FUNCTION FOLLOWS FORM: NOVEL STRUCTURED ENCAPSULATION MATERIALS VIA MICROFLUIDICS

BY
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DISSERTATION
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ABSTRACT

Materials are composed of more than just their atomical makeup. Everywhere we look in nature and in man-made materials, there is an essence of structure that imparts mechanical, physical, and chemical characteristics; a reason behind why bones are porous, ceramics are heat-resistant, and composites are resilient. Materials science students know this better than others as part of our core curriculum, yet there are many avenues and applications for which these principles have yet to be applied. My research, though a collection of diverse projects, has focused on how we can specifically engineer encapsulation materials with the desired properties simply by changing the hierarchy of said material, and how to better understand the sensitive interplay between structural parameters.

Beginning in Chapter 1, I focus on how from a single polymeric material core-shell microcapsule, we demonstrated the controllable, reversible, and pH-triggerable release of actives by tuning the pore sizes of the microcapsules, leading to mechanical measurements of individual microcapsules through the use of nanoindentation in Chapter 2. This chapter also documents my investigations into structure-mechanical property relationships toward a universal theory of capsule yield stress. Chapter 3 focuses on the development of a high-throughput tissue model system and investigates the effects of cellular encapsulation and geometric effects of vasculature on tumorous tissue behavior. Similar in the way that cells are directly influenced by the stiffness of their substrate, we found that tumors are also responsive to the shape of the nearby vasculature and other diffusional constrains and conditions.
AKNOWLEDGEMENTS

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CHAPTER 1

pH-DEPENDENT SWITCHABLE PERMEABILITY FROM CORE-SHELL MICROCAPSULES

1.1 Microencapsulation and Triggered Release

The encapsulation and triggered release of actives are important for applications in drug delivery\textsuperscript{1,2}, perfumery\textsuperscript{3}, lab-in-a-shell\textsuperscript{4}, nutrient preservation\textsuperscript{5} and self-healing materials\textsuperscript{6,7}. The ideal delivery vehicles are sufficiently stable to indefinitely store the payload and, following a specific triggering event or upon exposure to particular environments, release the cargo with a controlled profile. One such class of delivery vehicles includes polymeric core-shell microcapsules (µCs), which are mechanically strong, chemically resistant, and compartmentalize volumes of payload. Although many formulations of liquid-filled core-shell µCs have been studied\textsuperscript{7–9}, few combine favorable encapsulation properties and controlled payload release\textsuperscript{2,10–12} without destruction of the capsules or the use of multi-component systems. Here we fabricate core-shell µCs in which the shell is composed of acid-degradable poly(o-(α-methyl)vinylbenzaldehyde) (PMVB)\textsuperscript{13} with good barrier properties, yet tunable rates of cargo release by varying the pH and the shell-wall thickness. In comparison with other rapidly degrading polymer capsules\textsuperscript{14}, the PMVB system provides acid-catalyzed release characteristics with a change in shell wall porosity while maintaining capsule structural integrity. This unique degradation and pH-tunable release
may lend itself to future applications in drug delivery or other fields where multi-stage and reversible release kinetics from μCs of single polymeric material is desired.

Scheme 1. Proposed mechanism for the acid-catalyzed PMVB degradation to yield 3-methyl-1H-inden-1-ol.$^{15}$

Scheme 1 illustrates the acid-catalyzed mechanism of PMVB degradation. Protonation of the backbone oxygen and subsequent elimination generates indenyl alcohol, 3-methyl-1H-inden-1-ol.$^{13}$ We imagined this chemistry to be well-suited for the development of μCs with acid-triggered release.

1.2 Microfluidic Encapsulation

The core-shell μCs were fabricated using a flow-focusing glass capillary device as shown in Figure 1.1a.$^{16}$ PMVB polymer and rhodamine B were dissolved in chloroform to form the oil phase (middle fluid). As described by Phillips and co-workers, poly(vinyl alcohol) (PVA) was included in the inner (5 wt %) and outer (10 wt %) fluids to balance the osmotic pressure between the interior and exterior of the capsules.$^8$ Control over capsule morphology and shell-wall thickness was realized by judicious selection of the inner, middle and outer fluid flow rates. Hexagonally close-packed, monodisperse μCs (Figure 1.1c) were recovered upon evaporation of chloroform. An inner fluid of 25 mg/mL FITC-
Dextran (4 kDa) was used to monitor shell wall barrier properties and triggered release by acid.

Figure 1.1 (a) Optical image of a glass capillary flow-focusing device. Inner fluid consisting of 5 wt % PVA and 25 mg/mL FITC-Dextran (4 dKa), a middle fluid of 10 wt % PMVB with 1mg/mL Rhodamine B, and an outer fluid of 10 wt % PVA (b) hexagonally-packed and dried collected µCs (c) magnified view of ‘b’ and (d) confocal fluorescent image of the shell wall of capsules following isolation and drying.

The shell wall thickness of the µCs was measured by scanning electron microscopy (SEM) (Figure 1.2). Upon mounting the capsules on SEM stubs, they were ruptured with a razor blade for cross-sectional imaging, and coated with approximately 5 nm of gold/palladium. Ten capsules from each sample set were measured for size and shell thickness and averaged together for each flow rate.
Figure 1.2 (a-b) The effect of flow rate on the mean shell thickness determined by SEM. For each flow rate, 10 µCs were analyzed and observed thicknesses were averaged. Fluorescence labeling of capsules with (c) Rhodamine B staining of the middle fluid and polymer shell-wall, (d) FITC staining of the inner fluid core. (e) The overlay of image ‘c’ and ‘d’, (f) Rhodamine B and (g) FITC emission intensity plots of one capsule across the diameter determined by confocal fluorescence microscopy.

In order to confirm the formation of core-shell µCs and provide additional information concerning the shell-wall thickness, fluorescence measurements\textsuperscript{17} were performed separately at FITC and rhodamine B emission wavelengths to clearly distinguish the capsule inner core and shell-wall (Figure 1.2e-g). The flow-focusing technique readily formed µCs that contain aqueous interiors (FITC-stained) with polymeric shell walls.
(rhodamine B-stained), and was capable of generating capsules that could be suspended in an aqueous solutions of various ionic strength and buffer compositions.

For SEM and fluorescence characterization, 120 µm sized capsules were selected predominantly for monodispersity and stability. The combination of flow rates (inner-middle-outer: 1000-X-8000 in µL/hr) generated capsules with a high level of reproducibility so long as X remained between 400 and 1000 µL/hr. Further variations in flow rates resulted in a loss of stability in monodisperse capsule formation or significant changes in capsule size.

**Figure 1.3** Triggered release of FITC-DEX from acid-triggerable PMVB core-shell µCs when exposed to pTsOH solutions in water. (a-f) Optical images of fluorescence emission of 1130 ±5.6 nm shell wall for 170 capsules over 14 hours.
Figure 1.4 Graph of 4 kDa Dextran-FITC conjugate release upon exposure to different organic acids. Results were the average of over 300 capsules measured for each condition.

μCs were further characterized for selective dye release by exposing them to various test conditions as shown in Figure 1.3. Microcapsules were collected into 10% weight PVA solution and left gently stirring to prevent settling for three hours. They were then washed with 20 mL of distilled water four times to remove residual PVA. Immediately after washing, the microcapsules were transferred to 96 well plates to be imaged on the fluorescence microscope and simultaneously triggered with the addition of solution to the wells. The average fluorescence intensity per capsule was measured for each time point and averaged for each condition.
Figure 1.5 Graph of 4kDa Dextran-FITC conjugate release in response to SDS and KOH as controls. This shows that after adding an acid trigger solution followed subsequently by a base solution resulted in significantly reducing the payload release, until the following addition of more acid solution.

