

Encapsulated Fusion Protein Confers “Sense and Respond” Activity to Chitosan–Alginate Capsules to Manipulate Bacterial Quorum Sensing

Apoorv Gupta,^{1,2} Jessica L. Terrell,^{3,4} Rohan Fernandes,⁴ Matthew B. Dowling,¹ Gregory F. Payne,^{3,4} Srinivasa R. Raghavan,¹ William E. Bentley^{1,3,4}

¹Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, Maryland 20742; telephone: 301-405-4321; fax: 301-405-9953; e-mail: bentley@umd.edu

²Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland

³Institute for Bioscience and Biotechnology Research (IBBR), University of Maryland, College Park, Maryland 20742

⁴Fischell Department of Bioengineering, University of Maryland, College Park, Maryland 20742

ABSTRACT: We demonstrate that “nanofactory”-loaded biopolymer capsules placed in the midst of a bacterial population can direct bacterial communication. Quorum sensing (QS) is a process by which bacteria communicate through small-molecules, such as autoinducer-2 (AI-2), leading to collective behaviors such as virulence and biofilm formation. In our approach, a “nanofactory” construct is created, which comprises an antibody complexed with a fusion protein that produces AI-2. These nanofactories are entrapped within capsules formed by electrostatic complexation of cationic (chitosan) and anionic (sodium alginate) biopolymers. The chitosan capsule shell is crosslinked by tripolyphosphate (TPP) to confer structural integrity. The capsule shell is impermeable to the encapsulated nanofactories, but freely permeable to small molecules. In turn, the capsules are able to take in substrates from the external medium via diffusion, and convert these via the nanofactories into AI-2, which then diffuses out. The exported AI-2 is shown to stimulate QS responses in vicinal *Escherichia coli*. Directing bacterial population behavior has potential applications in next-generation antimicrobial therapy and pathogen detection. We also envision such capsules to be akin to artificial “cells” that can participate in native biological signaling and communicate in real-time with the human

microbiome. Through such interaction capabilities, these “cells” may sense the health of the microbiome, and direct its function in a desired, host-friendly manner.

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Introduction

Biopolymer-based capsules are being extensively studied for diverse applications in biotechnology ranging from traditional drug delivery to cell-targeting and reporting. These capsules are typically synthesized via electrostatic complexation or layer-by-layer (LbL) deposition of charged polymers (Ochs et al., 2011; Sato et al., 2012; Xie et al., 2009). Electrostatic complexation is particularly advantageous because the capsules can be prepared very easily, and the capsule formation and payload encapsulation processes can be accomplished in a single step. Two oppositely charged polymers are required for this strategy. Among the various choices, the polysaccharides alginate and chitosan find extensive use due to their low toxicity and biodegradability (Aiba, 1992; Hirano et al., 1989; Kumar et al., 2004; Skaugrud et al., 1999). Alginate is an anionic polysaccharide derived from brown algae. It is a linear copolymer comprised of 1 → 4 linked β-D-mannuronic acid and α-L-guluronic acid residues that are arranged as alternating or

Correspondence to: W. E. Bentley

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homopolymeric regions. Chitosan is the N-deacetylated form of chitin that is often derived from crustacean shells. It is a cationic linear polysaccharide comprised of β -(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine (Liu et al., 2010; Yi et al., 2005). Alginate–chitosan capsules have been shown to prolong viability of entrapped cells (Kanmani et al., 2011), improve fermentation yields of ethanol (Ylivero et al., 2011), release targeted compounds at local sites to treat gastrointestinal disorders (Metz et al., 2005), and deliver probiotic bacteria (Cook et al., 2011) or therapeutics (Elzatahry et al., 2009; Ramadas et al., 2000).

