of the polymer. Several models have been proposed to correlate the decrease in the relaxation time of elastomeric polymer networks with decreasing fraction of 'dangling' chains that are attached to the network at only one end.$^{141-145}$ All of these models use, to different extents, the concept proposed by deGennes: the relaxation of an entangled dangling chain in a polymer network occurs by its 'retracing', that is, the chain end diffuses towards the chain end, which is attached to the network.$^{142}$ All the models predict that the relaxation time follows a power law, but they differ in their predictions of the effect of crosslinking density on the power law exponent. In addition, the assumption that the concentration of agarose is proportional to the density of polymer crosslinking may not be valid.
Finally, agarose gels by the association of double helices, in contrast with elastomeric networks for which the models were proposed.
5. Characterization of Cell growth in Agarose Microgels

This section was done in collaboration with Ethan Tumarkin of the Kumacheva group; Department of Chemistry, University of Toronto.

The motivation behind this work is to encapsulate cells in microgels with varying mechanical properties, using a high-throughput approach, in order to examine the effects of the matrix properties on cell growth and activity. Acute myeloid leukaemia is a cancer of the myeloid line of white blood cells, which is characterized by the abnormal growth of cells that accumulate in the bone marrow before entering the circulatory system. Therefore, the study of the abilities of the cells to grow, as well as escape their environment is important in understanding the underlying mechanism of the effect of mechanical properties of the surrounding matrix on cell behaviour.

We studied the effect of matrix stiffness on the behaviour of the non-adherent acute myeloid leukemia-2 (AML2) cell line. We also report an approach to quantify the cell behaviour in microscopic and macroscopic agarose gels. The elasticities of the agarose gels were controlled by varying the concentration of the polymer as described previously.

5.1 Encapsulation of AML2 Cells in Macroscopic Agarose Gels

Cell-laden macroscopic gels with a volume of 20 µL and a C_{ag} of 1.0-5.0 wt% were created to study effect of matrix stiffness on cell viability. The volumes of the macroscopic gels were 3.0x10^5 fold larger than that of microgels prepared by the MF method. The initial cell concentration in the agarose solutions used to prepare the gels was 1.25x10^5 cells/mL. The cell-laden gels were cultured for 7 days in a cell medium composed of 85% Dulbecco's Modified
Eagle (DME) Medium, and supplemented with 15% Fetal Calf Serum (FCS), as described previously. Growth and viability of the cells within the gels was analyzed on days 0 and 7.

Figure 5.1 shows optical microscopy images of AML2 cells encapsulated in agarose gels at varying $C_{ag}$ on day 0 (the day of encapsulation) and 7 days later. On day 0, the cells are suspended in all agarose gels similarly, with no cell clusters apparent within the gels. By day 7, the cells grew differently in the gels with varying $C_{ag}$. In the weakest gel ($C_{ag} = 1.0$ wt.%) the cells showed the highest level of growth, and there were a larger number of cell colonies. The cells embedded in the 2.0 wt. % agarose gel grew to a similar extent as in the 1.0 wt. % agarose. In the 3.0 wt.% agarose gel, the number of cells increased by day 7, however, the degree of cell growth was markedly lower compared to cell growth in gels of 1.0 and 2.0 wt.% agarose. As in the 1.0 and 2.0 wt.% gels, the majority of cells in 3.0 wt. % were present in cell colonies, though both the size and number of colonies was smaller. In gels with a $C_{ag}= 4.0$ wt.%, cells showed less growth than at lower concentrations, and few colonies were visible in the gels. Lastly, in 5.0 wt.% gels, very few individual cells or cell colonies were observed in the sample.
**Figure 5.1** Optical microscopy images of cell laden agarose gels. Day 0: Images of single cells embedded inside agarose gels at varying $C_{ag}$. Images were acquired immediately after gelation. Day 7: Optical microscopy images of cell-laden agarose gels on Day 7 at varying $C_{ag}$. Scale bar is 100 µm.

