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Research Article

Single-Step Synthesis of Alginate Microgels Enveloped with a Covalent Polymeric Shell: A Simple Way to Protect Encapsulated Cells

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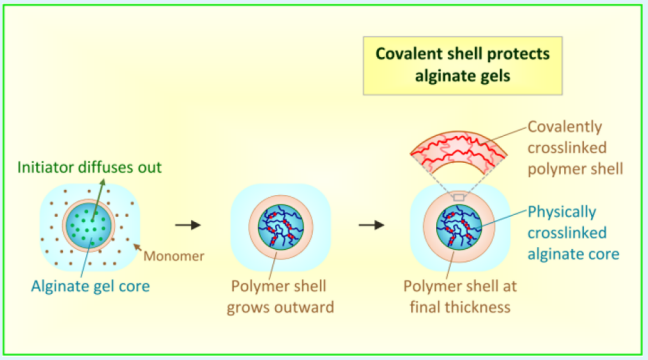
ABSTRACT: Microgels of biopolymers such as alginate are widely used to encapsulate cells and other biological payloads. Alginate is an attractive material for cell encapsulation because it is nontoxic and convenient: spherical alginate gels are easily created by contacting aqueous droplets of sodium alginate with divalent cations such as Ca^{2+}. Alginate chains in the gel become cross-linked by Ca^{2+} cations into a 3-D network. When alginate gels are placed in a buffer, however, the Ca^{2+} cross-links are eliminated by exchange with Na^{+}, thereby weakening and degrading the gels. With time, encapsulated cells are released into the external solution. Here, we describe a simple solution to the above problem, which involves forming alginate gels enveloped by a *thin shell of a covalently cross-linked gel*. The shell is formed via free-radical polymerization using conventional monomers such as acrylamide (AAm) or acrylate derivatives, including polyethylene glycol diacrylate (PEGDA). The entire process is performed in a single step at room temperature (or 37 °C) under mild, aqueous conditions. It involves combining the alginate solution with a radical initiator, which is then introduced as droplets into a reservoir containing Ca^{2+} and monomers. Within minutes of either simple incubation or exposure to ultraviolet (UV) light, the droplets are converted into alginate−polymer microcapsules with a core of alginate and a shell of the polymer (AAm or PEGDA). The microcapsules are mechanically more robust than conventional alginate/Ca^{2+} microgels, and while the latter swell and degrade when placed in buffers or in chelators like sodium citrate, the former remain stable under all conditions. We encapsulate both bacteria and mammalian cells in these microcapsules and find that the cells remain viable and functional over time. Lastly, a variation of the synthesis technique is shown to generate *multilayered* microcapsules with a liquid core surrounded by concentric layers of alginate and AAm gels. We anticipate that the approaches presented here will find application in a variety of areas including cell therapies, artificial cells, drug delivery, and tissue engineering.



KEYWORDS: *microcapsules, core-shell structures, multilayered capsules, liquid-core capsules, cell encapsulation*

INTRODUCTION

Cell encapsulation refers to the entrapment of live cells in polymeric scaffolds, with conditions tailored to ensure that the cells remain viable and functional.[1−4] This technique has found increasing application across various fields, particularly in the area of tissue engineering.[2,3] For cell encapsulation to be successful, both the type of polymer and the physical properties of the final scaffold are important. Typically, polymers form a hydrogel, i.e., a three-dimensional (3-D) network of cross-linked chains, with the cells entrapped in this network.[1−4] Hydrogels exhibit solid-like mechanical properties, which are important for keeping the cells protected and immobilized, while the large water content and porous nature of the gel network ensure that cells can exchange oxygen, nutrients, and waste with the external medium. It is important for cell encapsulation in gels to be performed under mild, physiological conditions to ensure cell survival. The polymer backbone must also be nontoxic and compatible with the cells. The above requirements are commonly met by naturally derived biopolymers such as polysaccharides (e.g., alginate, chitosan, agar, or hyaluronic acid) or proteins (e.g., gelatin or collagen).[3,4] Gels of the above biopolymers have been used to encapsulate various mammalian cell types such as pancreatic islets,[5] hepatocytes,[6] osteoblasts,[7] Jurkat cells,[8] and stem cells.[9] Gels have also been used to encapsulate microbial cells such as

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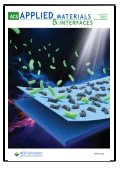

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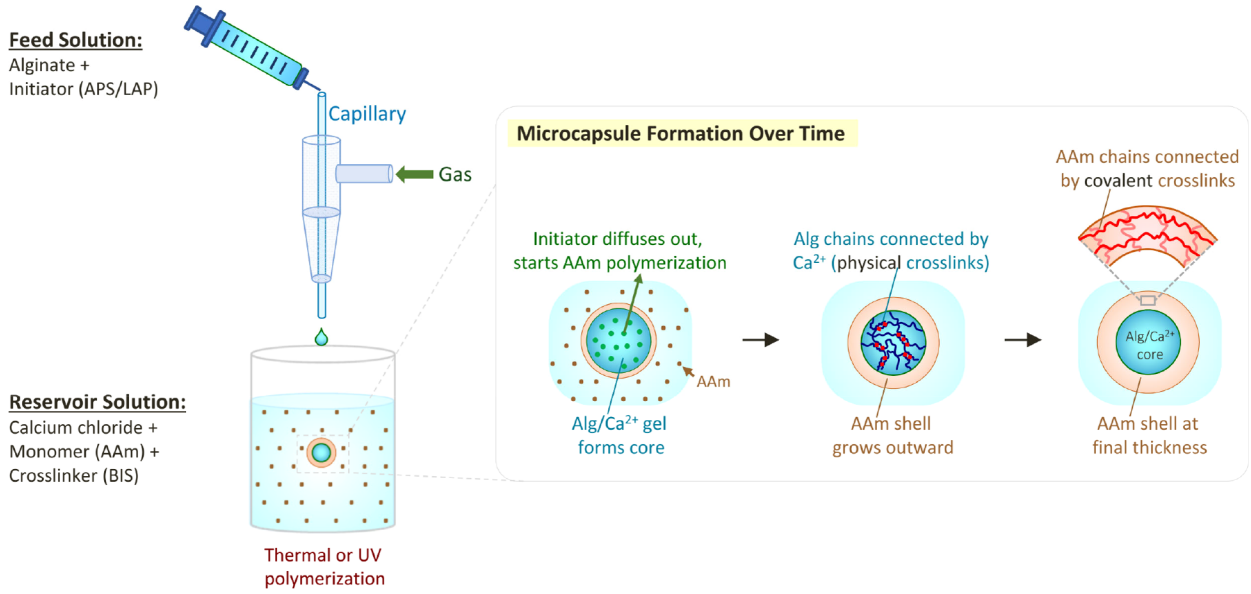

Figure 1. Schematic of the procedure used to synthesize alginate−polymer microcapsules. A feed of alginate (Alg) and the initiator is sent through a capillary into a reservoir containing monomers and Ca^{2+}. Pulses of gas shear off aqueous droplets from the capillary tip. As the droplets enter the reservoir, the inset shows the progression toward the final microcapsule structure. First, the Alg in the droplets is gelled by Ca^{2+} and this forms the core of the structure. The initiator then diffuses out and, upon activation by ambient heat or UV light, polymerizes a shell of AAm around the Alg core. The AAm shell grows outward and reaches its final thickness in a few minutes.

yeast or bacteria for soil fertilization,[10] removal of urea and ammonia,[11] or delivery of probiotics to the gut.[12]

Among the polymers mentioned above, the one that is most widely used for cell encapsulation is alginate due to its abundance, low cost, biocompatibility, and non-immunogenicity.[13,14] Alginate is an anionic polysaccharide derived from brown algae. It forms gels when combined with divalent cations such as calcium (Ca^{2+}) under mild conditions at physiological pH.[13,14] The gels arise because the Ca^{2+} cations bind with the carboxylate anions on adjacent alginate chains, thereby cross-linking the chains into a sample-spanning 3-D network.[14] To encapsulate cells in such a gel, one simply has to suspend the cells in an alginate solution and add this dropwise into a reservoir containing Ca^{2+}. The cell-bearing droplets then become converted into gels, and the sizes of these gels can be controlled by modulating the droplet size. Microscale gels (10−1000 μm), termed microgels or microbeads, are commonly used in applications.

Despite the many advantages of alginate for cell encapsulation, however, one major drawback is that alginate gels tend to degrade easily, i.e., they are chemically and mechanically weak.[15−17] For example, when these gels are stored in a buffer containing univalent cations such as sodium (Na^+), the Ca^{2+} cations get exchanged with the Na^+. This means a loss of cross-links from the alginate gels, which makes the gels weaker (i.e., decreases their elastic modulus). Moreover, a loosely cross-linked gel will tend to swell more in water, and if cross-links continue to be eliminated, the gel will completely degrade, i.e., the alginate will be solubilized. During this degradation process, cells encapsulated in the gel will be released into the external solution. Removal of Ca^{2+} cross-links from an alginate gel can also be induced by anions in the solution like citrate that can chelate (competitively bind and remove) Ca^{2+}. For this reason, cell-bearing alginate gels cannot be stored in citrate buffers as an example. The problem of premature degradation in cell-bearing alginate gels has been noted in numerous studies.[15−17] An underlying reason for the easy degradability of alginate gels is that they are formed by physical (ionic) bonds rather than by covalent bonds.