Upon addition of freshly prepared aqueous solutions 12 mM para-toluene sulfonic acid (pTsOH), 12 mM KOH, 0.1 M NaCl, 0.1 M sodium dodecyl sulfate (SDS), and 0.1 M phosphate buffer, fluorescent images were captured at regular intervals for at least 8 hours (Figure 1.6). At least 170 capsules were tracked and monitored for each test condition. The percentage of FITC-DEX detected in the capsules was normalized to 25mg/mL FITC-DEX emission standards. Sodium chloride solutions and phosphate buffer solutions were tested as controls: the NaCl solution was to determine whether osmotic pressure causes the µCs
to release FITC-DEX, and the phosphate buffer solution provided stable pH over the duration of the experiment.

\[
\text{Figure 1.6 Graph of 4 kDa Dextran-FITC conjugate release from capsules of various shell walls.}
\]

µCs exposed to acids began releasing FITC-DEX immediately, particularly when compared to the control. This release profile was significantly faster, compared to acid-sensitive polymeric µCs of other shell wall materials reported in the literature that typically take two days to release. Sodium chloride triggered very gradual release while phosphate buffer showed no significant release. The pH of the supernatants was measured for each of the conditions after the timed fluorescence measurements and was found to be 2.06 for \( p\text{TsOH} \), 4.92 for NaCl, and 7.47 for phosphate buffer. Increases in shell wall thickness corresponded to delayed payload release as well as asymptotic tendencies (Figure 1.6).
**Figure 1.7** (a) The percentage of 4 kDa FITC-DEX released from 1130 ± 5.6 nm shell capsules in response to single-instance triggering via aqueous pTsOH solutions of various concentrations. Power Law curve fits of pH payload release (b) Plot of $k_{\text{release}}$ and $n$ with respect to acid concentration.

The ESEM images indicate that exposure to acid tended to cause transverse cracks to propagate through the thickness of the PMVB. This was particularly evident in Figure 1.10f where the disjunctions traversed the thin polymer film from the acid-exposed side of the Franz cell to the FITC-DEX-containing side. Curiously, the cracks tended to be approximately 50 nm in gap distance regardless of the thickness of the sample or acid strength. In the Franz cell experimental samples, larger disjunctions formed, but they were predominately seen connected by smaller cracks 50 nm in width. Altogether, these cracks seemed to form a bi-continuous network of 50 nm spacing, which we believe is how the FITC-DEX transferred from the core to the environment.
A most interesting observation is that the polymeric μCs maintain most of their structural stability through routine sample handling procedures; the acid exposure does not significantly compromise the overall capsule integrity. In the absence of razor-blade destruction, both thin films and capsules displayed surfaces covered with longitudinal and latitudinal cracks (Figure 1.8).

**Figure 1.8** These SEM images show that for either a thin film (left) or a whole intact capsule (right), the cracking of the polymeric layer is not primarily due to the handling and sample preparation. Decreases in pH and increases in exposure time both caused an increase in the cracking density. SEM images were prepared by freeze drying samples for 24 hours and sputter coating 5nm of Au/Pd.
1.3 Modeling Triggered Release of Actives from Microcapsules

The system also demonstrated a strong correlation between acid concentration and the payload release rate. Figure 1.7 shows the rate of release increased with the hydronium ion concentration. It was observed that pH had an effect on the equilibrium payload release, even though all conditions were from the same batch of µCs with the same shell thickness. To quantify the release character of the capsules, the data was fit to a power law derived from Fickian diffusion (Figure 1.7). As shown below in Equation 1, $M_t$ and $M_\infty$ are the cumulative amount of payload released at time $t$ and $\infty$ respectively. The constant $k_{release}$ relates to the geometry of the microcapsule barrier, while $n$ describes diffusive vs. degradative release character. Values of $n$ closer to 1 tend toward degradation-induced, while lower $n$ values indicate Fickian diffusion.

$$\frac{M_t}{M_\infty} = k_{release} t^n (1)$$

<table>
<thead>
<tr>
<th>pH</th>
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<th>$n$</th>
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Table 1.1 Curve fit of Fig. 7 to the power law equation

From Table 1.1, it is evident that there is a strong relationship between hydronium ion concentration and the mechanism by which the payload is released. As shown in Figure 1.7, the correlation for both $k_{release}$ and $n$ to pH is non-linear.
Another unique characteristic of this system was the reversible release caused by pH changes that result in ‘on/off’ permeability behavior, as shown in Figures 1.5 and 1.9. The µCs were exposed to either neutral or acidic conditions by subsequent equimolar additions of pTsOH and KOH respectively. Upon acid addition, the µCs were in the open state, while neutralizing the pH with the addition of base significantly reduced the payload release rate. Typically, systems require non-covalent shell components to rearrange depending on environmental pH such as lipids\textsuperscript{20} or polyelectrolytes.\textsuperscript{21} In our case, even though trace PVA still remains despite the washing steps, PVA is fairly pH stable\textsuperscript{22} and does not seem to undergo drastic conformational changes within the range we have studied.\textsuperscript{23}
1.4 Switchable Triggered Release via Salt Crystal Formation

ESEM images (Figure 1.10a-f) were taken to further investigate the mechanism behind the release of FITC-DEX from the polymeric µCs. Images were taken of samples before (Figures 1.10a,c,e) and after acid exposure to pTsOH (Figures 1.10b,d,f). The capsule images (a-d) were taken after the timed fluorescence measurements and the thin film samples (e,f) were gathered before and after exposure to 0.1 M aqueous TFA from one side of a Franz cell over 24 hours. The thin films were prepared by drop-casting the polymer dissolved in 1,4-dioxane and allowing the resulting film to air-dry.

Figure 1.10. Environmental Scanning Electron Microscopy (ESEM) of capsules and thin films prior to and immediately following acid exposure. (a) Cross-sectional view of a capsule prior to acid exposure (b) Cross-section of the same sample exposed to 12 mM
**Figure 1.10 cont.** pTsOH and an example of crack formation within the capsules (c) Macroscopic view of μCs ruptured by razor blade prior to acid exposure (d) Macroscopic view of a μC ruptured via razor blade after acid exposure (e) Thin film cross-section before acid treatment (f) Thin film cross-section after 0.1M aqueous TFA treatment for 24 hours.

A change in mechanical properties can be inferred particularly in Figure 1.10a and 1.10b. Figure 1.10a shows an uneven fracture pattern, characteristic of elastic tearing in polymeric films, while Figure 1.10b shows the shell wall fracture characteristic of a brittle material. The data in Figure 1.10c and 1.10d also support this, as the original capsules tended to fold over and deform elastically, compared to the relatively brittle nature of acid-exposed μCs.

![Nanoindentation graph](image)

**Figure 1.11** Nanoindentation with a 100nm Berkovich 3-sided diamond pyramid tip using Hysitron TI-950 TriboIndenter
**Figure 1.12** Optical images taken on the Hysitron TI-950 TriboIndenter showing capsule integrity was observed for the control and acid-triggered samples. The Control sample was stored in phosphate buffer solution while the Acid-Triggered sample was stored in 12 mM pTsOH solution, both for 12 hours prior to mechanical testing. Indenting experiments were done with dry capsules in air, with their liquid core still intact.

Nanoindentation experiments (Figure 1.11&1.12) support this theory of embrittlement, with hardness increasing by more than an order of magnitude after acid exposure and the reduced Young’s modulus nearly doubling. Also, upon taking optical images of the individual capsules before and after the indents have been performed, it is evident that there is also significant plastic deformation occurring after acid triggering, as well as some of the inside aqueous core leaking out from the microcapsule. The relationship between these mechanical property changes and the chemistry shown in Scheme 1 are presently under
investigation, but further ESEM was conducted of the samples to form a theory as to how the microcapsules’ reversible capabilities. Figure 1.13 illustrates some preliminary results investigating the pores of the microcapsules. On the left hand image, the capsules were exposed to pTsOH acidic triggers as per previous experimental conditions, partially crushed to enlarge the microcapsule pores for ease of viewing, and immediately imaged under ESEM.

**Figure 1.13** ESEM images taken of 115 micron-sized microcapsules that had been triggered with acid (left) and capsules that have been passified with a pH 12 basic solution immediately following acid triggering (right). Scale bar is 10 microns.

The right-hand image represents the same batch of capsules that were exposed to acidic trigger, yet were passivated with pH 12 solution to achieve pH 7. As one can observe, the cracks after basic triggers following acidic triggers resulted in capsules with cracks that appear partially filled, compared to simply the acidic triggered capsules. This suggests that upon acidic triggering, the capsules reach an equilibrium with the acidic solution on the
inner core, and during the basification process, salt crystals form at the shell interface, thereby inhibiting the polymer-conjugated dyes from leaking out further.