Fluorescent dyes were added to the cell samples on the 7th day post-encapsulation, to distinguish between viable and non-viable cells. Hoescht, Calcein AM, and Ethidium homodimer-1 were added to stain for nuclear DNA of all cells (viable and non-viable), viable cells, and non-viable cells, respectively. Figure 4.4 shows the fluorescence imaging of AML2 cells encapsulated in 1.0 and 3.0 wt.% agarose slabs on day 7. Cell colony growth in the 3.0 wt. % agarose gel was less than colonies formed in the 1.0 wt. % gel, which confirmed our observations from optical microscopy measurements. Furthermore, a larger number of non-viable cells were observed in the 3.0 wt.% (as indicated by the fluorescent emission from Ethidium homodimer-1) in comparison to the 1.0 wt.% gel.
Figure 5.2 Fluorescent images of AML2 cells encapsulated in 1.0 and 3.0 wt.% agarose on day 7 of cell culture. (a,g) Ethidium homodimer-1 was used to observe red fluorescence in non-viable cells. (b,h) Viable cells labelled with Calcein AM (green). (c,i) Hoescht dye was added to stain DNA of both viable and non-viable dyes. (d) Bright field image of the cell colony shown in a-c, d-e. (e) Composite image overlaying fluorescence of both viable (Calcein AM) and dead (Ethidium homodimer-1) cells shown in a and b. (f) Composite image overlaying fluorescence of Hoescht stained cells and dead (Ethidium homodimer-1) cells appearing in a and c. (k) Composite image overlaying fluorescence of both live (Calcein AM) and dead (Ethidium homodimer-1) cells appearing g and h. (l) Composite image overlaying fluorescence of Hoescht stained cells and dead (Ethidium homodimer-1) cells shown in g and i. Scale bar is 50 µm.
Cell growth in macroscopic gels was also analyzed quantitatively on day 7, using a water-soluble tetrazolium (WST) salt. WST-8 is reduced through a reaction with the reduced form of a 1-Methoxy-5-Methylphenazinium Methyl Sulfate (1-methoxy PMS), an electron mediator, to assess viable cells. 1-methoxy PMS is found in the cytoplasmic side of the cell membrane and reacts directly with NADH (nicotinamide adenine dinucleotide reduced form). NADH is generated in live cells from NAD+ by the action dehydrogenase enzymes. Thus, the WST-8 tetrazolium salt is used as an indicator of dehydrogenase activity. The conversion of WST-8 to formazan dye with an absorption maximum at 450 nm, was then used to study the number of viable cells.

On day 7 post-encapsulation of AML2 cells in the macroscopic agarose gels, CCK-8 dye was added to each sample at a 1:10 ratio (CCK-8 dye: cell culture media). The samples with dye were incubated at 37 °C (5% CO2) for 3 hours. Similarly to the observational studies of cells grown in agarose with different concentrations (Figure 5.1), absorbance measurements at 450 nm showed a decrease in the number of metabolically active cells with increasing C_ag. Figure 5.3 shows that with increasing C_ag, the absorption at 450 nm decreased, due to lower conversion of WST-8 salt to the formazan dye. The average absorbencies were normalized relative to the absorbance at 450 nm of the sample composed of cell-laden 5.0 wt.% agarose on day 7, in order to quantitatively compare the relative fold increase in the absorbance at lower concentrations. Cells encapsulated in gels at C_ag of 1.0 and 2.0 wt. % agarose showed statistically equal absorbance, which was slightly lower than the level of absorbance in a control sample (cells in media without agarose). The 1.0 and 2.0 wt.% agarose samples both showed approximately a 3-fold increase in the relative absorbance the absorption maxima in comparison with cells encapsulated in gels with a 5.0 wt.% concentration of agarose. From the graph, it is evident that
upon increasing the agarose concentration beyond 2.0 wt.%, the number of metabolically active cells decreases.

Figure 5.3 Graph of the absorbance of Formazan Dye at varying concentrations of agarose (C_{ag}). Metabolically active cells convert WST to Formazan Dye, corresponding to an increase in the absorbance at 450 nm. 0 wt. % agarose represents a positive control of AML2 cells suspended in agarose-free cell media. Absorbance was normalized with respect to the average absorbance of Formazan Dye in 5.0 wt.% agarose.

5.2 Microfluidic Encapsulation of Leukemia Cells in Agarose Microgels

For the encapsulation of cells in agarose microgels, we used AML-2 leukemia cells suspended in the droplet phase to a concentration of 1.0 x 10^{6} cells/mL. The microgels were produced from a C_{ag} = 1.0 and 2.0 wt.%. The microgels had a round shape and an average
diameter that was approximately 10% smaller than the mean diameter of the corresponding droplets. The microgels had a narrow size distribution, with a polydispersity that did not exceed 2.5%. In our experiments we aimed to minimize the number of microgels containing multiple cells by reducing the initial concentration of cells in the agarose suspension.