We conceptualized an approach by which biopolymer-based capsules could perform biological functions beyond merely containing cellular or molecular entities. Specifically, we noted that capsules are essentially bottom-up fabricated artificial biomimetic “cells,” with the semipermeable capsule shell protecting the encapsulated contents and controlling the passage of small molecules in and out of the container. Such artificial cells may participate in biological activities, such as active communication with bacteria or other cells, producing desired responses. Cell-to-cell communication among bacteria has emerged as a “new frontier” to combat infections and antibiotic resistance (Hardie and Heurlier, 2008). It has been shown that bacteria have complex sensing and communication systems that allow them to “talk” to each other, act as multicellular entities, and coordinate cellular functions. This process, termed quorum sensing (QS) (Fuqua et al., 1994), is a cell-density-dependent process that is mediated by the production, release, and detection of specific signaling molecules, including autoinducers (Fuqua and Greenberg, 1998). QS affects processes including bioluminescence (Bassler et al., 1994; Chen et al., 2002b; Waters and Bassler, 2006), virulence gene expression (Higgins et al., 2007; Le Berre et al., 2008), and biofilm formation (Irie and Parsek, 2008; Yoshida et al., 2005). As bacteria become increasingly resistant to common antibiotics, it is necessary to develop novel detection and antimicrobial techniques through which bacterial pathogenicity and survivability can be controlled (Roy et al., 2011).

Recently, an approach originally proposed by LeDuc et al. (2007) was implemented whereby biological effector molecules could be synthesized local to the site of action through biological nanofactories (Fernandes et al., 2010). These nanofactory constructs are self-assembled, and consist of a targeting domain (antibody), and a fabrication + biosynthetic domain (HGLPT) that can synthesize the bacterial signaling molecule, autoinducer-2 (AI-2), from S-adenosylhomocysteine (SAH). AI-2 is believed to be a “universal” bacterial signaling molecule and is recognized by over 70 species of bacteria (Vendeville et al., 2005). The fusion protein, HGLPT, consists of an IgG-binding domain that allows assembly with a targeting antibody. Previous work has demonstrated that nanofactories can elicit bacterial QS responses in the absence of native AI-2, as well as target and trigger response from specific species in a mixed bacterial culture (Fernandes et al., 2010).

Furthermore, it has also been shown that when targeted to eukaryotic cell surfaces, these nanofactories can elicit bacterial responses in the vicinity, and thereby, modulate interkingdom signaling (Hebert et al., 2010).

In this study, we create bioactive capsules that can participate in communication with nearby bacterial cells. The liquid core of the capsules contains alginate while the shell is formed by chitosan crosslinked by multivalent tripolyphosphate (TPP) ions (Goycoolea et al., 2009). Alginate enables assembly of the spheroids due to its charge and hydrogel forming properties (Zhu et al., 2005); it also serves as a permissive scaffold for incorporation of proteins and/or cells (Li et al., 2007; Munarin et al., 2010). TPP-crosslinked chitosan confers mechanical strength and integrity to the capsule or “cell” wall (Fan et al., 2012). In these capsules, we entrap biological nanofactories capable of synthesizing AI-2 from the substrate SAH (Fig. 1, panel a). Locally available SAH diffuses into the capsule allowing the nanofactories to convert it into AI-2, as shown in Figure 1, panel a. The product diffuses out and directs QS responses from vicinal *Escherichia coli* present outside and around the capsule (Fig. 1, panel b). We note that while the capsule shell is permeable to small molecules, SAH and AI-2, it remains impermeable to macromolecules, such as the nanofactories. The capsules “sense and respond” to their environment, as they utilize the locally available substrate and create the QS signaling molecule, AI-2. Thus, the capsules (i.e., artificial “cells”) can be used to interact with bacteria and even direct their collective behaviors. By entrapping the relevant biological machinery, these capsules can be activated to perform a given task and are less burdened by the metabolic processes of a biological cell should it be tasked with the same function. By communicating with the internal