To address the above problem, many attempts have been made to increase the chemical and mechanical durability of alginate gels. One approach has been to coat the anionic alginate gels with cationic polymers such as chitosan.[18,19] However, coating procedures can be time-consuming due to multiple steps, and cationic polymers also tend to be toxic to cells.[18] Another approach is to blend alginate with other natural biopolymers such as agarose or gelatin.[20,21] More recently, interpenetrating networks (IPNs) of alginate and one or more biocompatible synthetic polymers have been synthesized. For example, an IPN of alginate and acrylamide (AAm) has been reported for encapsulation of stem cells.[22,23] In another example, an IPN of polyethylene glycol diacrylate (PEGDA) and alginate was used to encapsulate bacteria.[24] Lastly, alginate derivatives bearing covalently cross-linkable groups have been used to form gels.[25,26] The presence of strong covalent bonds can ensure that a gel remains intact even if the ionic bonds degrade. However, there are several problems with existing approaches. First, if a covalent network coexists with the alginate, the former could impair the growth of encapsulated cells. Second, the degradation of ionic bonds in an alginate gel will still induce the gel to swell appreciably (even if covalent bonds are also present) and cells in the gel can be released. Also, to form a second network, additional steps are usually required, which makes the encapsulation procedure more complex. Likewise, synthesizing alginate derivatives can be complex and laborious. In short, a simple and straightforward way to strengthen alginate gels has proved elusive.

In this study, we describe a simple technique for protection of alginate microgels, which involves enveloping the microgel in a layer of covalently cross-linked polymer gel. Importantly, the synthesis of such "microcapsules" is accomplished in a

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single step that is completed in a matter of minutes from start to finish. Briefly, our approach involves sending a feed solution of alginate and either a thermal- or a photo-initiator through a microcapillary into a reservoir containing both Ca^{2+} and monomers (e.g., AAm). This results in a core−shell structure with a core of alginate cross-linked by Ca^{2+} and a thin shell of cross-linked AAm formed by free-radical polymerization that occurs either by ambient heat or by short exposure to UV light. The thin polymer shell around the alginate core stabilizes the overall microcapsule. Even if the alginate core was to get degraded due to ion-exchange or chelation, the microcapsule remains intact because of its covalently cross-linked shell. The presence of such a robust shell differentiates our approach from other core−shell capsules that have involved alginate.[27−30] Using the above procedure, we encapsulate bacteria and mammalian cells in the core of the capsules and show that the cells remain viable. The thickness and chemistry of the polymer shell as well as the microstructure of the overall capsule can be varied systematically. We believe that the simplicity and versatility of our technique will allow it to be widely adopted to improve the properties of alginate microgels in myriad applications.

RESULTS AND DISCUSSION

Synthesis of Alginate−Polymer Microcapsules. The technique used to synthesize microcapsules with an alginate core and a covalent polymer shell is shown schematically in Figure 1. We use the concept of "*inside-out polymerization*", which was developed recently by our lab.[31] The idea is to include one component of a free-radical polymerization (the initiator) in a core structure, while the remaining components (monomers) are added to the surrounding solution. In that case, the initiator would diffuse outward from the core, where it would encounter the dissolved monomers and induce polymerization. A covalently cross-linked polymer shell would thereby grow outward from the core.[31] Here, we couple such polymerization with the gelation of alginate via ionic cross-links. To accomplish this at the microscale, we use a water-gas microfluidic device that employs pulses of gas to shear off aqueous droplets from the tip of a microcapillary.[32] Unlike other microfluidic approaches used for microcapsule synthesis,[17,24] this technique eliminates the use of oil or non-aqueous solvents. This is attractive because the technique is thereby more compatible with cell encapsulation.[30] Control of droplet size in the range between 80 and 500 μm is made possible by modulating the aqueous flow rate and the gas pressure.[32]

The single-step process in Figure 1 begins with a feed solution that contains both alginate (2 wt %) and a free-radical initiator. In the case of thermal polymerization, we use ammonium persulfate (APS), typically at 2 wt %. Any payload to be encapsulated in the final structure (such as cells) would also be included in this feed. The reservoir contains the rest of the chemicals needed for polymerization, which include a monomer (e.g., acrylamide, AAm, 10 wt %), a cross-linker (e.g., *N*,*N*′-methylene-bis-acryl-amide, BIS, 0.15 wt %), and an accelerant (tetramethylethylene diamine, TEMED, 1.5 wt %). In addition to the above, the reservoir also contains 1.6 wt % (150 mM) calcium chloride. When the device is switched on, the feed solution flows through a capillary of diameter 200 μm at its tip. Pulses of gas (air or nitrogen) are sent around the tip of the capillary, and for each pulse of gas, a droplet of the feed is sheared off from the tip.[32] As this droplet enters the reservoir, the alginate in the droplet is cross-linked by Ca^{2+} ions almost instantaneously, thus converting the droplet into a gel. At the same time, the APS initiator diffuses out of this gel into the solution. The APS induces the polymerization of the AAm monomer, resulting in a thin layer of poly(AAm) around the alginate gel.[31] Due to the use of the accelerant TEMED, the entire polymerization is completed in about 5 min at room temperature.

A variation of the above process is used in the case of UV polymerization. The thermal initiator is replaced with a photoinitiator such as lithium phenyl-2,4,6-trimethylbenzoyl-phosphinate (LAP), typically at 1 wt %. Otherwise, the feed is identical to the above. The reservoir solution is also identical, except that TEMED is omitted. As droplets from the feed are collected in the reservoir, UV light is shone around the reservoir for 60−90 s. This is sufficient to complete the formation of the covalent poly(AAm) shell around the alginate core. Photos of the setup for thermal polymerization are shown in Figure S1A and for UV polymerization in Figure S1B (see the Supporting Information). The LAP photoinitiator was particularly chosen because it is known to be relatively nontoxic to cells,[33,34] and this will be particularly useful in the encapsulation of mammalian cells in the above structures.

Microstructure and Mechanical Properties of the Microcapsules. Figure 2 shows optical (brightfield) micrographs of structures obtained by the above procedures via thermal or UV polymerization. All structures are microcapsules with a distinct core (a gel of alginate cross-linked by Ca^{2+}) and a thin shell (a gel of a covalently cross-linked polymer). The reason that we obtain core−shell structures is because the rate of alginate cross-linking by Ca^{2+} is much faster than the rate of AAm polymerization.[31] If the alginate was mixed with monomers prior to contact with Ca^{2+}, one would obtain a composite (IPN) of alginate and AAm rather than a core−shell structure.[23] Instead, in our case, the core contains only alginate, which is conducive for cell encapsulation. At the same time, the shell provides protection to the alginate core, as will be shown below. The technique presented here is simple yet versatile. It allows the average sizes of the core and shell to be varied independently. Moreover, instead of AAm, the shell can be made from other monomers with a C═C bond that can be cross-linked by free radicals. As an example, we have formed shells of polyethylene glycol diacrylate (PEGDA) around the alginate core, as shown in Figure 2B. PEGDA, like AAm, is a monomer that is commonly used in biomedical applications.[3,24] In addition to optical microscopy, we have also used SEM to analyze the capsules. SEM images (Figure S2) confirm that there are two distinct layers to the capsules—an outer layer of the covalent gel around a core of the alginate gel.

We have also compared the mechanical properties of alginate microgels versus microcapsules with an alginate core and a thin (~40 μm) covalent shell of AAm. The tests were performed under compression, and the data (Figures S3 and S4) reveal that the AAm shell makes the microcapsule significantly more robust than the microgel. When the alginate microgel (control) is compressed to about 50% strain, it is irreversibly squished from a sphere to a pancake shape and does not recover its original shape when the compression is stopped (Figures S3A and S4A). On the other hand, when the alginate−AAm capsule is compressed to a higher strain (~55%), the capsule recovers its original shape after compression (Figures S3B and S4B). Moreover, the peak stress measured during the compression of the alginate

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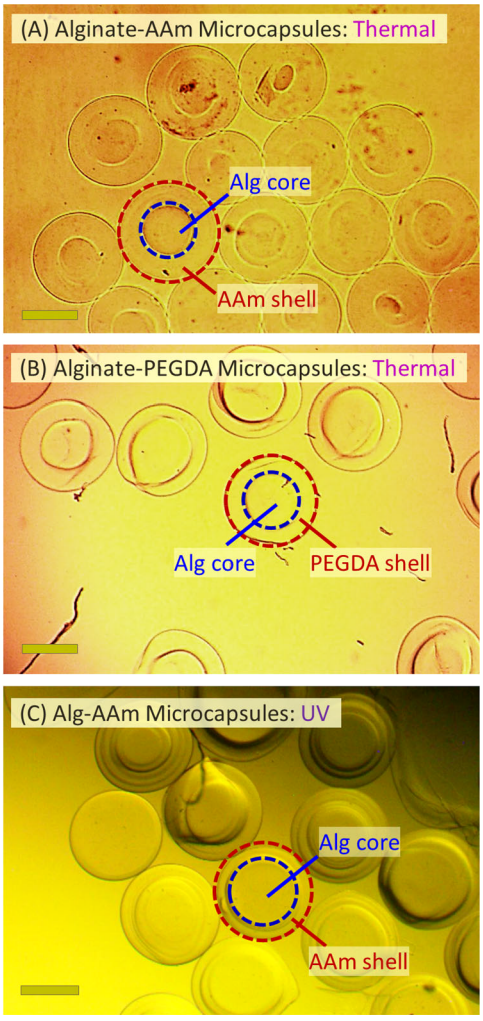