Figure 5.4 shows a schematic of the MF device with the suspension of cells in the agarose solutions in the disperse phase. As the disperse phase is introduced into the MF device, it travels and enters the T-junction. Droplet formation occurs due to the shear force imposed on the disperse phase by the continuous mineral oil phase. In our experiments, the total flow rate of the disperse phase was maintained at 0.1 mL/hr. The flow rate of the continuous phase was tuned, in order to produce droplets with a mean diameter of ~110 µm, which would lead to microgels with a mean diameter of ~100 µm.

**Figure 5.4** Schematic of the MF device for the generation of cell-laden agarose microgels with tunable elasticity.
Figure 5.5 shows a theoretical and experimental comparison of the distribution of the number of cells in agarose microgels directly after encapsulation. Theoretical values for the percent of the number of cells per microgel were calculated using the Poisson distribution (described in Chapter 3), based on a droplet diameter of 110 µm, and an initial cell concentration of $1 \times 10^6$ cells/mL. For precursor droplets with a mean diameter of 110 +/- 2.8 µm, we achieved an overall encapsulation efficiency of 45.4 +/- 3.9%, with 34.2 +/- 5.0% of the microgels containing one cell, 9.0 +/- 2.6% two cells and 54.6 +/- 3.9% containing no cells (empty). A comparison with the theoretical results of the distribution of cells per gel shows a high similarity to the experimental results (Figure 5.5).

**Figure 5.5** Comparison of the number of encapsulated cells per droplet achieved experimentally (●) to the number of cells theoretically expected by the Poisson distribution (■). Cells were encapsulated in 110 µm-diameter droplets, using an initial suspension of $1 \times 10^6$ cells/mL in 2.0 wt.% agarose. The theoretical Poisson distribution was determined using identical parameters.
Approximately 300 microgels were analyzed to determine the encapsulation efficiency. The diameter of the precursor droplets was 110 +/- 2.8µm. \( Q_O = 1.2 \text{ mL/hr}, \ Q_{ag} = 0.1 \text{ mL/hr}. \)

Images of AML2 cells encapsulated in agarose microgels with 1.0 and 2.0 wt.% \( C_{ag} \) are shown in Figure 4.8 on day 0 and on day 7. Upon the encapsulation of the cells in agarose microgels cell viability was determined to be 64 ± 1.2% using a standard trypan blue staining and quantification using a haemocytometer. A comparison of the images of cell-laden microgels on day 7 with images taken directly after encapsulation, shows that cell growth appeared to be minimal in microgels composed of both 1.0 or 2.0 wt. % agarose. The reasons for the limited cell growth are not well understood and remain under investigation.

**Figure 4.8.** Optical microscopy images of AML2 cells encapsulated in 1.0 and 2.0 wt.% agarose microgels on day 0 and day 7. The concentration of cells in the feed suspension was \( 1 \times 10^6 \) cells/mL. The scale bar is 100 µm.
5.3 Discussion

The experiments on the high-throughput encapsulation of an acute myeloid leukemia cell line demonstrate the capability of the MF generation of tunable cellular microenvironments. The microgels can be used for subsequent cell culture, thereby enabling further studies of the effect of mechanical properties on cell growth, proliferation, motility and secretions. The properties of cells can be examined within the microgels using various spectroscopic techniques such as absorption (UV-Vis) and fluorescence microscopy (fluorescent microscopy, confocal microscopy). Furthermore, the cells can be studied upon their release from the microgel by digestion of the agarose gel using enzymatic cleavage (e.g. agarase).

In our current work, AML2 cells were encapsulated in microgels with $C_{ag} = 1.0$ and 2.0 wt%. Future work will involve the generation of cell-laden microgels with increasing Young’s modulus (up to 5.0 wt.%), to investigate the effects of the local mechanical properties of the microenvironment on cell behaviour. Further optimization of the conditions for cell viability within the microgels will need to be established to ensure successful sample preparation and analysis of cell behaviour.

The study of cellular behaviour in both bulk and microscale agarose gels with different $C_{ag}$, may also be useful in comparing the effects of localized and delocalized cellular environments. The use of two inlet channels for two independent disperse phase solutions will allow for the high-throughput generation of agarose microgels with varying elastic properties. Previous measurements of the mechanical properties of agarose microgels will be extended to cell encapsulated microgels, to study the correlation between varying elasticity of the microbeads and cell behavior. Furthermore, the mechanical properties of the gel will be studied throughout
the cell culture period to study the change in the properties with time (e.g., the effect of cells in changing the microenvironment).
6. Conclusions and Outlook

We have developed a MF method for the continuous, high-throughput generation of agarose-based 3D cellular microenvironments with precisely controlled dimensions and varying mechanical properties. Using this method, large combinatorial libraries of microenvironments with different mechanical properties can be generated and used in studies of the effect of elasticity of the surrounding extracellular matrix on the behaviour of cells. These studies can be achieved by culturing cells in microgels and subsequently cell assays using optical fluorescence microscopy or flow cytometry experiments without dissolution of agarose microgels.