Figure 1.14 Schematic of dye permeability reversibility experiments

In conclusion, we have demonstrated that PMVB μCs can be manufactured via microfluidic flow-focusing devices with robust barrier properties in the absence of acidic triggers. Interestingly enough, the degradation mechanisms of this system is unique compared to other stimuli-responsive systems in that it is able to rapidly release active cargo while maintaining core-shell structure. The reversibility of the μC permeability also opens up possibilities for use as a highly sensitive microencapsulation method utilizing acid as a trigger to release payload or capture sample for analysis in response to environmental cues. Mechanistic and size-selective release studies are currently under investigation and results of these studies will be published in due course.

1.5 Experimental Details

Polymer Preparation

Poly(α-(α-methyl)vinylbenzaldehyde) was synthesized according to published procedure. (8)
o-(α-methyl)vinylbenzaldehyde was distilled twice with Kugelrohr apparatus to ensure monomer purity. 2.5 mol % BF₃.OEt₂ was used as an initiator and excess pyridine was employed to quench the polymerization. Mₙ = 53.1 kDa, PDI = 2.4.

**Diffusion Measurements with Side-By-Side Diffusion Cell: Film Preparation**

A solution of poly(o-(α-methyl)vinylbenzaldehyde) (3 mL in 1,4-dioxane, 15mg/mL) was cast onto Teflon Petri Dish Liner (diameter of the liner: 5 cm, purchased from Welch Fluorocarbon) and stored at RT for 12 hours. Resulting polymer film was further dried in a vacuum oven at 65 °C for 12 hours to give a film with a thickness of 25 +/- 1.2 μm.

![Fluorescence Intensity vs Time graph](image)

**Figure 1.15** Thin film triggered release diffusion measurements of fluorescent actives.

Square-shaped poly(o-(α-methyl)vinylbenzaldehyde) film (1cm x 1cm) was sandwiched between donor and receptor compartments of Side-Bi-Side diffusion cell (purchased from PermeGear, compartment volume: 3.4 mL, orifice diameter: 5 mm). Donor compartment
was charged with FITC-Dextran (4 kDa) solution in DI water (25mg/mL) and the acceptor compartment was charged with 0.1 M $\rho$TsOH in DI water. Diffusion of FITC-Dextran through the thin film was monitored with fluorescence measurements (sampling volume: 50 $\mu$L, sampling interval: 30 mins). Each measurement was performed for 3 times and averaged to better reflect the accuracy of the experiment. For a control experiment, only DI water was charged into the donor compartment of the diffusion cell. Diffusion experiments were conducted at RT.

![GPC traces after 5 minutes and 60 minutes](image-url)

Figure 1.16 Gel Permeation Chromatography Traces of Polymer Degradation
Poly(o-(α-methyl)benzaldehyde): Mn = 28 kDa, Mw = 63 kDa PDI = 2.2

10 wt % polymer subjected to 0.1 M acid solution in DCM

1.6 References


(7) Esser-Kahn, A. P.; Odom, S. A.; Sottos, N. R.; White, S. R.; Moore, J. S. 


CHAPTER 2

MECHANICAL PROPERTIES OF MICROCAPSULES VIA AUTOMATED NANOINDENTATION

2.1 Abstract

From the difficulty and investment required of making polymeric core-shell microcapsules, there is a need for a theory that accurately predicts the mechanical properties of a capsule based on its intrinsic measurements. This development could potentially save resources and allow for prediction of engineered microcapsules (μCs) for targeted formulation and product development. Herein we report the mechanical probing of core-shell μCs and thin films via multi-array nanoindentation, and our progress toward relating capsule size and shell thickness with the stiffness of the microcapsule as a function of depth. By developing a novel capsule array holder, we are able to apply high-throughput μC analysis with minimal human involvement to a nanoindentor, and by comparing several polymeric barrier materials, we aim to develop a generalized and unified theory.
2.2 Introduction

**Figure 2.1** Thin film of PMVB (left) and nanometer-sized core-shell capsules of PMVB produced by microfluidic devices (right)

It is relatively easy to make thin films;\textsuperscript{1,2} it doesn’t take much equipment other than a liquid material and a spin coater. Making micro and nano-sized core-shell encapsulants can be much more involved.\textsuperscript{3} Often times, encapsulation involves processes like stirring and ultra-sonication\textsuperscript{4–7} or microfluidic devices\textsuperscript{8–13}, both of which are highly sensitive to shell material viscosity, hydrophilic-hydrophobic interactions, and active sensitivity. Whichever the route, encapsulating materials can often times be a trial and error process, and has to be tailored to the specific active and shell material composition, with compatible surfactants selected.\textsuperscript{14} To compound the situation, making mechanical measurements of individual particles can also be troublesome. Work done by the Sottos group has demonstrated the feasibility of isolating and performing compression testing of singular microcapsules, coupled with optical microscopy.\textsuperscript{15} However, the compression punch system used lacks sensitivity because the piezo resister used was fairly rudimentary and was optimized for the yield stress significantly larger and thicker capsules. Atomic force
microscopy (AFM), on the other hand, has been used with a high level of sensitivity, but has been mostly limited to significantly smaller encapsulants in the nanometer range that are relatively soft.\textsuperscript{16} Nanoindentation, though often applied for organic and inorganic thin films,\textsuperscript{17–21} combines the strength of both techniques, with nano-scale precision, high load capabilities, and the ability to pair mechanical property measurements with XYZ dimensionality and optical microscopy.\textsuperscript{22} For these reasons, nanoindentation has recently started to become a technique applied to microcapsules,\textsuperscript{23–26} yet it still requires a large amount of direct human involvement to align the nanoindentor probe tip with each individual capsule. With so much time and effort required to first produce encapsulated materials, it is little wonder why in the industrial setting microcapsule production is considered a significant time investment and are not produced unless the target shell material has been decided. Often times the capsules mechanical properties are not measured at all, even though in many applications, core-shell microcapsules rely on mechanical failure or rupture at a specific engineered yield stress. One particular example is the encapsulation of enzymes for low-temperature laundry detergent. For product stability purposes, manufacturers wouldn’t want their encapsulated active to be released prior to laundry washing, but at the same time, they don’t want the capsules to be too strong that they fail to rupture and release the enzymes during the wash cycle. Therefore, having an estimate of a microcapsules properties based on intrinsic thin film measurements would give manufacturers a good approximation with which they could design encapsulated materials with targets in mind, compared to the trial and error system currently in place. Therefore, the goals of this project were two-fold, to first develop a technique by which hundreds of capsules could be measured in a semi-autonomous manner, and to use said
technique to develop a theory by which intrinsic properties from a thin film material could be used to estimate the mechanical properties of core-shell microcapsules.

2.3 Automated Nanoindentation

Nanoindentation is a highly-sensitive mechanical measurement with XY and Z sensitivity; however there are major hurdles that make it unfeasible as a general purpose quality control measurement. For one, measuring capsules requires near-perfect alignment with the probing tip and the capsule, which requires a great deal of human interaction and involvement with the instrument for each individual measurement. Secondly, in industrial settings, many capsule measurements are desired for large statistically-relevant populations, and nano-indentation is not currently a high-throughput measurement. To address these obstacles and to lead for better quantification of capsule mechanical properties, we have developed a method in which we align the capsules in a microarray using a patterned substrate with shallow holes. These holes are tuned to the order of size of the microcapsules (100-250 µm), which allow the capsules to sit half exposed when their dry powder is applied to the microarray device. As shown in Figure 2.2, the powder of capsules is then
Figure 2.2 Microarrays for high-throughput micromechanical measurements

shaken to remove capsules that have not successfully seated within the microarray of divots, and tape or PDMS is used to transfer the array of capsules to a glass slide where the nanoindentation can occur. In practice, as shown in Figure 2.3, not all capsules seat perfectly within the microarray of divots, especially with polydisperse microcapsule samples. This is because the capsule geometries may not fit well within the divots or they may extricate themselves during the shaking step to remove extraneous capsules if the tolerances aren’t just right. For monodisperse capsules made using microfluidic devices, the results are far more consistent, but future work will focus on how to better engineer the microarray divots to further enable better capture and retention of microcapsules into a homogenous array format.
Taking into account that this technique often produces missing parts of the array where a capsule should be present but isn’t, the overall goal of the technique development is still achieved. As an user can instruct the instrument to make periodic, region-regular indents within an XY plane using the existing software, missing capsules show up in the data as air-indents, which can then be removed from the data pool with simple macros.