Figure 2. Images of microcapsules with a covalent polymer shell around an alginate core. (A) Shell of acrylamide (AAm) and (B) shell of polyethylene glycol diacrylate (PEGDA), both via thermal polymerization. (C) Shell of AAm via UV polymerization. In all cases, the core and shell are distinctly visible and are marked around a specific capsule for clarity. Scale bars: 200 μm.

microgel is only around 13 kPa, which is much lower than that for the microcapsule (71 kPa). The data confirm the contribution of the thin polymer shell to the capsule elasticity and strength. Similar differences in mechanical properties have been reported in the case of macrosized (~5 mm) alginate gels with and without a polymer shell from our previous study.[31]

Tuning the Microcapsule Core and Shell Sizes. The growth of the polymer shell around the alginate core is controlled by the amount of initiator present in the core (i.e., in the feed solution). Once the droplets enter the reservoir, the shell grows over 1−2 min and reaches its final thickness. With longer incubation in the reservoir, there is no further growth of the shell, i.e., the growth is self-limiting. This is shown by Figure 3, where the concentration of the initiator (APS) in the feed is varied. If the initiator is low (1 wt %), no visible shell of the polymer (AAm) is found around any of the alginate cores (Figure 3A). Upon increasing APS to 1.5 wt %, shells become visible around a few alginate cores (Figure 3B). A further increase in APS to 2 wt % results in all alginate cores having uniform, discernible AAm shells (Figure 3C). However, when APS is further increased to 3 wt %, the shell elongates into a tail in some cases, indicating that polymerization is not merely confined to the volume around the cores but extends into the solution (Figure 3D). The tail may also reflect polymerization in the wake of the droplet as it falls in the reservoir. If APS is increased above 3 wt %, soon after droplets of the feed enter the reservoir, the entire solution is gelled into a solid block. Overall, the results in Figure 3 imply that there is an optimal APS concentration around 2 wt % for forming polymer shells by thermal polymerization, and we have fixed this concentration for the studies below. In the case of 1 and 1.5 wt % APS, although shells are not visible, the structures do resist degradation by chelators, as discussed below. This means that thin shells are present in those cases too, and additionally, it implies that the shell thickness can be tuned by the initiator concentration.

We can also vary the core diameter independent of the shell thickness. This can be done by changing the flow rate of the feed, which alters the droplet size (and thereby the core size), as shown in our previous study.[32] Here, we performed experiments at various flow rates with a feed of 2% alginate and 2% APS, with the reservoir containing AAm and 150 mM Ca^{2+}. At the lowest feed flow rate of 5 μL/min, the microcapsules have a core diameter of 88 μm and a shell thickness of 25 μm (image 1 in Figure 4). If the flow rate is increased to 20 μL/min, the core diameter increases to 132 μm with the shell thickness being 28 μm (image 2). With further increase in the flow rate to 40 μL/min, the core diameter reaches 142 μm and the shell thickness is 37 μm (image 3). These data show that the main effect of increasing the flow rate is to increase the core size, while the shell remains at about the same thickness. Based on Figures 3 and 4, the core size can be controlled via the flow rate and the shell thickness via the initiator concentration.

Microcapsule Stability and Swelling. Our main reason for adding a polymer shell around alginate gels was to prevent their degradation or swelling when contacted with certain ions or chelators. We now proceed to test these aspects. First, we placed bare alginate microgels and alginate−polymer microcapsules in 100 mM ethylene diamine tetracetic acid (EDTA), a well-known chelator of Ca^{2+} ions. As expected, the bare alginate microgels completely degrade within 30 min (Figure 5A). As per the schematics, the degradation is because Ca^{2+} cross-links are removed by EDTA, leaving behind linear alginate chains that are no longer part of a 3-D network.[14] In contrast, alginate−AAm and alginate−PEGDA microcapsules both maintain their spherical shape even after 24 h in the EDTA solution (Figure 5B). In these cases, the alginate cores are expected to get degraded into linear alginate chains, but the polymer shells stay intact because they are held together by covalent bonds. Similar results are found if sodium citrate is used as the chelating agent instead of EDTA.

While chelators can cause complete degradation, alginate gels can also suffer partial degradation when placed in buffers. For example, when placed in phosphate buffered saline (PBS), some of the Ca^{2+} cross-links are exchanged with Na^{+}, which reduces the mechanical rigidity of the gels. In turn, a decrease in cross-link density will induce the gel to swell in water, which creates a vicious cycle because the swollen gel will be even weaker. If the gels are to be used for encapsulating biological payloads, their ability to maintain their mechanical integrity under physiological conditions will be crucial. To test the degree of swelling, we placed alginate microgels and alginate−polymer microcapsules in PBS (pH 7.4) and monitored their

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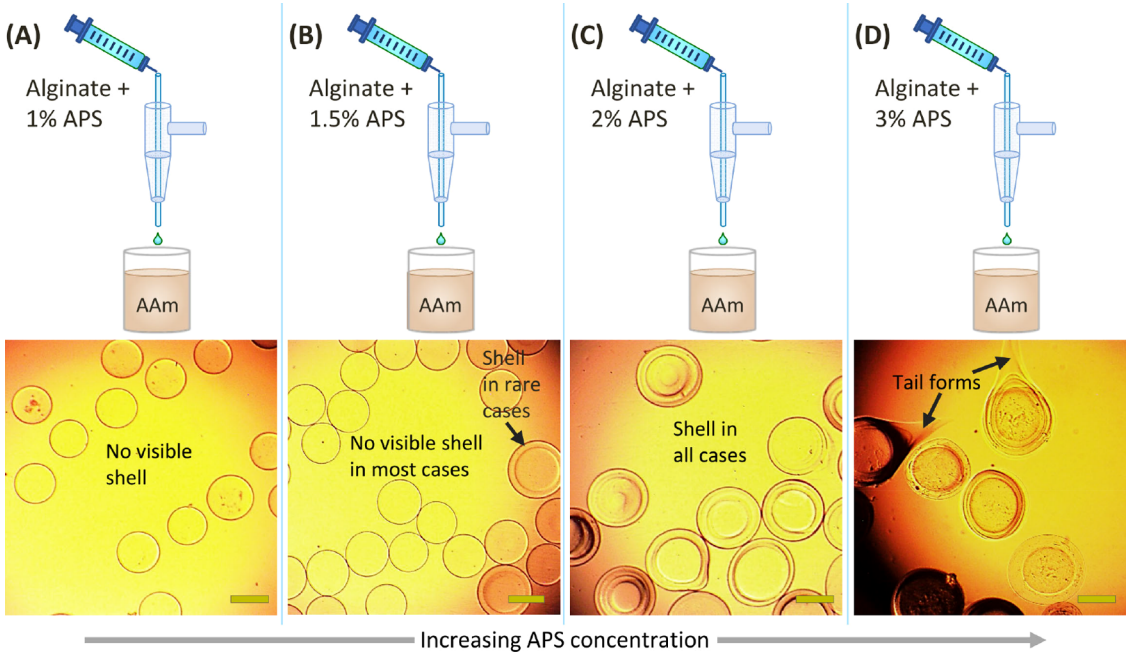
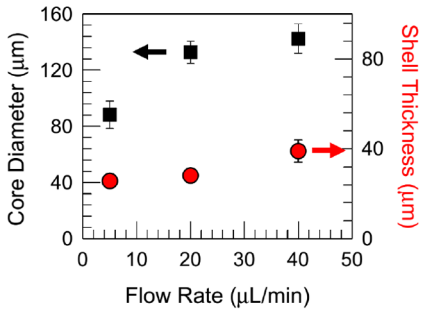


Figure 3. Effect of initiator (APS) concentration on the formation of alginate–AAm microcapsules by thermal polymerization. (A) 1 wt % APS: no AAm shells are visible; (B) 1.5 wt % APS: shells visible only around a few cores; (C) 2 wt % APS: visible shells around all cores; and (D) 3 wt % APS: tails around some cores and the capsules clump together. Scale bars: 200 μm.



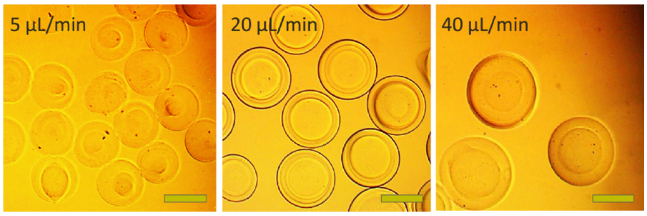


Figure 4. Effect of increasing the feed flow rate on microcapsule sizes. Increasing the flow rate increases the alginate core diameter while maintaining about the same AAm shell thickness (all structures made by thermal polymerization). This is shown by the plot above and the images below. Scale bars: 100 μm. The error bars represent the standard deviation of the distribution (n = 3).

size over time up to 10 h. As expected, the alginate microgels (without any shell) show a 57% increase in size (data in Figure S5). In comparison, alginate–PEGDA microcapsules swell by 42%, while the alginate–AAm microcapsules swell only by 28%. These results confirm that the presence of a polymer layer hinders swelling of the capsule. Among these two polymer shells, AAm appears to provide greater resistance to swelling, possibly indicating that the AAm network is more densely cross-linked than the PEGDA network. Note that AAm and PEGDA are both nonionic polymers that will not be affected by the ionic strength or pH of the buffer solution. Thus, regardless of any loss of cross-links from the alginate core, the polymer shells will ensure that the microcapsules preserve their structural integrity.