The proposed method is based on the high-throughput mixing of two precursor polymer solutions, which can be used for the generation of microgels of other biopolymers (collagen, fibronectin, elastin, k-Carrageenans, etc.) Physical gelation of the polymers can be achieved by thermosetting or ionic crosslinking, in the case of a high temperature sensitivity of the cells. In addition, the use of two individual inlet channels allows for the generation of combinatorial microgels from multiple polymers within the matrix. The use of specific polymers will allow control over both the mechanical and chemical properties of the cell microenvironment. Furthermore, precursor solutions may contain different protein molecules, e.g., growth factors, drugs, or DNA molecules.

Our work shows that the AFM approach can be efficiently used to examine the Young’s modulus and the relaxation time of the microenvironment surrounding cells. The distinct feature of our work is that the mechanical properties of the matrix are probed on the length scale of the encapsulated cells, which can secrete proteins, ions and products of cell metabolism, and locally change their microenvironment. The ability to study cell feedback very favourably contrasts our
work with studies of the elastic modulus of two-dimensional films prior to-cell seeding or the examination of the elasticity of bulk three-dimensional materials.

Future work will include an increase in the concentration of agarose in the microgels, which would be extremely important in studies of the formation of colonies by cancer cells. Cell culture experiments will shed light on how different cell lines are influenced by the elasticity of the matrix. A study of cell “feedback” mechanism will be accomplished by studying the mechanical properties of the microgels in the course of cell culture experiments. The examination of the structure of the agarose matrix by cryotoming it and inspecting it using transmission electron microscopy will enable us to understand the relation between the permeability of the gel matrix and its mechanical properties.

This high-throughput approach to the development of tunable microenvironments for cells will impact tissue-engineering and cell based screening. The main focus of tissue engineering has been to produce suitable tissue replacements; however, a more immediate contribution of tissue engineering may be the generation of 3D in vitro models for research and drug testing. Currently, cell-based screening and validation of therapeutic compounds conducted on large-scales are carried out almost exclusively on cells cultured on rigid multiple-well plates. As mentioned earlier, this rigid substrate environment is not representative of the physiological or pathological tissue environment. The development of an approach to screen cellular responses within tunable microenvironments would provide a more relevant representation of normal biological processes.
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8. References


43 Ng, C.E.; Keng, P.C.; Sutherland, R.M. J. Cancer 1987, 56, 301.
46 Sutherland, R.M. Science 1988, 240, 177.
50 Suresh, S.; Acta Biomater. 2007, 3, 413.
57 Hutmacher, D.W. Nat. Mater. 2010, 9, 90.
Weaver, V.M.; Petersen, O.W.; Wang, F.; Larabell, C.A.; Briand, P.; Damsky, C. 


Shield, K.; Ackland, M.L.; Ahmed, N.; Rice, G.E. 

Frankel, A.; Buckman, R.; Kerbel, R.S. 

Lee, G.Y.; Kenny, P.A.; Lee, E.H.; Bissell, M.J. 


Bissell, M.J.; Radisky, D. 

Cukierman, E.; Pankov, R.; Yamada, K.M. 


Sodek, K.L.; Ringuette, M.J.; Brown T.J. 

Casey, R.C.; Burleson, K.M.; Skubitz, K.M.; Pambuccian, S.E.; Oegema, T.R.; Ruff, L.E.; Skubitz, A.P. 

Helmlinger, G.; Netti, P.A.; Lichtenbeld, H.C.; Melder, R.J.; Jain, R.K. 

Lu, H.F.; Targonsky, E.D.; Wheeler, M.B; Cheng, Y.L. 


Tan, W.H.; Takeuchi, S. 


94 Chang, T.M.S. *Science* 1964, 146, 524.


Hochmuth, R.M. *J. Biomech.* **2000**, *33*, 15


Swift, C. Image adapted from: *The Opensource Handbook of Nanoscience and Nanotechnology*, **2010**.


141 Mckenna, G.B; Gaylord, R.J. Polymer 1988, 29, 2027.