In addition, we tested whether or not the act of handling the microcapsules using the microarray method would impart significant damage to the microcapsules by testing microcapsule populations in three ways. First, as shown in Figure 2.4, we conducted quasi-static load functions with a maximum force of 100,000 μN and at a constant strain to microfluidically-produced capsules of approximately 115μm in diameter. The nanoindentor was furnished with the high-load Omniprobe piezo resistor to achieve higher probe deflections at the expense of smaller-order deflection capabilities. We also chose to use a 392 μm cylindrical sapphire punch tip as the probe to simplify the tip-to-contact area calculations. This was possible because most surface contact calculations have been well established for spherical probes impinging on a flat thin film surface. In our case by using

Figure 2.3 Preparing an array of polydisperse microcapsules from dry powder using our device
a flat probe, we were able to simply reverse the situation with a flat probe impinging on a spherical surface. The results of 10 capsules were tested and averaged to produce the following data. As can be seen, there is negligible differences in slope in both the loading and unloading curves compared to capsules that have undergone the microarray handling process in Figure 2.4b.

**Figure 2.4** Quasi-static load functions with constant strain for (a) ~115 micron diameter capsules prior to microarray mounting (b) capsules after microarray mounting (c) capsules after vacuum shock followed by array mounting.
However, we did notice that the capsules that went through the microarray handling were more compliant than their counterparts, which we hypothesize is due to the liftoff transfer process, which we found the slightly larger capsules within the population were prone to adhere better to the scotch tape compared to the slightly smaller and hence stiffer capsules. Liftoff with poly(dimethylsiloxane) (PDMS) stamps also showed similar results. That being said, the integrity of the capsules were not compromised at all through the microarray transfer process, especially when compared to a structurally-impaired population of capsules (Figure 2.4c). In this case, the capsules were exposed to house vacuum for 10 seconds and then were checked under an optical microscope to make sure that the capsules did not rupture significantly. Through ESEM, it was found that 10 seconds was enough time to cause small hairline cracks in the microcapsules but not damage the overall integrity. With this population of capsules, the quasi-static loading curves were significantly different, as there were many instances of buckling, folding, and plastic deformations observed with both loading and unloading features, indicative of small micro-fractures as a result of the vacuum shock.\textsuperscript{27}

This technique has proven invaluable for the second part of this project, as it requires large statistical populations to generalize and formulate a theory that can predict from intrinsic properties the geometric-dependent properties of microcapsules.
2.4 Relating Capsule Size and Shell Wall with Stiffness Profile

![Stiffness profile is dependent on the depth of the probe](image)

Figure 2.5 Stiffness profile is dependent on the depth of the probe

Making core-shell microcapsules is not a trivial process, and often times during product development and formulation, predicting how a capsule will behave mechanically before synthesis can be an invaluable estimation. Therefore, the central goal of this project was to develop a predictive theory to link measured intrinsic material properties of thin films, and how they relate to the geometric properties of core-shell microcapsules, such as capsule size and shell wall thickness. As shown in Figure 2.6, we used the observation that the stiffness profile changes in core-shell microcapsules depending on the depth of the indent.\(^{28-30}\) Intuitively, it can be imagined that at small probe distances into the capsule surface, the capsules will not deform significantly, and therefore the forces will primarily be compressive. At higher strains or larger impinging distances, the forces will be both compressive and tensile as the entire capsule deforms to adjust to the force. In practice, we were able to produce monodisperse microcapsules via microfluidic devices with constant shell walls and tune the overall capsule diameter. Figure 2.6 illustrates the capsules at low probe depths converge on an intrinsic material value, and how the stiffness drastically increases the further the indenter probe is in the z-direction with regards to smaller capsule diameters. This can be explained that at smaller probe depths, the intrinsic properties of the
Figure 2.6 Stiffness profiles in relation to probe depth for 2 µm-thick PMVB capsule shell walls for three capsules sizes (each capsule size was averaged among 30 different microfluidically-produced capsules). Capsule size and shell thickness was measured by SEM.

material are dominating the stiffness readout, but larger depths start to depend more on the overall shape of the capsule and how much tension the capsule can withstand not just locally but on a macro scale, as well as the viscoelastic nature of the core material. Smaller capsules, therefore, are expected be stiffer on the macro scale of measurements. From this, two distinct regions within the plot stand out. For displacements under 10 microns from the surface, or roughly 3% of the capsule diameter, intrinsic effects dominate, and for larger displacements, capsule geometric effects dominate. The stiffness/depth profile for each of the capsule diameters can be fit under the following equation characterized by Table 2.1.
\[
y = A_1 + (A_2 - A_1) \left[ \frac{p}{1 + 10^{(\log x_01 - x)h_1}} + \frac{1 - p}{1 + 10^{(\log x_02 - x)h_2}} \right]
\]

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<td>Span</td>
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</table>

**Table 2.1**: Parameterized equation based on curve fitting Figure 2.6

**Figure 2.7** Modeling the effects of capsule size on the Stiffness/Depth profile
By simply interpolating the assumed linear relationship between capsule diameter size and the Log(x) values of the parameterized equation, we were able to mathematically model the stiffness/depth characteristics of capsule size, as shown in Figure 2.7. Even though these are fairly small changes in capsule diameter sizes, the preliminary data supports the hypothesis that the geometric characteristics of microcapsules can be modeled and characterized to simulate yield and rupture properties of microcapsules. Much work still is required to test the universality of these observations. Firstly, microcapsules of additional shell material will be required, as well as larger variations in capsule diameter and shell thickness. To address these obstacles, we switched polymer shell material systems from the PMVB previously used to 4 kDa poly(ethylene) which was a stronger shell material and could be purchased directly from Sigma-Aldrich instead of synthesizing on milligram scale.

In parallel, we had also begun to use our automated nanoindentation system to explore shell thickness geometric effects on the mechanical characteristics of core-shell microcapsules using the poly(ethylene) dissolved in chloroform as the shell material. Figure 2.8 represents an averaged quazi-static loading curve for microfluidically-produced capsules made from 4 kDa poly(ethylene) dissolved in 10% by weight chloroform which is almost twice as stiff at same probe depths as the PMVB microcapsules. We were also able to use significantly larger forces on these capsules due to their less compliant nature, and from the quasi-static loading curve, observe primary and secondary rupture points. For this particularly thick capsule of poly(ethylene), the first rupture occurs at approximately 45 µm depth, and the secondary at 60 µm. From the unloading curves we are also able to calculate out reduced Young’s modulus and stiffness, as it relates to the probe displacement.
This is calculated according to Oliver-Parr methods, which is derived from Hertzian theory of contact mechanics.\textsuperscript{31}

\[
E_r = \frac{\sqrt{\pi}}{2} \frac{S}{\sqrt{A_p}}
\]

\(A_p\) is the projected contact area of the probe, \(S\) is the slope of the unload curve at the maximum displacement point (\(h_{\text{max}}\)) and therefore the stiffness. Stiffness was calculated by modeling and curve-fitting the region between 40\% and 90\% of \(h_{\text{max}}\). The resulting reduced modulus is the combined modulus of the substrate deformations as well as the probe deformations. However, because in our case we are nanoindenting polymeric capsules, the sapphire tip is orders of magnitude stiffer than our substrate and we can assume that it is negligible. By conducting these measurements for hundreds of capsules, we are able to build a more detailed picture of how complex geometric aspects are with regards to shell thickness.

\[ \text{Figure 2.8 Quazi-static loading curve for 71 µm–thick poly(styrene) capsules with aqueous core} \]
At very low displacements in Figure 2.9, the nanoindentation quasi-static load curves follow the predicted trend of thicker capsule shell walls behaving stiffer and more like the 75 µm spin coated thin film of poly(styrene). However, this predictable trend degrades the further one probes into the core-shell microcapsules, suggesting that buckling and plastic deformations within the shell of the microcapsules are dominating and are much more complicated than previously thought.\textsuperscript{27}

\textbf{Figure 2.9} Averaged nanoindentation of a thin film of 12 kDa poly(styrene) and 115 µm in diameter microcapsules made of the same material with different thicknesses