Encapsulation of Cells in Microcapsules. We proceeded to study the encapsulation and culture of both bacteria and mammalian cells in the microcapsules. Culture of bacteria in alginate gels can be problematic because the gels can degrade either due to ion-exchange in growth media (similar to degradation in buffers, as discussed above) or because of rapid bacterial growth (bacteria often escape out of the gel matrix and spread to the outer solution).[16] In this regard, the polymer shell around the alginate core can protect the cells and also help maintain the cells in the core. Note that the polymer shell, being a porous gel, does allow diffusion of small molecules in and out of the core, which is essential for cell viability.

We conducted the encapsulation studies with a strain of *E. coli* that had been genetically engineered to express a red-fluorescent protein (DsRed) when isopropyl β-D-1-thiogalactopyranoside (IPTG), a small molecule "inducer", is present in its environment.[35,36] The procedure for cell encapsulation is that from Figure 1, with the feed containing both alginate/APS and the cells in a mixture of PBS and Luria Broth (LB) media. We used AAm as the monomer in the reservoir and after thermal polymerization, we obtained microcapsules with bacteria in the alginate core and encased by an AAm shell (Figure 6). Following synthesis, the microcapsules were placed in culture media and incubated overnight (18 h) in a shaker at 37 °C. Two culture conditions were explored: one without IPTG and the other in the presence of 1 mM IPTG. The images show that both non-induced and induced microcapsules maintain their structural integrity during the overnight incubation. Non-induced capsules show a low level of red fluorescence, indicating low expression of DsRed (Figure 6B). In comparison, the induced capsules show significant red fluorescence, indicating that the presence of IPTG has indeed

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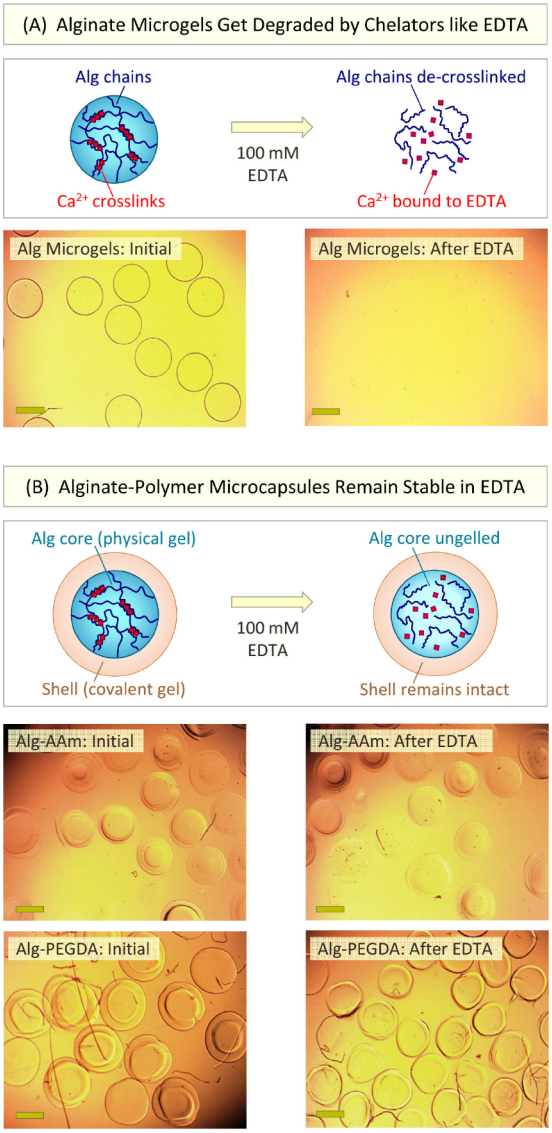

Figure 5. Stability of alginate–polymer microcapsules to chelation. (A) Alg microgels (control) are rapidly degraded within 30 min when placed in 100 mM EDTA. The schematics indicate that degradation is due to Ca^{2+} cross-links being removed from the gels. (B) Alg–AAm and Alg–PEGDA microcapsules remain intact in 100 mM EDTA even after 24 h. The schematics indicate that degradation occurs in the cores of the capsules, but the polymer shells remain intact.

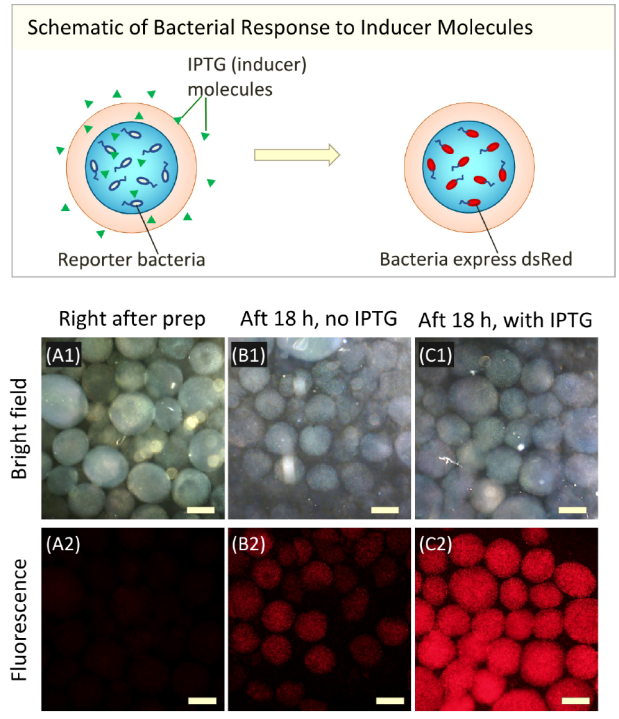

Figure 6. Culture of genetically engineered bacteria in alginate–AAm microcapsules and their response to an inducer. The bacteria (*E. coli*) are engineered to express a red fluorescent protein (DsRed) when exposed to a molecular inducer (IPTG), as indicated by the schematics. Brightfield and fluorescence micrographs are shown for the microcapsules: (A1, A2) Right after preparation; (B1, B2) cultured without IPTG; and (C1, C2) cultured with IPTG. Red fluorescence is higher in C2 than B2. Scale bars: 200 μm.

stimulated the cells to express the fluorescent DsRed protein (Figure 6C). Thus, the results confirm that the bacteria are viable and that they follow their genetically programmed response (i.e., they remain functional).

The same bacteria were also cultured over a longer time (4 days), and the results are shown in Figure S6 for the control alginate microgels (no shell) and in Figure S7 for microcapsules of alginate–AAm polymerized thermally (S7A) or by UV (S7B). In the absence of the shell, the alginate microgels completely disintegrated after overnight incubation (Figure S6). On the other hand, both sets of alginate–AAm microcapsules remained intact over the 4 days of culture (Figure S7). Higher cell growth is observed in the UV-polymerized capsules, which is the mode that we have employed for culture of mammalian cells as well (see below). Note also that the cells remain largely contained in the capsules even over the longer culture period.

Next, we explored the encapsulation and culture of mammalian cells, specifically those of the human epithelial cell line, Caco-2 (Figure 7). In this case, we used UV polymerization to prepare the capsules—for this, a feed of alginate/LAP + cells in PBS was sent into a reservoir containing AAm and exposed to UV for 90 s, as shown in Figure 1. As mentioned earlier, the LAP photoinitiator was chosen because it is relatively nontoxic to cells.[33,34] The images in Figure 7 show that the capsules remain intact and maintain their spherical shape over the entire 7 day culture period. Moreover, we employed live/dead staining to infer the state of cells in the capsules, and the data were quantified using ImageJ. The cells are mostly live at all time points, as indicated by the predominantly green fluorescence in the images. Cell viability is calculated to be more than 85% after 4 days and more than 80% after 7 days. These results are very encouraging and show that our approach is suitable for encapsulation of both bacteria and mammalian cells. Note that the entire encapsulation of cells is done at room temperature without involving an immiscible oil phase, which is usually needed in other microfluidic approaches. Because mammalian cells grow at a slower rate than bacteria, maintenance of capsule integrity in growth media over a long period of time is arguably more important for these cells.

Multilayer Microcapsules with Liquid Cores. Lastly, we present a variation of our synthesis scheme that gives rise to a distinct structure for the microcapsules. In this case, rather

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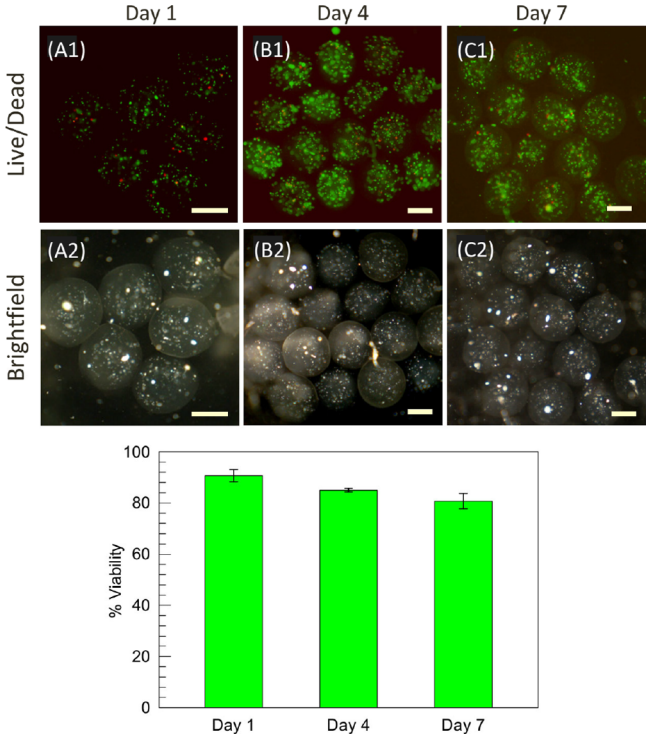