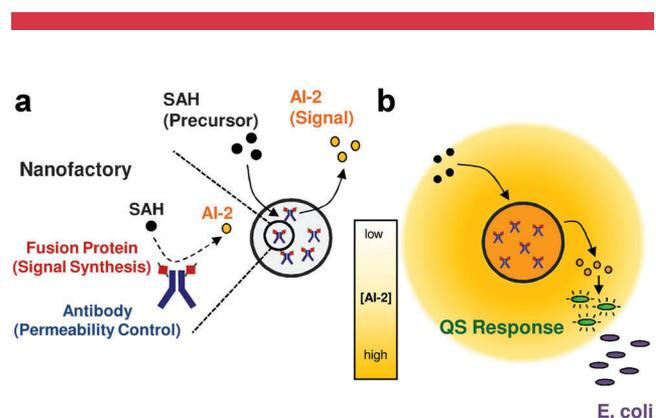


Figure 1. Encapsulation of signal-producing nanofactories in chitosan–alginate capsules allows participation in bacterial quorum sensing. Capsules contain a liquid alginate interior bounded by a chitosan shell that is crosslinked by tripolyphosphate (TPP). The biological nanofactory is assembled in vitro and composed of a fusion protein for autoinducer-2 (AI-2) biosynthesis, and an antibody that confers targeting in some applications (Fernandes et al., 2010), but here confers decreased permeability of the complex through the capsule membrane. **a:** S-adenosylhomocysteine (SAH) diffuses into the capsule to be converted to AI-2 that diffuses out. **b:** AI-2 concentration gradient results due to localization of the nanofactory, and is detected by nearby bacteria that elicit a quorum sensing (QS) response.

microbiome via QS and its effector molecules, such bioactive structures may eventually be used to detect the health of the internal populations, intercede in cell–cell signaling to guide population “trajectories,” as well as identify and selectively steer cells otherwise committed to pathogenic activity, without the collateral damage associated with indiscriminate antimicrobial therapies.

Results and Discussion

Visualization of Capsule Structure and Quantification of Chitosan Shell Thickness

Capsule structure and assembly were characterized by visualization with fluorescent and confocal microscopy. Chitosan and alginate were fluorescently labeled to aid visualization before assembly into spheroids. Chitosan (1% w/v) was labeled with NHS-Rhodamine, an amine-reactive molecule that emits orange-red visible light upon excitation. Alginate (1% w/v) was labeled, during capsule creation, with amine-modified fluorescent microspheres that emit green visible light. In order to synthesize capsules, TPP was diluted into alginate to a final concentration of 0.1% (alginate–TPP). Fluorescent microspheres were then mixed with the alginate–TPP solution to a final concentration of 0.1%. The labeled alginate–TPP was dropped into stirred fluorescently labeled chitosan and maintained for

3 min. They were then washed with deionized water and placed in 1.5 M NaCl solution until further analysis.

A fluorescent image of an intact capsule (Fig. 2, panel a) shows that the green alginate interior and red chitosan shell are readily apparent. There is a fairly homogenous alginate interior that is circumscribed by a remarkably uniform chitosan shell. In order to further characterize the capsule and the location of each polysaccharide, a capsule was mechanically punctured and fluorescent images were taken (Fig. 2, panel b). The image shows the viscous alginate interior exuding from the collapsing and wrinkled chitosan shell. There was minimal mixing of alginate and chitosan as there was no visible invasion of green alginate into the red shell and vice versa. In our application, alginate functions as both a scaffold for inclusion of biomolecules and as a mediator of structural integrity of an intact capsule. Other capsule assemblies were attempted including alginate (w/no TPP) added to stirring chitosan, and chitosan added to stirring TPP. Neither process yielded uniformly spherical capsules with a clean shell that retained structural integrity. The capsules made with only alginate dropped into chitosan were fragile, and ruptured with minimal contact, indicating the importance of TPP in the structural integrity.