However, at displacements under 25 microns, the slopes of the unloading curves and thus the stiffness follow the predicted pattern: the 75 µm thick poly(ethylene) thin film was the stiffest sample, followed by the 71 µm thick capsule, the 48 µm, the 9.6 µm, and finally the 4.6 µm capsule as the most compliant. In fact, the thin film and 71 µm capsule were nearly identical and their force-displacement curves until approximately 3% dimensional capsule strain, similar to the previously described measurements with PMVB core-shell capsules with regards to capsule diameter. It also became evident that capsule rupture was occurring during the compression tests (Figure 2.10).
Figure 2.10 (a) Quasi-static load function schematic of nanoindentation of microcapsules to the point of rupture (b) Enlarged force-displacement curves for poly(ethylene) microcapsules 115 μm in diameter and 51 μm thick (c) Optical images of poly(ethylene) core-shell microcapsules in various states of rupture before and after nanoindentation probing. Scale bar is 40 μm.
Through both \textit{in situ} optical imaging immediately following each load-displacement curve for quasi-static loading functions and corroborated by analyzing discrepancies in the load displacement curves (Figure 2.10b), we were able to determine both primary and secondary rupture. By plotting the rupture depth of the capsules with the maximum stiffness of the capsules with the primary rupture onset depth normalized to shell thickness, we found a linear relationship. In Figure 2.11, the 71 \textmu m thick capsule was significantly stiffer than all of the other capsules tested, followed by the remaining capsules in decreasing thickness. Though the thickness between the microcapsules varied, upon normalization, the primary rupture depth differences became even more pronounced. Thinner capsule shell walls intuitively were much more compliant, but they also tended to undergo primary rupture far earlier, which we hypothesize is due lack of sufficient material for plastic deformation. The
successful linearization of shell thickness correlating with maximum stiffness of the capsules suggests that parameterization of core-shell capsule shell is scientifically sound.

2.5 Nanoindentation as a Method to Measure Shell Cross-linking Density

![Figure 2.12 Schematic of pH-triggerable polyamide microcapsules formed by interfacial polymerizations.](image)

In collaboration with Hsuan-Chin Wang in Professor Zimmerman’s group, we have also begun to utilize our nanoindentation platform for microcapsules to determine whether differences in cross-linking density could be modeled and correlated. One type of pH-responsive materials that can be designed to react to both high and low pH are polyampholytes, which consist of polymers that contain both cationic and anionic groups. Stimuli-responsive polyampholytes have been prepared in different forms, such as gels, films, and thin-shell capsules. Polyampholyte capsules are typically formed around a template or at a liquid-liquid interface. However, as is common for polyelectrolyte assemblies, preparation of stable, liquid-core-thin-shell ampholyte capsules generally require multiple steps that could include core template dissolution, shell crosslinking for
structural stabilization or cargo encapsulation by post-fabrication loading. Furthermore, the currently known amphoteric encapsulants have yet to demonstrate controlled, extended release of small molecule payloads. In this work, we fabricated in one step a high capacity polymeric microcapsule that remained stable in a non-aqueous environment but demonstrated sustained release of a small molecule dye at neutral pH and accelerated release at acidic and basic pHs.

The microcapsules were prepared by interfacial polymerization based on a slight modification of procedure reported by Fréchet and co-workers. As shown in Figure 2.1, a saturated solution of pyromellitic diester diacid chloride (PDDC) in toluene was emulsified with an aqueous 0.4 w/w % polyvinyl alcohol solution with magnetic stirring to produce a suspension of oil-in-water emulsion droplets, whose shapes and sizes determine the final capsule dimensions. Dropwise addition of an aqueous solution of the hydrophilic monomers, N,N’,N’’-tris(2-aminoethyl)-1,3,5-triazine-2,4,6-triamine (triazine) and diethylenetriamine (DETA) initiated the polycondensation reactions at the water-oil interface to form a cross-linked polyamide shell. Maturation for 60 min afforded a suspension of microcapsules that were vacuum filtered, rinsed with DI water and acetone, and allowed to dry in air to give a white, free flowing powder.

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This free-flowing powder was then subjugated to the same automated nanoindentation procedure described in previous sections. Populations of over 150 capsules per sampling set were measured via load-constant quasi-static load functions with a 309 cylindrical sapphire tip. From calibrated optical microscopy, the capsules were measured to range from 50 to 400 µm in diameter with shell thicknesses on average 1 µm. As shown in Figure 2.13, increasing the feed ratio of trifunctional triazine monomer threefold resulted in a nearly doubling of stiffness for similar probe depths. This was corroborated by the observation that increases in triazine also resulted in a decrease in dye leakage rate from the inner core. Though preliminary, additional work is required because the ability to

Figure 2.13 Stiffness measurements of 3:1 and 1:1 triazine:DETA feed ratio capsules.
correlate capsule stiffness profiles with the cross-linking density would be an invaluable technique, especially as it can be difficult to measure experimentally from interfacial cross-linked networks.

2.6 Nanoindentation as a Method to Measure Phase-Change Materials

In collaboration Dr. Jinyun Liu in Professor Braun’s research group, we have also produced microcapsules using microfluidic devices to encapsulate saturated aqueous solutions of sodium acetate trihydrate (SAT). SAT, which is a common phase change material (PCM) has become popularized of late as the main component of reusable hand warmers. These simply resemble super-saturated solutions of SAT encapsulated in large poly(ethylene) bags containing a trigger inside that serves as a seed crystal for crystallization. Due to a high latent heat of fusion when transitioning from liquid to solid, it can store and then release large amounts of thermal energy as the solution crystallizes during the liquid-solid transition. There are numerous applications of PCM materials for optical triggers and thermal storage, yet we were interested in the mechanical property changes the capsules with PCM cores.

To first construct capsules with PCM cores, we turned to microfluidics. Compared to traditional stirring and interfacial polymerization techniques to form water-in-oil-in-water (WOW) double emulsions, we can achieve 100% encapsulation efficiency. This is especially non-trivial when considering encapsulating super-saturated solutions, as they can be readily triggered with stimuli such as sonication and slight amounts of evaporation. Microfluidics also offered control over the shell wall thickness, the capsule diameter, as
well as differential heating of the core, shell, and outer fluid material. The core consisted of a saturated SAT solution extracted directly from a consumer HotSnapz handwarmer package and filtered, and the sheath outer fluid was 10% 13 kDa poly(vinyl alcohol) in water, similar to the procedure discussed in Chapter 1. Dreve Fotoplast was chosen as the shell material because it can quickly cure to form tough acrylic polymer shells in under 10 seconds under 365 nm UV light. The capsules were on average 105 µm in diameter and a flow rate of 220 µL/hr inner fluid, 180 µL/hr middle fluid, and 4500 µL/hr outer fluid. The capsules were cured in-line with 365 nm UV lamps for approximately 30 seconds before collected and rinsed with DI water three times. These capsules were then dried and mounted with the capsule microarray for nanoindentation.

Figure 2.14 Crystallization of sodium acetate as a result of nanoindentation at room temperature. Scale bar is 50 µm.

Optically, it was evident that after quasi-static loads at room temperature, the capsules appeared to crystallize in response to the pressure of nanoindentation, as seen in Figure
2.14 and 2.15. When comparing capsules with SAT solution to the control sample of DI water, the crystallizing capsules dramatically increased their stiffness at indents greater than 8 microns in depth. This corresponded to approximately 10 μN of force on the individual capsules, and we hope to continue to use nanoindentation as a tool to further probe phase change materials and their transitions. Though these are preliminary results, we hope to further increase the SAT concentration within the microcapsules and perform differential scanning calorimetry and dynamic mechanical analysis tests on both the capsules as well as capsules embedded within polymer matrices in the future.

![Graph showing stiffness calculations from quasi-static load functions for capsules with sodium acetate cores and DI water cores](image)

**Figure 2.15** Stiffness calculations from quasi-static load functions for capsules with sodium acetate cores and DI water cores

### 2.7 Experimental Details

Nanoindentation experiments were conducted with a Hysitron TI-950 TriboIndenter™ with a 10 mN two-axis capacitive transducer. Micro-indentation experiments were
performed using the 3D OmniProbe™ transducer. All measurements employed standard 390 \( \mu \text{m} \) cylindrical indenters in the load-control mode. Tip-shape calibration was based on determination of the tip-area function. Indent load was varied from 1000 \( \mu \text{N} \) to 100,000 \( \mu \text{N} \) for the calibration. Calibrations were conducted in air, quartz, aluminum, and wax substrates prior to microcapsule indentation.