Figure 7. Encapsulation and culture of Caco-2 epithelial cells in UV-polymerized alginate−AAm microcapsules. Fluorescence and brightfield micrographs are shown over the 7 day culture, with live/dead staining (live = green and red = dead), indicating the state of the cells in the former. (A1, A2) Day 1; (B1, B2) day 4; and (C1, C2) day 7. A bar graph of the cell viability at days 1, 4, and 7 is shown below the images, with the error bars representing the standard deviation from multiple (n = 3) experiments. Scale bars: 200 μm.

than feeding a solution of alginate into Ca^{2+} to make gels or capsules, we reverse the order and use Ca^{2+} as the feed and alginate in the reservoir. Previous attempts using this scenario[37−39] have recognized that there are two problems. First, when a drop of Ca^{2+} solution encounters the alginate solution, the drop rapidly loses its spherical shape and dissolves away. In that case, there would be insufficient time to gel the droplet. To prevent this problem, researchers have added sucrose,[37] methyl cellulose,[39] or xanthan gum[38] to increase the viscosity of the Ca^{2+} solution. Even with a viscosity increase, the second problem is that structures formed by adding Ca^{2+} to alginate are weaker mechanically compared to structures made by the "normal" route of adding alginate to Ca^{2+}.

Our approach (Figure 8) avoids both the above problems. Here, the feed is a solution of 1.6 wt % Ca^{2+} combined with the initiator (2 wt % APS) and with glycerol (50 wt %) as the thickener. The reservoir contains 0.3 wt % alginate and the monomers (AAm and BIS). In this case, when the feed droplet enters the reservoir, the Ca^{2+} and alginate will immediately come into contact, resulting in a shell of the alginate gel around the liquid droplet.[37−39] Next, APS will diffuse out and polymerize the monomer, resulting in a second shell of AAm. Thus, the final structure will be a multilayer microcapsule with an outer shell of AAm, an inner shell of alginate, and a liquid core. If capsules with a liquid core (rather than a gelled core) are desired, this approach provides a convenient way toward that end. Note that, glycerol, being a small molecule, will diffuse out of the core over time into the external solution. Also, note that we use a much lower alginate concentration in the reservoir as compared to the feed solution in Figure 1. The reason is to maintain a relatively low viscosity of the reservoir solution. If the reservoir is highly viscous, the droplets from the capillary tip tend to splatter as they hit the reservoir and capsules are not formed.

Figure 9 shows optical micrographs (under brightfield and fluorescence) of the resulting microcapsules. To confirm the location of the alginate relative to the AAm, we used a fluorescent derivative of alginate, which was synthesized by attaching fluorescein isothiocyanate (FITC) to the alginate backbone.[40] The alginate thus appears green under fluo-

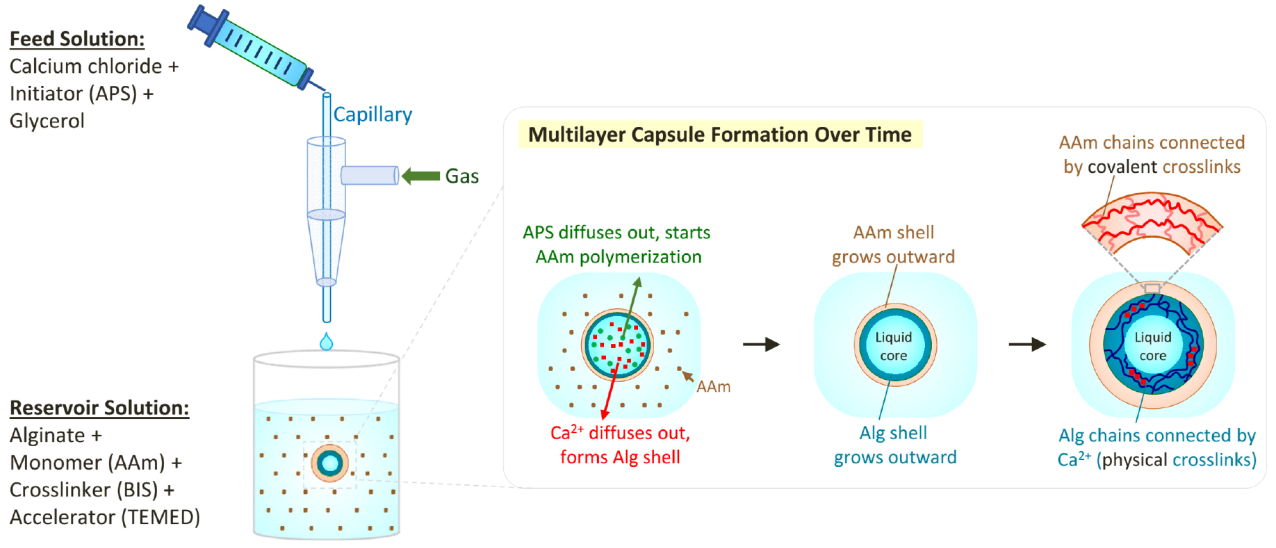

Figure 8. Schematic of the procedure used to synthesize multilayer capsules. A feed of Ca^{2+}, initiator (APS), and glycerol is sent through a capillary into a reservoir containing alginate (Alg), monomers (AAm and BIS), and an accelerator. Pulses of gas shear off aqueous droplets from the capillary tip. As the droplets enter the reservoir, the inset shows the progression toward the final multilayer capsule structure. First, Ca^{2+} diffuses out and an Alg/Ca^{2+} shell forms around the liquid core. APS then diffuses out and polymerizes a shell of AAm around the Alg shell. The final structure thus has two layers (Alg and AAm) and a liquid core.

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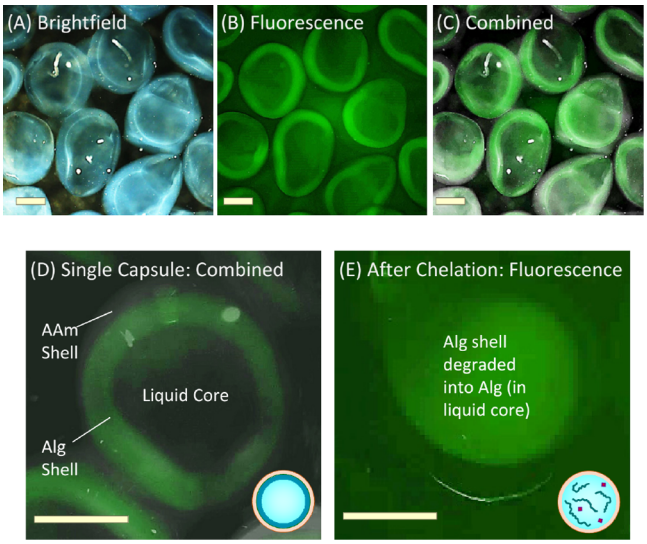

Figure 9. Images of multilayer microcapsules with concentric alginate and polymer layers around a liquid core. Images from (A) brightfield and (B) fluorescence microscopy are combined in (C). (D) Enlargement of a single capsule from (C). The green fluorescence is from alginate labeled with FITC. As indicated in (D), the capsules have a liquid core followed by an alginate shell (green) and then a polymer (AAm) shell. (E) When these capsules are subjected to chelation using sodium citrate, the alginate shell degrades and the fluorescent alginate is now found in the liquid core. Scale bars: 300 μm.

rescence microscopy. From the combined images (Figure 9C,D), it is clear that the capsules do have a liquid core (which appears dark because alginate is not present) surrounded by a shell of the alginate gel (green) and then a shell of AAm. The images thereby confirm the structure to be consistent with the schematic in Figure 8. When the capsules are submerged in solutions of a chelator (100 mM EDTA or 500 mM sodium citrate) overnight, they remain intact, but the inner alginate shell disappears and the green fluorescence is now observed in the core (Figure 9E). This finding implies that the alginate shell is degraded by chelating away the Ca^{2+} cross-links. The resulting alginate chains are large enough that they remain inside the capsule core rather than diffusing out through the AAm shell into the external solution.

CONCLUSIONS

We have developed a new single-step technique to synthesize alginate microgels enveloped by a thin shell of a covalently cross-linked polymer gel. The resulting microcapsules combine the attractive features of alginate (biocompatibility and suitability for cell growth) with those of covalent polymers (robustness and resistance to degradation due to chelators or ionic species). The polymer shell is formed by "inside-out" polymerization, where an initiator in the core diffuses outward and reacts with monomers present externally. The thickness of the shell and the size of the core can be controlled by varying the concentration of the initiator and the flow rate of the feed solution, respectively. Various monomers that can be cross-linked via free-radical polymerization using heat or UV light can be used to generate the shell. As the synthesis is done at room temperature in aqueous media, both bacterial and mammalian cells can be readily encapsulated in the microcapsules. Studies with cells show that the microcapsules remain intact over a culture period of up to 7 days, whereas alginate microgels without a shell disintegrate in less than a day under the same culture conditions. Lastly, we have also developed a variation of the synthesis method to generate microcapsules with multiple shells (alginate and polymer) around a liquid core. The simplicity and versatility of our approaches allow them to be broadly used as tools to enhance the properties of alginate microgels.