A confocal image of an intact capsule reconstructed from z-stacks using IMARIS software was obtained (Fig. 2, panel c). The rendered image confirms the homogeneity of both the alginate interior and the outer chitosan shell. Similar to the fluorescent images in Figure 2, panel a and b, there

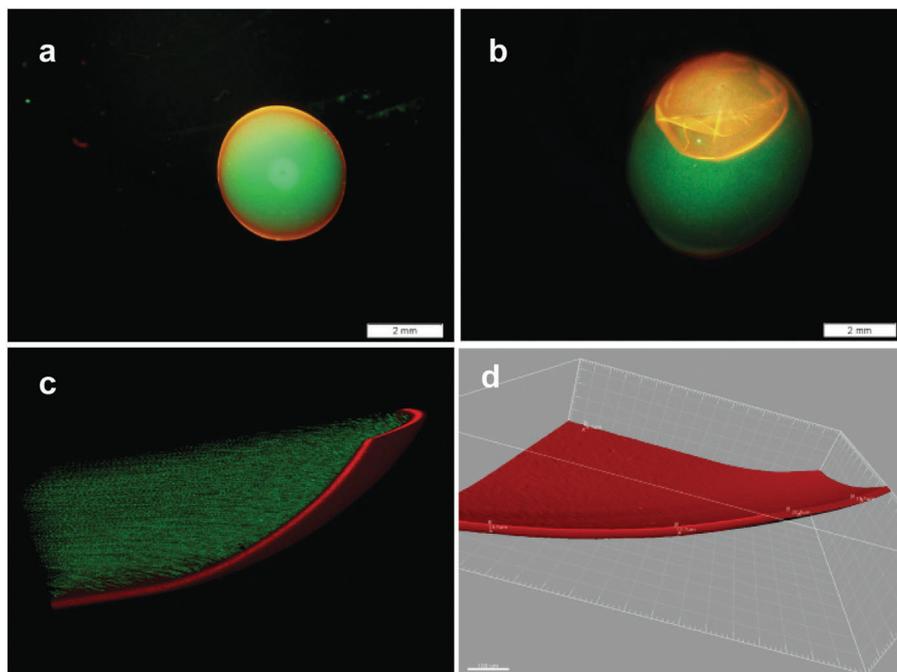


Figure 2. Visualization of capsule assembled with NHS-Rhodamine labeled chitosan shell (red) and amine-modified microsphere-labeled alginate interior (green). Overlaid fluorescence images taken under red and green fluorescence filters of (a) an intact capsule, and (b) a ruptured capsule with alginate emerging from the receding chitosan shell. c: Rendered confocal z-stack image visualizing a section of an intact capsule (d) Chitosan shell, calculated to be $20 \pm 2 \mu\text{m}$ thick using the IMARIS software.

appeared to be minimal diffusion of one polysaccharide into the other. The thickness of the chitosan shell was quantified with IMARIS (Fig. 2, panel d) and was $20 \pm 2 \mu\text{m}$ as measured across different points using multiple capsules. The consistency of the chitosan shell thickness validates the regularity and robustness of the assembly process and supports the concept that these capsules may serve as reliable carrier structures due to their uniform characteristics.

Containment of Biomolecules

Capsules were tested for their propensity to retain biomolecules based, in part, on size. As described in Experimental Section, capsules containing labeled biomolecules were placed in an isotonic saline solution and periodically sampled for leakage. Specifically, fluorescently labeled bovine serum albumin (BSA, MW ~ 60 kDa) and goat anti-mouse IgG (antibody, MW ~ 150 kDa) were encapsulated and placed into 1.5 M NaCl. The Concentration of saline solution was chosen to minimize the swelling of the capsules. Capsules were incubated at room temperature for the indicated times and assayed for biomolecule leakage by measuring the fluorescence of the surrounding solution. Additionally, after all measurements were taken, intact capsules were resuspended in fresh saline solution and punctured to release contents. Resultant fluorescence of the surrounding solution was measured. This yields the level of biomolecules that had been retained in the capsule during the incubation experiment.