2.8 References


14. Madene, A., Jacquot, M., Scher, J. & Desobry, S. Flavour encapsulation and


CHAPTER 3

RAPID 3D EXTRUSION OF SYNTHETIC TUMOR MICROENVIRONMENTS

3.1 Abstract

Solid tumors house an assortment of complex and dynamically changing microenvironments in which signaling events between multiple cell types are known to play a critical role in tumor progression, invasion, and metastasis. To deepen our understanding of this biology, it is desirable to accurately model these structures in vitro for basic studies and for drug screening, however, current systems fall short of mimicking the complex organization of cells and matrix in vivo. Here we demonstrate the generation of spatially-organized 3D hydrogels of cells and matrix produced from a simple concentric flow device in a single step. Multiple cell types are pre-seeded in different spatial domains such as concentric regions of vessel-like tubular structures to reproducibly establish heterotypic cellular environments in 3D. Using macrophages and breast adenocarcinoma cells as an example of a paracrine loop that regulates metastasis, we explored the effects of clinical drug treatments and observed a dose-dependent modulation of cellular migration. This versatile and tunable approach for tissue fabrication will enable a means to study a wide range of microenvironments and may provide a clinically-viable solution for personalized assessment of patient response to therapeutics.
3.2 Introduction

Anti-cancer drugs are typically assayed on tumor cell lines grown on tissue culture plastic with efficacy measured by growth inhibition or cell death. However, tumor progression in vivo is mediated by dynamic microenvironments where spatiotemporal control of signaling between diverse cell populations is responsible for growth and dissemination. Metastasis of breast cancer, in particular, is partially regulated by a paracrine loop between tumor cells (TC) and macrophages (Mϕ) in the primary tumor.\textsuperscript{3,4} This interaction enhances the motility of both cells and primes the TC to intravasate into the bloodstream, thus playing a key initiating event in disease progression. This heterotypic cell interaction pair has been directly observed in vivo using intravital microscopy\textsuperscript{5} and in vitro using a variety of 2D and 3D culture platforms.\textsuperscript{6,7} The development of therapeutic regimens that target heterotypic interactions preceding metastasis is an emerging area for development in cancer therapy. However, there is a deficit of in vitro systems that generate reproducible tissue morphology for a quantitative assessment of heterotypic signaling suitable for therapeutic development.

Compared to traditional 2D culture in a petri dish, 3D culture allows more accurate replication of natural tissue and matrix organization.\textsuperscript{8–10} In vitro models developed for drug screening have demonstrated differences in cell proliferation, morphology, and drug response for 3D compared to 2D systems.\textsuperscript{11,12}

Microfluidic devices provide a means to organize 3D microenvironments such as cysts and tubules, which mimic the basic building blocks of epithelial tissue and allow high-surface-area interfaces between chemically or biologically distinct domains of tissue.\textsuperscript{9} Kang et al. developed a process in which they vary chemical composition and topography as a fiber is extruded\textsuperscript{13} and Onoe et al. have pioneered a hydrodynamically-focusing method for
generating cell-encapsulated fibers on a large scale. However, single channel fibers are limited in geometry, and rely on post-processing methods to achieve geometric variability and structural control. Additionally, nearly all of the current synthetic vasculature techniques have predominately focused on producing straight vasculature, yet if one were to look within nature, most vasculature is in fact not straight. Intestinal tissue vasculature form mesh-like structures, while breast tissue tends to be more serpentine in shape.

![Intestinal Tissue vs Breast Tissue](image)

**Figure 3.1 Comparing the vasculature of human intestinal tissue and breast tissue**

We demonstrate a versatile approach to multi-domain tissue mimetics by extruding alginate fibers under controlled flow rates to modulate flapping instabilities. Controlling the fiber arrangement in a single fluidic extrusion step allows integration of multiple cell types in distinct and controllable spatial domains. We demonstrate the scope of this approach for modeling tissue-mimetic interactions *in vitro* by filling the inner channel of the fiber with macrophages and incorporating tumor cells in the surrounding peptide-modified alginate. We characterize the 3D segregation of this cell pair over time and show how
pharmacological inhibitors of migration or TC-Mϕ signaling disrupt the normal spatiotemporal organization.

Figure 3.2 Schematic of dual-cultured hydrogel fiber production.

The strategy we present to make high-throughput co-cultured alginate fibers in a single step is illustrated in Figure 3.2. First, human breast adenocarcinoma (MDA-MB-231; hereafter TC) and mouse macrophage (RAW 264.7; hereafter Mϕ) cells are labeled with CellTracker and then mixed into pre-prepared 3.2% weight alginate and 0.046 g/mL CaCl₂ in Dulbecco’s Modified Eagles’ Media respectively. We conjugate the pentapeptide sequence Tyr-Ile-Gly-Ser-Arg (YIGSR) with EDC/NHS to the alginate to support cell-adhesion (Figure 3.3).¹⁶
Figure 3.3 NMR of alginate conjugated with YIGSR peptide. Peak a correlates with the 4 backbone protons from YIGSR and peak b correlates with the manuronic and gluronic acid ring protons.\textsuperscript{27,28} We calculate approximately 7% conjugation of peptide to the alginate backbone.

After being placed in syringe pumps, the solutions are extruded in the microfluidic device in the desired three geometries as illustrated in Figure 3.4, and collected in 45 mg/mL CaCl\textsubscript{2}
aqueous solution. These fibers are then cut into pieces suitable for mounting as flowable tissue culture chips (Figure 3.5), or used in 96-well plates for long term culture.

**Figure 3.4** (a) Architecture and pattern amplitude spacing dependence on outer fluid/inner fluid ratio (b) Optical images of select patterned fiber structures with 200 µm scale bars

**Figure 3.5** The flow-focusing device used to extrude the fibers is assembled by gluing glass capillary tubes with inner diameters 100, 700, and 2000 µm in a concentric pattern. Each tube is connected to a separate inlet channel as shown in (a). Once extruded, fibers can be mounted in a similar manner to flow fluid through the inner channel (b). Scale bars are approximately 3 mm.
3.3 Control and Characterization of Vasculature Geometry

Just as vertically extruded soft-serve ice cream twists into swirls when the end is seated in an ice-cream cone, hydrodynamically-focused alginate fibers are manipulated by an analogous push-back force that packs the extruded material into specific hierarchical conformations. Due to the shear-thinning nature of the alginate solution as it is extruded,\textsuperscript{17} the solution increases in its ability to bend and twist to accommodate for the stiffness exerted from the gelled, downstream middle fluid. By running the middle fluid at significantly higher volumetric fluid flow rates compared to the outer fluid, the middle fluid tends to pack the extra volume by flapping back and forth in periodic arrangements.\textsuperscript{15,18} Based on results shown in Figure 3.4a, decreases in the outer fluid/inner fluid volumetric flow rates result in tighter packing and form a single concentric co-flow device.

We are able to demonstrate ‘1D’, ‘2D’, and ‘3D’ architectures by simply changing the flow rate and thus the periodicity. However, this phenomena was only observed at very high flow rates (middle fluid = 20 mL/hr). Using this strategy, we are able to tune the flapping frequency on-the-fly and create hollow-channel fibers with multiple types of patterns on a continuously hollow calcium alginate hydrogel strand. Because sodium alginate is shear-thinning, we use a slower gelating outer fluid (sat. CaSO\textsubscript{4}) to maintain temporal phase separation behavior until the gelator (45 mg/mL CaCl\textsubscript{2}) in the inner channel diffuses radially throughout the fiber to ‘lock’ the structures into their respective architecture.
Figure 3.6 Confocal fluorescence scan of fluorescently-conjugated alginate hydrogel in the ‘3D’ vasculature pattern. Scale bar is 100 µm.

Figure 3.7 Extruded alginate fibers fastened vertically in a Perkin Elmer 7e dynamic mechanical analyzer (DMA) were measured for frequency sweeps of storage and loss
Figure 3.7 cont. modulus at room temperature. The samples were approximately 3 cm with 0.5 cm of clamping distance.