EXPERIMENTAL SECTION

Materials. The following chemicals were obtained from Sigma-Aldrich: alginate (medium viscosity alginic acid, sodium salt from brown algae), acrylamide (AAm), *N*,*N*′-methylenebis(acrylamide) (BIS), calcium chloride dihydrate ($CaCl_2$), ammonium persulfate (APS), tetramethylethylene diamine (TEMED), polyethylene glycol diacrylate (PEGDA, M_W 575), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), 1× phosphate buffered saline (PBS), glycerol, sodium citrate, and ethylene diamine tetraacetic acid (EDTA). Deionized (DI) water was used in all experiments. Luria Broth (LB) was obtained from Life Technologies. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS (DPBS), 0.25% trypsin−EDTA, fetal bovine serum (FBS), live/dead kit for mammalian cells, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from ThermoFisher.

Capillary Device for Microcapsule Synthesis. The device for microcapsule synthesis was fabricated in a manner similar to that from our earlier work.[32] Briefly, a capillary with a 200 μm inner diameter (ID) (Vitrocom 8320, 5 cm long) was inserted into the center barrel of a multibarrel glass capillary (from World Precision Instrument) and was fixed with epoxy glue. A P1000 plastic pipette tip was cut and placed around the multibarrel capillary, leaving 3 mm of the 200 μm capillary extruding out from the pipette casing. The pipette tip was glued to a male Luer adapter tee (Cole-Parmer, UX-45508-00) with epoxy glue. Next, a female Luer hose barb adapter (Cole-Parmer, UX-45508-00) was inserted to one end of Tygon tubing (15 cm, ID 3/16″) and joined with epoxy glue. The part of the capillary protruding from the male Luer adapter tee was inserted to the other end of the Tygon tubing to connect the tubing with the assembled capillary. The rest of the setup, including a syringe pump, a function generator, and a gas flow regulator, were identical to those from our previous study.[32] Before each experiment, the 3 mm capillary tip was immersed in a hydrophobic coating (Rainex from Home Depot). To form droplets of the feed solution, nitrogen gas was flowed through the Tygon tubing around the capillary tip (Figure 1). The nitrogen pressure was kept at 5 psi, and pulses of gas were sent at a frequency of 0.5 Hz.

Synthesis of Microcapsules (Thermal Polymerization). The feed solution was made in DI water containing varying amount of APS initiator and 2 wt % alginate. The reservoir solution in the case of alginate−AAm microcapsules was made by dissolving 10 wt % AAm, 0.034 wt % cross-linker (BIS), 1.5 wt % accelerant (TEMED), and 1.6 wt % (150 mM) $CaCl_2$ in DI water. The feed was loaded into a syringe and fed through the capillary device mentioned above, with the feed flow rate typically being 25 μL/min. Droplets were sheared off from the capillary tip by pulses of nitrogen gas and these entered into an unstirred reservoir solution, whereupon they became capsules (Figure 1). Typically, collection was done for 5 min (yielding a total of 600 capsules), and the capsules were washed three times with DI water to remove residual chemicals. Capsules were then suspended in DI water for storage. For alginate−PEGDA microcapsules, the procedure was identical, but the reservoir solution was made with 20 wt % PEGDA instead of AAm and BIS.

Synthesis of Microcapsules (UV Polymerization). The feed solution was made in DI water with 1 wt % LAP initiator and 2 wt % alginate. The reservoir solution in the case of alginate−AAm microcapsules was identical to the above (10 wt % AAm, 0.034 wt % BIS, and 1.6 wt % $CaCl_2$ in DI water). The feed was loaded into a syringe and was fed through the capillary device at a flow rate ∼30 μL/min. Droplets from the capillary tip entered into an unstirred reservoir solution, whereupon they were exposed to UV light from an

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Ionica 36 W UV lamp that generates wavelengths from 350 to 400 nm. The droplets were exposed to UV light for 60−90 s, whereupon they were converted into capsules (Figure 1). The capsules were washed three times with DI water to remove residual chemicals and then suspended in DI water for storage.

Optical Microscopy. Brightfield images of the microcapsules were obtained using an inverted optical microscope (Zeiss Axiovert 135 TV) using a 2.5× objective. Images under brightfield and fluorescence of the microcapsules with encapsulated cells were obtained using an Olympus MVX10 microscope. To better visualize the shell, the capsules were observed under slight under-focus in some cases.

Scanning Electron Microscopy (SEM). A suspension of alginate−AAm microcapsules (5 μL) was pipetted onto a double-sided carbon tape that in turn was attached on an SEM stub. A drop (3.5 μL) of ionic liquid (Hilm IL 1000, Hitachi) was added and gently mixed using the micropipette. The sample was set to dry in air on a Petri dish for about 30 min. Excess liquid was then removed using filter paper, and the sample was then dried overnight. The dried sample was examined on a Hitachi SEM (SU-70) with an accelerating voltage of 5 kV.

Mechanical Properties of Microcapsules. Alginate microgels and alginate−AAm microcapsules were compressed between parallel plates using a MicroSquisher (CellScale Inc.). A beam with a thickness of 0.3 mm and a length of 60 mm attached to a 2 × 2 mm^2 metal plate was used for the compression. All samples were kept in PBS for 24 h prior to testing. Samples were compressed by 55% with a load time of 40 s and a 10 s hold followed by a recovery time of 40 s. Data acquired was for the force required to compress the sample; this force was converted to stress by dividing by the cross-sectional area of the capsule.

Synthesis of Multilayer Microcapsules. The feed solution in this case contained 50 wt % glycerol, 1.6 wt % $CaCl_2$, and 1.5 wt % APS, the rest being DI water. The reservoir solution consisted of 10 wt % AAm, 0.3 wt % alginate, 0.034 wt % BIS, and 1.5 wt % TEMED dissolved in DI water. As above, droplets get sheared off from the capillary tip and fall into a reservoir solution that is stirred using a magnetic stir bar at 700 rpm. The droplets thereby transform into multilayer capsules, as discussed in Figure 8. Collection was again done for 5 min, and the capsules were washed and stored as before.

Synthesis of Fluorescent Alginate. Fluorescent alginate conjugated with FITC was synthesized following a previous report.[40] Briefly, 120 mg of alginate was dissolved in 10 mL of sodium acetate buffer (pH 5). After 10 min, 50 mg of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and 30 mg of *N*-hydroxy succinimide (NHS) were added and stirred at 700 rpm for 30 min at room temperature. The mixture was then precipitated by adding 100 mL of isopropanol. The precipitate was dissolved in 100 mL of sodium bicarbonate buffer (pH 8.5). After 10 min, 0.5 mg of FITC was added and the mixture was stirred for 4 h at room temperature. Acetone (100 mL) was added under vacuum filtration to precipitate the alginate−FITC derivative. The resulting derivative was dried under vacuum. The alginate−FITC was combined with the regular alginate in a 1:100 ratio for studies involving fluorescence microscopy.

Bacterial Strain and Growth Conditions. The *E. coli* reporter strain BL21(DE3)-(pET-DsRed) was used for the cell encapsulation study.[35,36] These bacteria were grown in LB media overnight with 50 μg/mL kanamycin at 37 °C with 250 rpm shaking, reinoculated by a 1:100 dilution, and grown to an approximate optical density (OD) of 0.4 at 600 nm.

Encapsulation of Bacteria in Microcapsules. After reinoculation, 15 mL of cell culture was spun down at 4 °C and 3000 rpm for 20 min. Cell pellets were resuspended in 400 μL of PBS and 100 μL of LB. For preparing cell-bearing capsules, the feed contained 500 μL of the cell culture, 500 μL of DI water, and 1 mL of 2 wt % alginate that was dissolved in PBS. Droplets of this feed were introduced into the reservoir solution containing AAm to create alginate−AAm capsules with bacteria in the core. The capsules were first washed three times with PBS (pH 7.4) and kept on ice. Washed capsules were placed in 1 mL Eppendorf tubes to which 800 μL of PBS, 200 μL of LB, and 1 μL of 1 M IPTG were added. The capsules were then cultured overnight (for 18 h) in the case of Figure 6 or over 4 days in the case of Figures S6 and S7. In both cases, culture was done at 37 °C with 250 rpm shaking.

Encapsulation of Mammalian Cells in Microcapsules. Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin (100 units/mL), and streptomycin (0.1 g/mL) in a 37 °C incubator with 5% CO_2 and passaged two times a week. Prior to encapsulation, cells were grown to 80% confluence, trypsinized, and centrifuged at 300 rpm for 15 min at 4 °C. Cell pellets were resuspended in 1 mL of DMEM and kept on ice. Cell density was checked using a hemocytometer, and the density was adjusted by adding PBS until it reached 1 × 10^6 cells/mL. Next, an initiator solution was made by dissolving 0.012 g (1.2 wt %) of LAP and 0.2 g of sodium alginate in 1 mL of PBS. For cell encapsulation, 0.15 mL of the cell suspension was mixed with 0.85 mL of the initiator solution to make the feed. The rest of the procedure followed that described in the above section on capsule synthesis by UV polymerization.

Mammalian Cell Culture and Live/Dead Assay. Microcapsules containing Caco-2 cells were placed in a sterile 24-well Petri dish and cultured in a 37 °C incubator with 5% CO_2 for 1 week. For live/dead staining, capsules from one well were transferred to a 1 mL Eppendorf tube. Capsules were washed with DPBS three times. After washing, capsules were transferred to 1 mL of DPBS containing 2 μM ethidium homodimer-1 (dead stain) and 1 μM calcein AM (live stain), incubated at room temperature for 15 min, and washed with DPBS before imaging. The number of cells in the images were counted and normalized using ImageJ software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.0c20613.