Capsule Retention of Smaller Proteins—BSA Encapsulation

As in Experimental Section, BSA-containing capsules were created by mixing fluorescently labeled BSA with alginate-TPP, and adding the mixture drop-wise to stirred chitosan (“+BSA”). Controls included capsules with no encapsulated BSA (“-BSA”) as well as BSA (calculated to match the amount of BSA encapsulated per capsule) dissolved in saline solution only (“+BSA (no capsule)”). Permeability of BSA through the capsule wall was monitored by taking fluorescence measurements of the surrounding solution for 24 h (Fig. 3, panel a). The fluorescence of the “+ BSA” and “+ BSA (no capsule)” groups were notably higher than those from the “-BSA” group. Measurements for the “+BSA” group increased over time, presumably along with diffusion out of the capsule, and stabilized near those from the “+BSA (no capsule)” control. This indicated nearly complete BSA leakage. As noted above, capsules were resuspended in fresh saline, punctured, and then fluorescence was measured. Punctured capsules from the “+BSA” group indicate negligible retention of BSA, as their fluorescence values were near those of punctured capsules from the “-BSA” group.

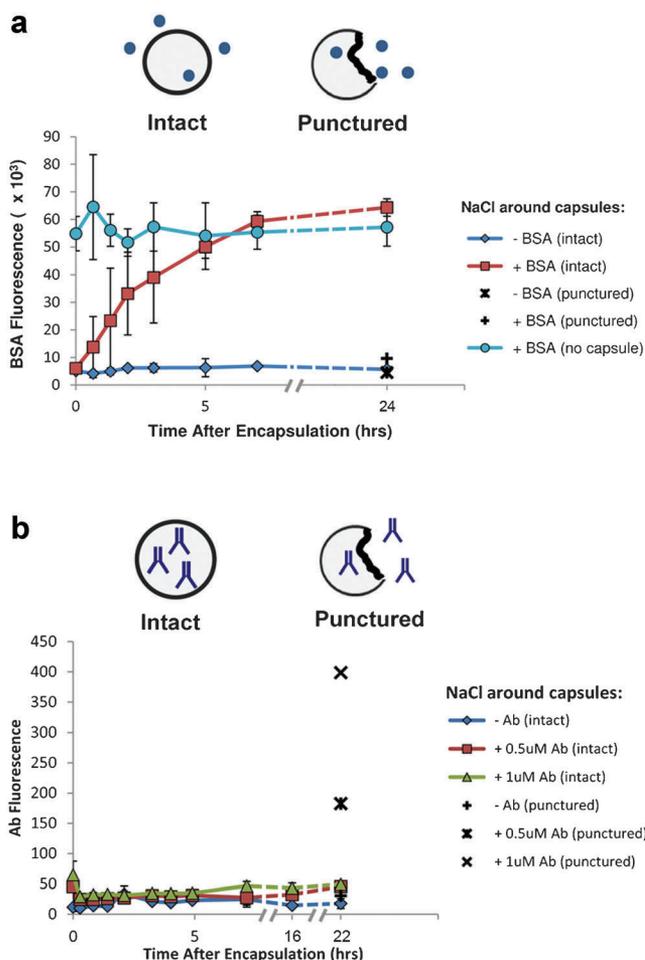


Figure 3. Capsule performance in retaining small and large proteins. **a:** A representative small-sized protein, fluorescently labeled BSA, was encapsulated. A time-course plot compares protein effluence between intact versus punctured capsules by measuring fluorescence of the surrounding solution. **b:** A representative large-sized protein, fluorescently labeled antibody (Ab), was encapsulated. The time-course plot compares intact versus punctured capsules at two concentrations.