Figure 3.8 (d-i) Calcium-Alginate fibers prior to culturing with cells (d) Close-up ESEM image of tightly-cross-linked inner channel membrane indicating the smaller, partially collapsed pores of the Calcium-Alginate matrix (e) ESEM of the bulk aspect of the alginate fiber matrix (f) Diagonal slice of a patterned alginate fiber showing intersecting inner channel segment (g) ESEM of patterned fiber close to the opening of the inner channel (h) Macro image of straight fiber near inner channel opening (i) Calcium-Alginate network freeze-dried after culturing with macrophages for four days (control media sample)

To characterize the geometric structure and porosity of our hollow channels, we use a combination of high speed video recording (Figure 3.4b), confocal fluorescence microscopy of the hydrogel covalently conjugated to fluorescein (Figure 3.6c), dynamic mechanical
analysis (Figure 3.7), and environmental scanning electron microscopy (ESEM) (Figure 3.8d-i). ESEM of the freeze-dried alginate demonstrates that pore size is homogenous within the bulk structure (Figure 3.8e) with the exception of an approximately 2 micron crust of tightly-cross-linked hydrogel that surrounds every hollow inner channel (Figure 3.8g-h). We note that this crust could prove advantageous for applications where several levels of spatial cellular organization are desired (e.g. endothelial perfusion on the channel wall). Inner channel periodicity does not affect cross-linking or porosity in the bulk. Significant structural changes of the hydrogel fibers suggestive of remodeling is seen after 4 days culture with macrophages (Figure 3.8i).\textsuperscript{19} We find that over 90% of encapsulated cells were viable within the first week of culture (Figure 3.9), with no significant changes in viability over 3 weeks.

![Graph showing viability of encapsulated cells.](image)

**Figure 3.9** Viability of encapsulated cells determined by Live-Dead assay (percent cells alive)
3.4 Quantifying Macrophage and Tumor Cell Migration

Tumor cells *in vivo* will attract macrophages that secrete epidermal growth factor (EGF) to enhance the metastatic phenotype, thereby priming the tumor cells to intravasate into the vasculature. This paracrine interaction is proposed as a central event mediating metastasis.\(^3,4,6\) We hypothesized that our alginate hydrogels would provide a simple model system of TC-Mϕ co-culture for optimizing pharmacological compounds that disrupt this clinically relevant interaction. To test this hypothesis, we supplemented our co-culture with Gefitinib (GEF), an epidermal growth factor receptor (EGFR) inhibitor,\(^20\) zoledronic acid (ZA), a bisphosphonate that targets osteoclasts and macrophage cells, and a Rac1 inhibitor (RAC) as a broad spectrum modulator of cell migration.\(^21\) We used CellTracker\textsuperscript{TM}-labeling and confocal fluorescence microscopy to quantify the co-localization of the tumor cells and macrophages in our co-culture over time. Correlation is calculated by the following equation, where each pixel intensity corresponding to a fluorescent marker is compared to the other

\[
Correlation = \frac{\sum_i(S1_i - S_{1aver}) \times (S2_i - S_{2aver})}{\sqrt{\sum_i(S1_i - S_{1aver})^2 \sum_i(S2_i - S_{2aver})^2}}
\]

![Figure 3.10 Demonstrating the co-localization plugin in ImageJ Fiji for proteins](image)

60
channel. The program then compares pixel-by-pixel how likely each fluorescent intensity is to be in the same three-dimensional space. Typically, this analysis is reserved for proteins and receptors within a cell (Figure 3.10), however we apply it to fluorescently-labeled cells within our extruded fibers.

![Figure 3.11 Co-localization analysis performed on extruded vascularized fibers](image)

To calculate correlation factor, we used ImageJ Fiji with the Coloc2 plugin. The images were split into separate channels for macrophages (3.11a) and breast cancer (3.11b) cells and despeckled to remove noise. We ran the colocalization test with threshold values of 12 for channel a, and 30 for channel b. The reported Pearson’s R-value above our threshold is used for correlation factor. To test the control conditions of co-localization analysis, we incubated the two fluorescently-labeled cell types on two distinct glass slides as well as mixing the cells together and imaging (Figure 3.12). When the cells are localized in distinct and segregated populations, the Pearson correlation value was negative, while cells that were interspersed with one another had positive values. This was also evident in the spectroscopic plot of pixel intensities, with each of the axis representing the two imaging channels ranging in intensity from 0 to 255. Segregated cells produced spectras with very few intensities in common, and thus localized along the axis.
At Day 0, the fiber samples are very distinct and the macrophages are exclusively located in the hollow channels of the fibers (Figure 3.13a). However, after four days of incubation in media or vehicle control, the macrophages became interspersed amongst the entire calcium-alginate hydrogel with a high degree of co-localization with tumor cells. When the co-cultures are treated with GEF, ZA, and RAC, there is a distinct impairment of migration and co-localization with the majority of macrophages remaining in the channel interior.
Figure 3.13 (a) 3-D reconstructions of macrophages (Mφs) in green and tumor cells (231) in red stained with CellTracker, and the location of the hollow inner channel indicated in dotted grey lines. Scale bar is 400µm. (b) Co-localization scatter plots indicating a trend toward correlated pixel values between CellTracker fluorescent channels, which can be partially reversed upon incubation at certain drug concentrations. The yellow arrow indicates the presence of anti-correlated pixel intensities between the two channels. (c) Plot of the calculated Pearson Correlation factors for the three drugs at three concentrations at low[1], medium[2], or high[3] concentrations (d) Plot of the RAW cell to 231 cell volume ratio per fiber
We quantitated the co-localization of cell-specific CellTracker™ fluorescence signal using the Coloc2 plugin of ImageJ Fiji.\textsuperscript{22} At Day 0 when the two cell types are localized in distinct regions, there is a strong anti-correlated band indicated by the yellow arrow in Figure 3.13b, which corresponds with a negative calculated correlation factor. After 4 days, when significant numbers of macrophages migrate within the alginate matrix, we see a disappearance of this anti-correlation band and an increase in the calculated correlation factor (Figure 3.13b, 3.13c). When our cultures were treated with drug concentrations at the approximate IC50 of all three inhibitors (50 µM for ZA and Rac1; 50 nM for GEF), we see the calculated correlation factor decrease. In particular, the inhibitor of Rac1 which is expected to impair cell migration between channels, leads to a return to anti-correlation comparable to initial seeding (Day 0). Supplementation of the cultures with drugs well below or above the IC50 fails to abrogate the co-localization.
3.5  Vascular Geometry Effects on Cellular Response

Next we explored how the ratio of macrophages to tumor cells vary over time in our system (Figure 3.14). Because the counting is done in a 3D stack, we add the volume of all the macrophage and cancer cells respectively and divide by the average cell size to calculate the TC/Mϕ ratio. There is an increase in the TC/Mϕ ratio from Day 0 to Day 4 in all of the samples, presumable because macrophage growth rates are nearly double that of the 231 tumor cells (Figure 3.13d).23,24

![Image](image_url)

**Figure 3.14** To count cells, macrophages (a) and breast cancer (b) cells are split into separate channels and thresholded to highlight cells (c, d).

Cells exposed to RAC show significantly higher TC/Mϕ ratios compared to the other samples, which we speculate is due to continued proliferation in the channels upon motility inhibition;21 however we cannot discount 231 cell death as a contributing factor. By moving
from the ‘1D’ fibers to the ‘2D’ patterns, the TC/Mφ ratio increases, which correlates with our fast camera observations. Comparing fibers with straight ‘1D’ channels to fibers with ‘2D’ wave-like architecture, we see that the straight fiber samples consistently show higher correlation factors and thus more migration of macrophages to the alginate than their patterned counterparts (Figure 3.15a). This trend is in contrast to the ratio of TC/Mφ where the straight fibers result in a 50% decrease in the fraction of macrophages to tumor cells. This is presumably because of the lower volume of the inner channel in the ‘1D’ compared to ‘2D’ fibers.

Figure 3.15 (a) Comparison of averaged RAW / 231 cell ratios to RAW-231 correlation factors calculated for straight and patterned fibers respectively after drug and media exposure (b) Plot relating the RAW-231 correlation with the RAW / 231 ratio, with labels and a line drawn to illustrate the best performing conditions inhibiting macrophage migration

When we compare the TC–Mφ correlation to the Mφ/TC ratio for our fibers treated with pharmacological inhibitors, we can readily identify drug treatments that show the highest
inhibition of migration and co-localization (Figure 3.14b; GEF and RAC). We hypothesize that the different behavior of cells in the “1D” fibers versus the “2” fibers may be on account of increased interactions, not only due to the increase in macrophages but also from the directionality of the signaling (see Figure 3.16b).