Experimental setups for synthesizing alginate−polymer microcapsules (Figure S1); SEM of alginate−AAm microcapsules (Figure S2); results of compression tests on alginate microgels and alginate−AAm microcapsules (Figure S3); images from the above compression tests (Figure S4); swelling of microcapsules vs microgels in PBS buffer (Figure S5); alginate microgels containing *E. coli* after overnight incubation (Figure S6); and alginate−AAm microcapsules containing *E. coli* after 4 day incubation. (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information for:

Single-Step Synthesis of Alginate Microgels Enveloped with a Covalent Polymeric Shell: A Simple Way to Protect Encapsulated Cells

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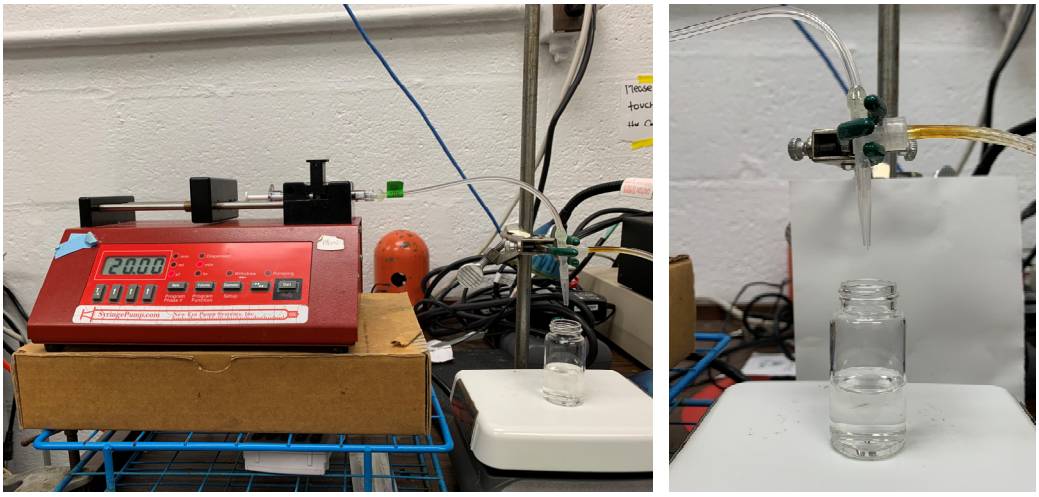
Contents

(A) Set up for thermal polymerization



(B) Set up for UV polymerization

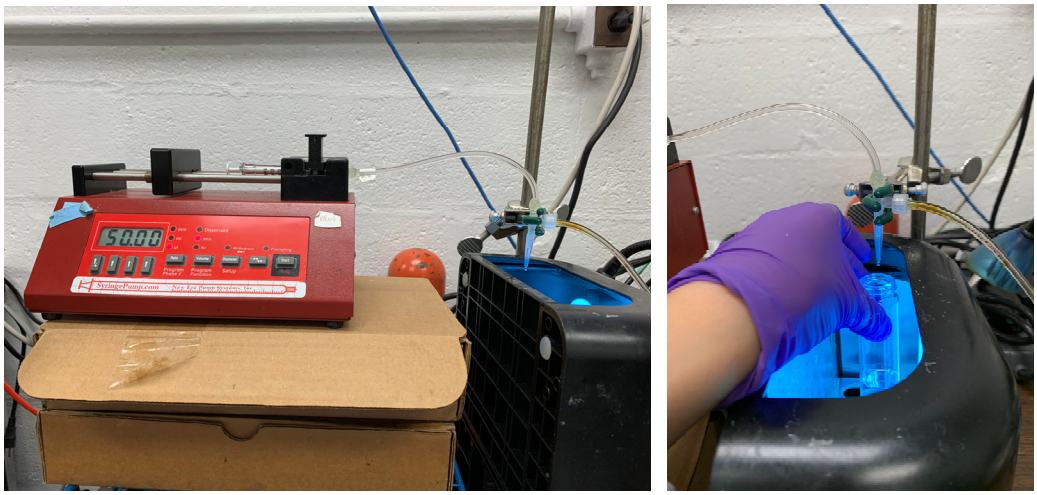


Figure S1. Experimental setups for synthesizing alginate-polymer microcapsules. (A) Setup for capsule synthesis using thermal polymerization. Droplets of feed with alginate and thermal initiator (APS) are collected in a reservoir (vial) containing monomers, calcium chloride, and accelerator. (B) Setup for capsule synthesis using UV polymerization. Droplets of feed with alginate and photoinitiator (LAP) are collected in a vial containing monomers and calcium chloride while the collecting vial is exposed to UV light.

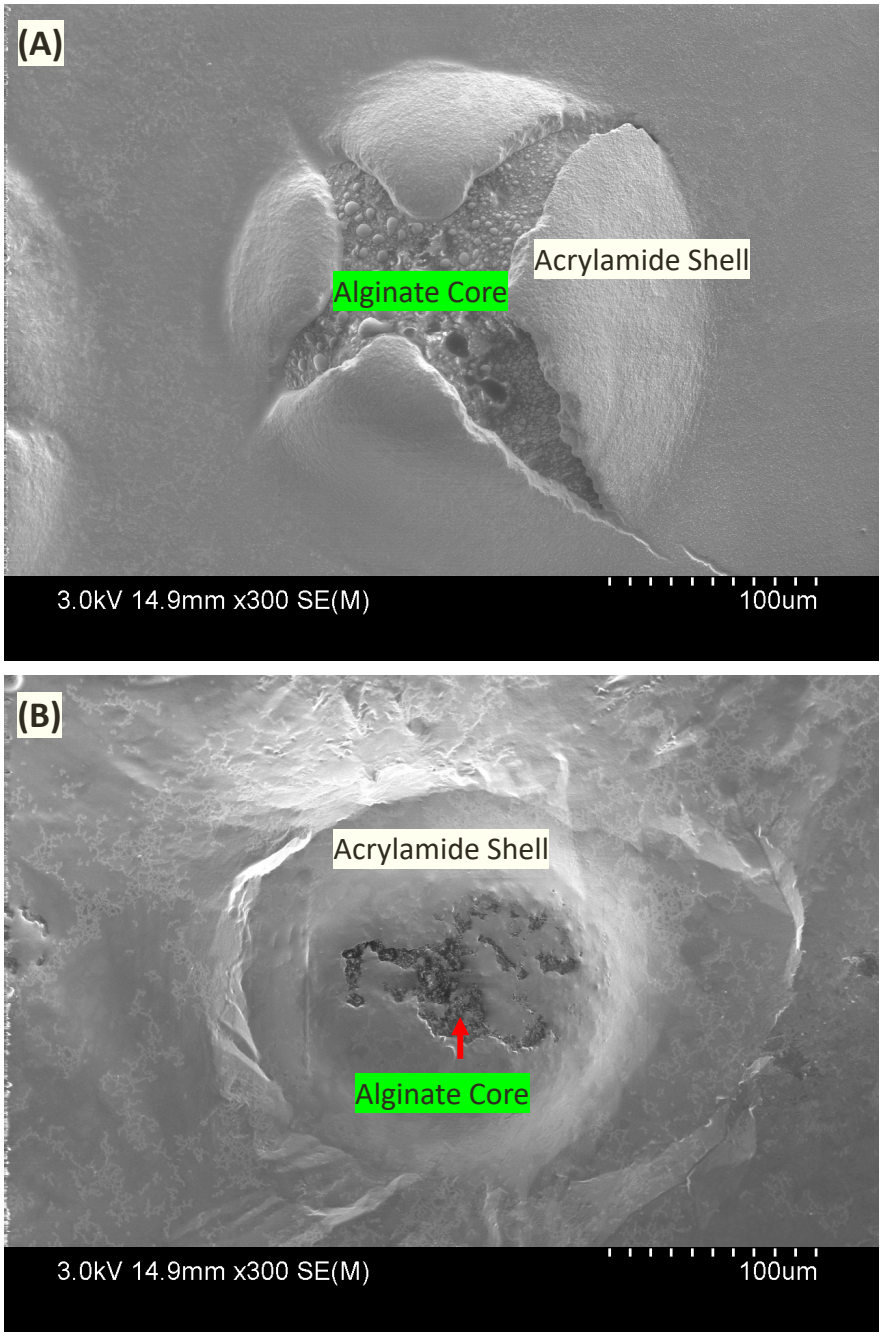


Figure S2. Scanning Electron Micrographs (SEM) of alginate-AAm microcapsules. (A) Microcapsule synthesized by thermal polymerization. (B) Microcapsule capsule synthesized by UV polymerization. In both images, the capsule is cracked open, thus revealing a distinct core (alginate microgel) and shell (AAm).