Capsule Retention of Larger Proteins—Antibody Encapsulation

Antibodies, which are significantly larger than BSA, were identically tested. Capsules containing fluorescently labeled antibodies at different concentrations (“+Ab”) were prepared similarly as the BSA-containing capsules above, and incubated at room temperature. Negative controls included capsules with no antibody (“-Ab”). Leakage was monitored through periodical fluorescence measurements of the surrounding solution over ~ 22 h (Fig. 3, panel b). Similar fluorescence profiles of solutions surrounding the “+Ab” and “-Ab” capsules indicate complete antibody retention for the full time-course. After the last measurement, capsules were placed in fresh saline solution and punctured. Fluorescence measurements were taken to gauge the level of retention during the incubation period. Capsules

in the “-Ab” group showed no increase in fluorescence, while the “+Ab” capsules showed a marked increase, as expected. We ran antibody experiments at two different concentrations. The punctured capsules made with 1 μ M antibody in alginate-TPP showed $\sim 2\times$ the fluorescence intensity of capsules with 0.5 μ M antibody.

Bioactivation of Capsules by Encapsulated Nanofactories

The biological nanofactories are composed of a targeting domain and a biosynthesis domain; the domains can be assembled into a nanofactory in vitro. The biosynthesis domain is composed of the 57 kDa fusion protein HGLPT (His-protein G-LuxS-Pfs-Tyr) (Fernandes and Bentley, 2009), which includes enzymes Pfs and LuxS to synthesize the bacterial QS molecule, AI-2, from SAH (Schauder et al., 2001). The targeting function of the nanofactory is enabled by the protein G domain of the fusion protein. That is, after its purification, HGLPT is incubated with an antibody of choice to allow assembly, conferring targeting and placement on an antigen. The complete nanofactory construct totals ~ 210 kDa, significantly larger than the antibody used above. We hypothesized that the addition of the targeting module (antibody) would help retain the biosynthesis module within the capsules, since the HGLPT is ~ 57 kDa—slightly smaller than BSA. We also conjectured that this capsule-nanofactory complex would provide an internal catalytic component.

Nanofactories were encapsulated and monitored for leakage. Nanofactories were assembled by mixing the fluorescently labeled antibody with HGLPT in a 1:1 molar ratio. The complex was then mixed with alginate-TPP and dropped into stirred chitosan (“+NF”). Control capsules contained only labeled antibody (“+Ab”). Each capsule was washed and placed in a separate vial containing saline solution for 2 h to allow any leakage of HGLPT. BSA and HGLPT have similar molecular weights, suggesting that they have similar transport rates across the capsule membrane. Therefore, any leakage of HGLPT should be detected within 2 h of encapsulation. In addition, leakage of the antibody component from “+NF” capsules was monitored by taking fluorescence measurements of the solution surrounding each capsule, similar to the antibody leakage experiment above. After the encapsulation period, the solution surrounding each capsule was removed and saved. In order to test for the presence of HGLPT, 1 mM SAH was added to each solution and incubated at 37°C for 2 h to allow AI-2 synthesis. AI-2 activity was detected using the *Vibrio harveyi* BB170 reporter strain that luminesces in response to AI-2 (Surette and Bassler, 1998). Additionally, capsules were resuspended in fresh saline and punctured to release the retained contents. The presence of HGLPT was assayed, as explained above, to measure the level of retention of the nanofactory complex in the capsule during the incubation period.

In the “+NF” group, antibody fluorescence of solutions surrounding intact capsules was much lower than that of solutions surrounding punctured capsules (Fig. 4, panel a). Thus, there was considerable retention of the antibody in the capsule with minimal leakage, consistent with Figure 3, panel b. Further, as indicated in Figure 4, panel a, the solutions surrounding “+Ab” control capsules showed no difference in AI-2 activity between the intact and punctured samples; when no HGLPT was present, no AI-2 was synthesized. In contrast, for “+NF” capsules, there was a considerably higher AI-2 activity in solutions around punctured capsules, and none from solutions incubated with the intact capsules. These data indicate presence of HGLPT *inside* the capsule. Consistent with antibody fluorescence data, there was minimal leakage of HGLPT from the capsules. Neither the HGLPT nor the antibody showed permeability across the capsule membrane, validating the integrity of the encapsulated nanofactory complex. It was also apparent that the HGLPT retains its biosynthesis capability, even after encapsulation or capsule puncture, as it actively synthesizes functional AI-2 that can stimulate bacterial QS.