**Figure 3.16** (a) Straight and patterned hollow alginate structures formed in our devices with 200 µm scale bars (b) Cartoon illustrations comparing how the geometric spatiality of cells may affect their signaling in naturally-occurring architectures and model systems.

**Figure 3.17** (a-c) Simulations of 2D anisotropic diffusion through finite difference method for channels of (a) zero, (b) one, and (c) two periodic patterns. (d) Cross-sectional plots of simulated diffusion away from inner channels for increasingly periodic patterns.
By simulating the diffusion of chemical signaling that may take place from the macrophage positioning within the fiber, it is evident that the patterning periodicity plays a more dominant role in how the diffusant disperses within a fiber than the initial chemical signal concentration (Figure 3.17a-c). To supplement the model, we injected Cy3-conjugated streptavidin through the hollow channel and found increased diffusion out of the channel for the serpentine architecture compared to the straight fibers (Figure 3.18).

![Figure 3.18 Dye simulation experiment with ‘1D’ and ‘2D’ fibers](image)

‘1D’ and ‘2D’ fibers were injected with a 0.1 M solution of Cy3-conjugated streptavidin through the hollow channel and images were taken every fifteen seconds in the plane of the hollow channel to measure the amount of dye diffusing over time. This supports are model simulations demonstrating the importance of vascular architecture.

In living systems, structures develop during normal morphogenesis and pathological processes to adopt a breadth of curvilinear and fractal-like forms (e.g., blood vessels, respiratory buds, mammary ducts), where diffusional distances and spatial positioning of cells are critical for function. Our tissue-mimetic fibers may better emulate the nonlinear
architecture in living systems,\textsuperscript{27} indicating that this technique may find broad applicability in fabricating model tumor architectures for therapeutic development.

We present a twist on traditional microfluidic concentric flow spinning methods using fiber packing minimization to produce a variety of structures in a single simple device. The ability to quickly tune the packing of vascularized alginate multi-cell tissue scaffolds may lend itself for use as a model system to study other heterotypic interactions. Indeed, we believe this system will prove particularly useful for modeling metastasis because the vessel architecture can be tuned on-the-fly. Not only are the scaffolds easily manufactured, they also offer tremendous potential as model systems for high-throughput screening of drug efficacy, as well as flow-able and vascularized lab-on-a-fiber platforms. We imagine that this packing is not limited to the gelation of calcium-alginate hydrogel fibers but is applicable to a wide range of experimentation required for fast-patterning vasculature in the future.

### 3.6 Experimental Section

**Co-culture of adenocarcinoma and macrophage cells within the fibers.** MDA-MB-231 human adenocarcinoma cells (ATCC) and RAW 264.7 mouse macrophage cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Fisher) supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (p/s), media changed every 2 days and passaged at ~80% confluency using 0.05% Trypsin:EDTA
CellTracker Green CMFDA dye and CellTracker CM-Dil dye (Life Technologies) were used to label RAW and 231 cells respectively according to manufacturer’s instructions. We dispersed labeled 231 cells in the alginate solution at a concentration of $5 \times 10^6$ cells/mL. Labeled RAW cells were dispersed in CaCl$_2$ solution at a concentration of $4.5 \times 10^7$ cells/mL. After fiber generation, fibers were cut into approximately 20 mm sections and stored in 24-well cell culture plates containing media and pharmacological drugs. For pharmacological inhibition studies, we used Gefitinib (G-4408, LC Labs) at 10, 50, and 100 nM, Zoledronic acid (Cayman Chemical) at 10, 50, 100 µM, and Rac1 Inhibitor II (CAS 1090893-12-1, Calbiochem) at 10, 50, 100 µM concentrations. A vehicle control of 2% DMSO in cell culture media was also used. The cells were incubated at 37°C, 5% CO$_2$ environment, with media changes every 2 days.

**Covalently-conjugating alginate fibers.** Sodium alginate (71238 Sigma, 1.5 g) was dissolved overnight stirring in 150 mL of PBS at room temperature. EDC (E1769 Sigma, 0.597 g) and sulfo-NHS (56485 Sigma, 0.418 g) were added and stirred for 5 minutes, followed by addition of YIGSR (T7154 Sigma) and stirred for 24 at RT under nitrogen. The solution was dialyzed in Millipore-filtered water for 5 days and lyophilized for 8 days. Conjugation was calculated to be 7% from $^1$H NMR in D$_2$O (Supplemental Figure 2). To image the fiber patterns in the absence of cellular additives, the 406 mg sections of sodium alginate were added to a solution of EDC (10.5 mg), sulfo-NHS (4.83 mg), in 7 mL of PBS and stirred for 5 minutes at room temperature. Then fluoresceinamine (201626 Sigma, 1.4 mg) was added and stirred for 24 hours. The fibers were then washed three times with PBS and fluorescently imaged. These procedures was adapted from Mooney *et al.*$^{26}$
**Concentric glass capillary microfluidic device manufacture.** Glass capillary tubes from Vitrocom were purchased with inner diameters of 100 µm, 700 µm, and 2000 µm. They were glued in a concentric pattern with Loctite 5 Minute Epoxy and washed several times with water and isopropanol.

**Producing 1D, 2D, 3D architectures.** A 3.2% weight solution of sodium alginate was left to gently stir at 3°C for 5 days in PBS for the middle fluid, a 45 mg / mL solution of CaCl$_2$ in media was prepared for the inner fluid, and a saturated solution of CaSO$_4$ in PBS for the outer fluid. The solutions were extruded from Harvard Apparatus PhD 2000 syringe pumps and collected in a bath of inner fluid solution without cells.

**Environmental scanning electron microscopy of alginate fibers.** After fiber production in the absence of cellular additives, the sections of hydrogel were cut into 20mm sections and submerged in liquid nitrogen for 10 minutes, fractured, and then immediately lyophilized in a LABCONCO Freezone 4.5 Liter Freeze Dry system for 36 hours. The fiber sections were then sputter-coated with ~80nm of Au/Pd for imaging.

**Cell count and viability assay.** Cell viability was measured every day for 7 days. A 20 mm section of cell fiber was collected in a centrifuge tube and suspended in 2 mL of 0.5M ETA solution for 30 minutes at 37 °C to dissolve the alginate followed by the addition of 100 µL 0.05% Trypsin and incubation at 37°C for an additional 5 minutes to form single cell suspension. The solution was centrifuged for 5 min at 300 rcf and the resulting cell pellet was re-suspended in 1mL of fresh cell culture media. A 1:1 mixture of cell suspension and 0.4% Trypan blue solution (Life Technologies) was prepared and counted
with a hemocytometer to determine the number of live (unstained) compared to dead (blue stained) cells. 3 counts were averaged for each day.

**Cellular migration and correlation analysis.** Confocal image stacks of fiber samples were opened in ImageJ Fiji using the Coloc2 plugin with threshold values consistently set throughout samples for the green (macrophage channels) and the orange (231 cell channels). The outputs of the plugin are displayed as Pearson Correlation Factors above the threshold values, and the 2-D pixel intensity correlation plot.

**Immunocytochemistry of co-cultured fibers.** After 4 days in culture, sectioned cell fibers were fixed in 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 for 30 minutes before blocking with 1% bovine serum albumin (BSA, Sigma) for 1 hour. Primary antibody labeling was performed in 1% BSA in PBS overnight at 4°C with mouse anti-cortactin (abcam ab33333, 1:500 dilution), or mouse anti-CD44 (abcam ab6124, 1:500 dilution). Secondary antibody labeling was performed in 5% goat serum containing 1% BSA with Alexa Fluor 647-conjugated anti-mouse IgG antibody (Life Technologies A-21236, 1:500 dilution) and DAPI (1:5000 dilution) for 1 hour at room temperature. Immunofluorescent images were taken on a Zeiss 710 multiphoton confocal microscope.

**Simulation of Diffusion:** The diffusion equation through finite element analysis was simulated and solved implicitly for a grid mesh of $40 \times 50$ in 2D, using the central difference as the spatial derivatives. For each initial condition in Figure 4e–h, the total amount of initial concentration and point sources was kept constant to illustrate the ideal case of comparing the diffusion profiles of different inner channel geometries while keeping simulated number of macrophages constant. Increasing point sources in relation
to length of simulated inner channel did not produce significant differences in the diffusion profile shape. The code from the MATLAB FileExchange was adopted and repurposed to accommodate more geometrically relevant models.
3.7 References