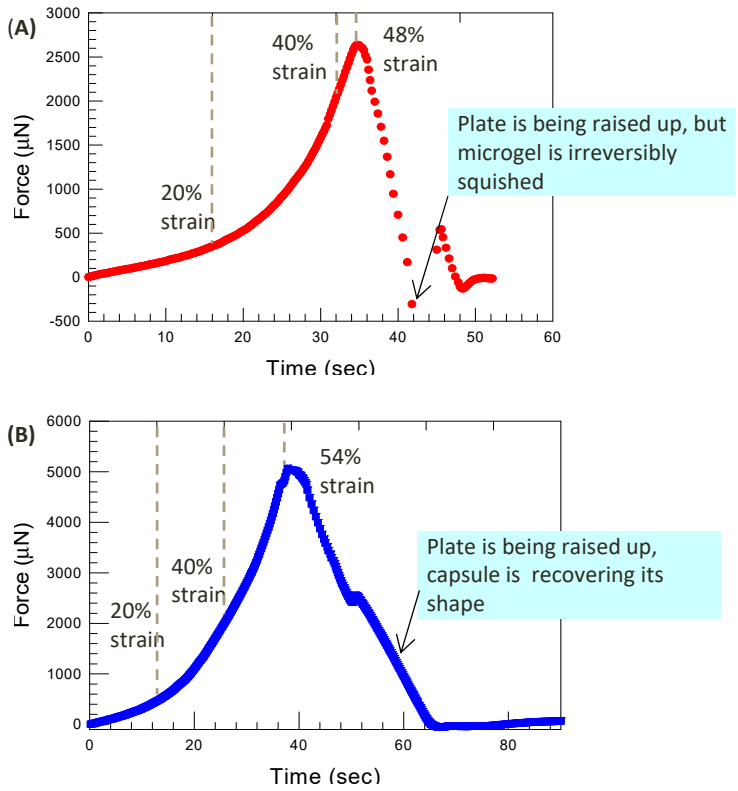


Figure S3. Results of compression tests on (A) alginate microgels and (B) alginate-AAm microcapsules. The graphs are plots of the force required for compression vs. time during steady compression. When compressed to 48% strain, alginate microgels fail to recover their shape whereas the alginate-AAm microcapsules can be compressed to 55% strain and still recover their original shape. The stress at the peak force is 13 kPa for alginate microgels and 71 kPa for Alg-AAm microcapsules. The compression tests thus show that the AAm shell improves the mechanical response of alginate microgels.

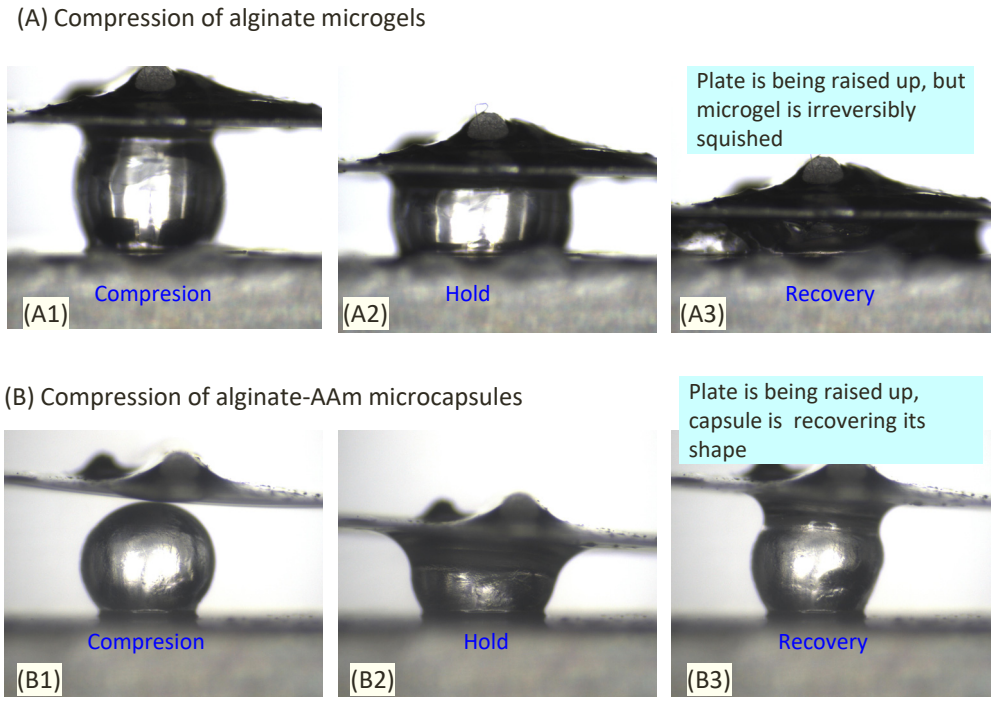


Figure S4. Images from compression tests on alginate microgels and alginate-AAm microcapsules. The tests are done using a MicroSquisher (CellScale Inc.). Upon compression, alginate microgels irreversibly collapse (A1-A3) whereas alginate-AAm microcapsules recover their shape after compression (B1-B3).

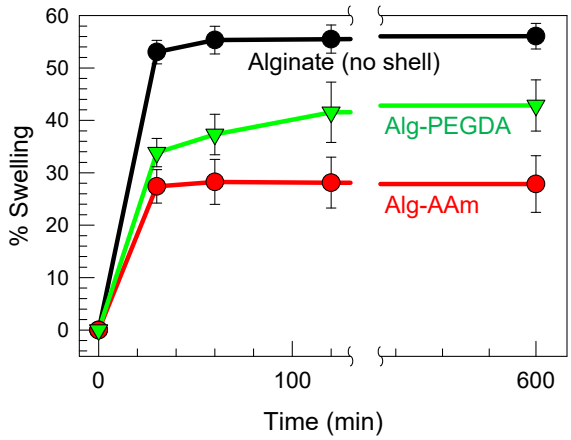


Figure S5. Swelling of microcapsules vs. microgels in PBS buffer. The extent of swelling over 10 h is plotted for Alg microgels (no shell), Alg-AAm microcapsules and Alg-PEGDA microcapsules. The presence of a polymer shell reduces the extent of swelling. The error bars represent the standard deviation from multiple experiments (n=3).

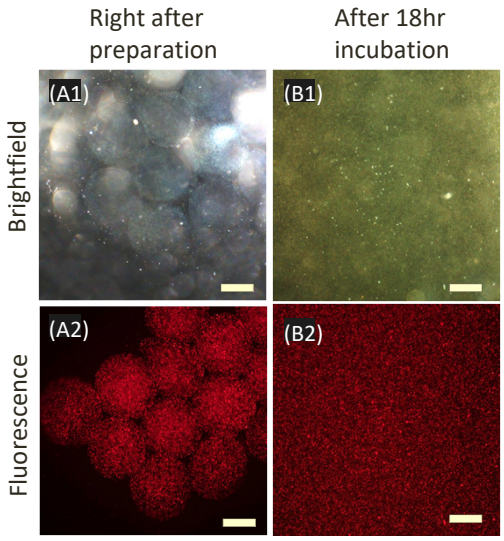


Figure S6. Alginate microgels containing *E. coli* after overnight incubation. (A1) Brightfield image of alginate microgel containing *E. coli* expressing red fluorescence (DsRed) and corresponding fluorescence image (A2) after preparation. (B1) Same microgel after overnight incubation in LB broth at 37ºC. The microgels completely disintegrate and no trace of the original structures can be found. The fluorescence is thus spread over the entire field of view (B2). Scale bars: 200 µm.

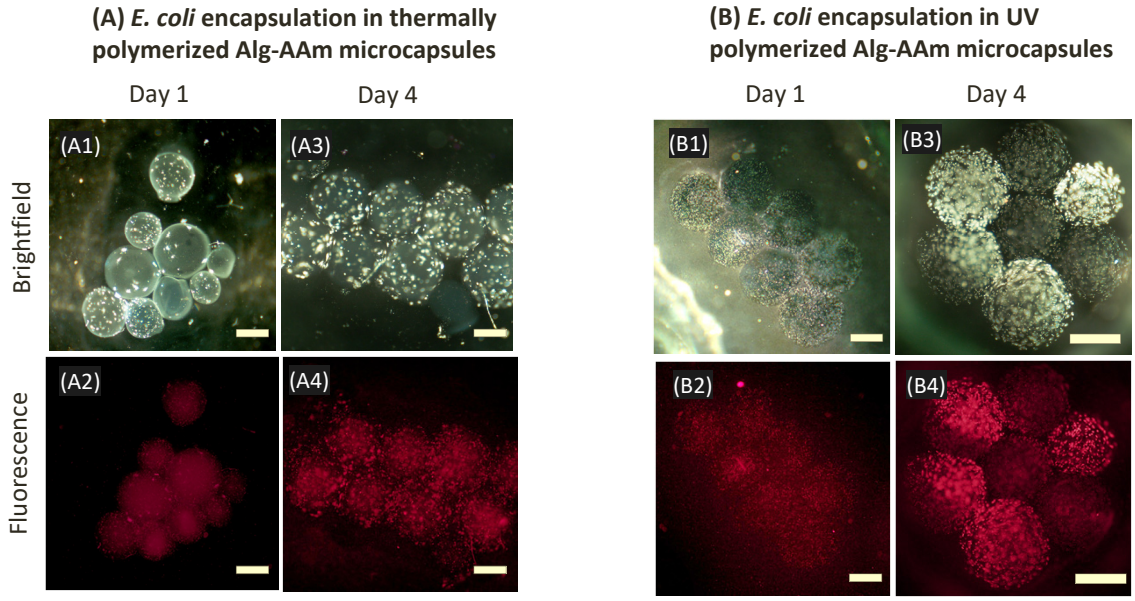


Figure S7. Alginate-AAm microcapsules containing *E. coli* after 4-day incubation. Alg-AAm microcapsules containing *E. coli* expressing red fluorescence (DsRed) are synthesized using (A1-4) thermal polymerization and (B1-4) photopolymerization. After 4-day incubation, both capsules remain intact under culture conditions. Cell growth is higher in capsules formed using UV polymerization. Scale bars: 200 µm.