Synthesizing Communication Signals in Capsules From Locally Available Substrates

The biocompatibility of the capsule in preserving the stability and functionality of the large nanofactory complex suggests its potential application as a medium of communication with microbial populations. The capsule acts as an artificial cell that synthesizes critical signaling molecules from locally available substrates to participate in bacterial QS. Substrates would diffuse into the capsule to be acted upon by the cargo enzyme, and products would diffuse out of the capsule to act on vicinal targets. The localization of the enzyme within the domain of the capsule has advantages in that the local concentration of AI-2 at the capsule/culture interface is high (Hebert et al., 2010). Bulk addition of the nanofactory to cell culture fluids has far less response and it is not targeted (Fernandes et al., 2010).

In order to demonstrate the in-capsule synthesis of AI-2 using externally available SAH, nanofactories were encapsulated using the procedure outlined above (“+NF”). Control capsules contained only the antibody (“+Ab”). The saline solution surrounding each capsule was supplemented with 1 mM SAH, and AI-2 synthesis was carried out for 2 h at 37°C. Following synthesis, the surrounding solution was removed from each vial without rupturing the capsules and tested for AI-2 activity using the *V. harveyi* BB170 bioluminescence assay (Fig. 4, panel b). AI-2 activity in solutions surrounding “+Ab” capsules was negligible due to the absence of HGLPT. In contrast, AI-2 activity in solutions surrounding the “+NF” capsules was markedly higher. In-capsule functionality of the nanofactory was demonstrated, as biologically active AI-2 was produced using locally available SAH added to the capsule exterior.

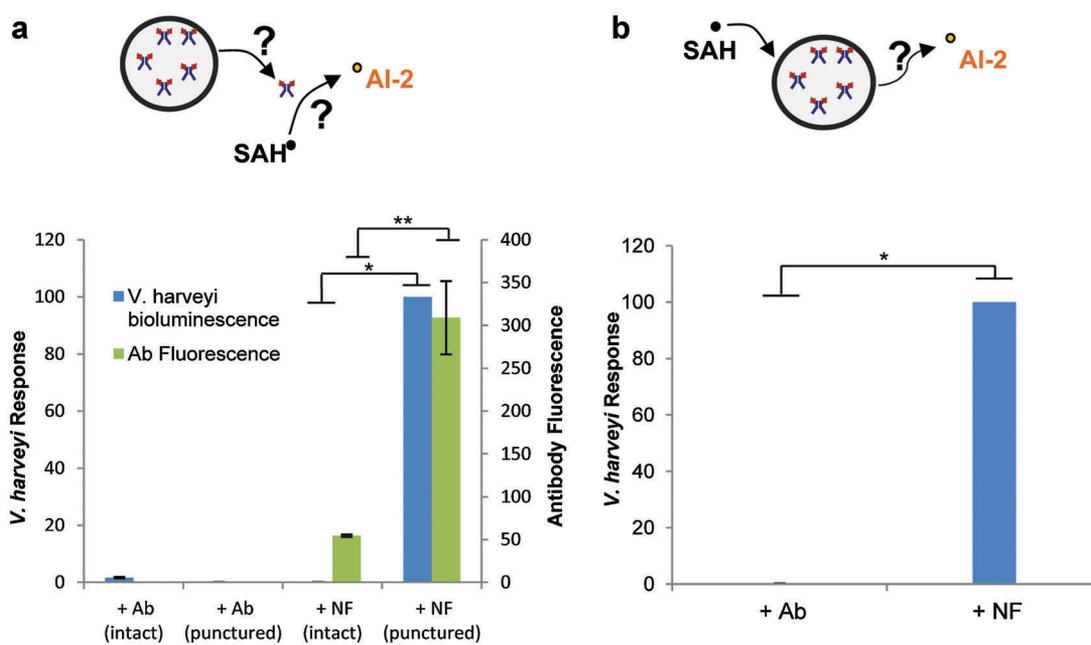


Figure 4. Conferring bioactivity to capsule interior by ensuring retention and functionality of encapsulated nanofactory. **a:** Solution surrounding punctured and intact capsules containing either antibody (“+Ab”) or nanofactory (“+NF”) were tested for presence of HGLPT, by synthesizing AI-2 through introduction of SAH and incubating with *V. harveyi* for expression of AI-2 mediated bioluminescence. *V. harveyi* bioluminescence values are normalized to AI-2 synthesized in solution around punctured “+NF” capsules (“+NF (punctured)” = 100). Fluorescence measurements were also taken of solutions around intact and punctured capsules to evaluate capsule retention of the “+NF” antibody component. **b:** Capsules containing antibody (“+Ab”) or nanofactory (“+NF”) were placed in SAH-supplemented solutions to allow synthesis and efflux of AI-2 from intact capsules. AI-2 presence in the solution surrounding the capsules was detected by *V. harveyi* response. Values are normalized to the *V. harveyi* response to AI-2 in the solution surrounding the “+NF” capsule (“+NF” = 100). Statistical analysis using a *t*-test is denoted for compared data sets ($n=3$). * $P < 0.05$; ** $P < 0.01$.

Directing Bacterial Quorum Sensing With “Bioactive” Capsules That Synthesize Small Molecules

One potential implication of encapsulating a biomolecule is the “bioactivation” of the capsule so it can actively interact with other biological entities. For example, capsules containing the nanofactory that produces AI-2 can be used to communicate with internal microbial populations and thereby direct their behaviors in desired manners by modulating the QS response (Fig. 5, panel a). Localized signal synthesis would allow localized response and require smaller quantities of signal. In order to demonstrate the capability of the capsule to communicate with bacteria, the AI-2 responsive *E. coli* strain, CT104 (pCT6-amp^r + pET-EGFP-kan^r), was used. This strain does not synthesize its own AI-2 (*luxS* mutant), but produces GFP in response to AI-2. Capsules containing either antibody only (“+Ab”) or nanofactory (“+NF”) were produced as in previous experiments. These were added to cultures of CT104 bacteria (OD 0.4) suspended in a mixture of NaCl and Luria–Bertani (LB) medium supplemented with SAH and antibiotics (NaCl/LB, 1.5 M NaCl + 10% LB (v/v) + 50 μg mL⁻¹ kanamycin + 50 μg mL⁻¹ ampicillin + 1 mM SAH). Our hypothesis was that nanofactory-containing capsules would produce sufficient AI-2 to elicit a QS response in nearby bacterial populations. Samples of culture surrounding the

capsules were harvested by taking out 150 μL of culture and fixing in 2% paraformaldehyde until analysis. Flow cytometry was used to measure *E. coli* QS response to the AI-2 produced by encapsulated nanofactories (Fig. 5, panel b). At the 6 h time point, a considerable difference in the population fluorescence appeared between cells exposed to “+Ab” capsules and those exposed to “+NF” capsules. Cells exposed to “+NF” capsules showed a higher fluorescence than control cultures that were exposed to “+Ab” capsules. We estimate that 32% of cells were fluorescent in cultures surrounding the “+Ab” capsules, while 85% of cells were fluorescent in cultures exposed to “+NF” capsules. We also note that ~2 h are needed for GFP maturation (Uemura et al., 2008); hence the QS response was on the order of 4 h. The shift of the population towards the “on” QS state indicates that AI-2 producing capsules can stimulate a response from a bacterial population.

Since many species of bacteria produce and respond to AI-2, we envisioned our capsule to be potentially used for interacting with native cell communication. Strain W3110 (pCT5-amp^r + pET-GFPuv-kan^r) (Tsao et al., 2010), produces GFP in response to AI-2, but also retains its native ability to synthesize its own AI-2. As described previously, capsule containing either antibody only (“+Ab”) or nanofactory (“+NF”) were synthesized and placed in cultures of W3110 bacteria (OD 0.4) suspended in NaCl/LB

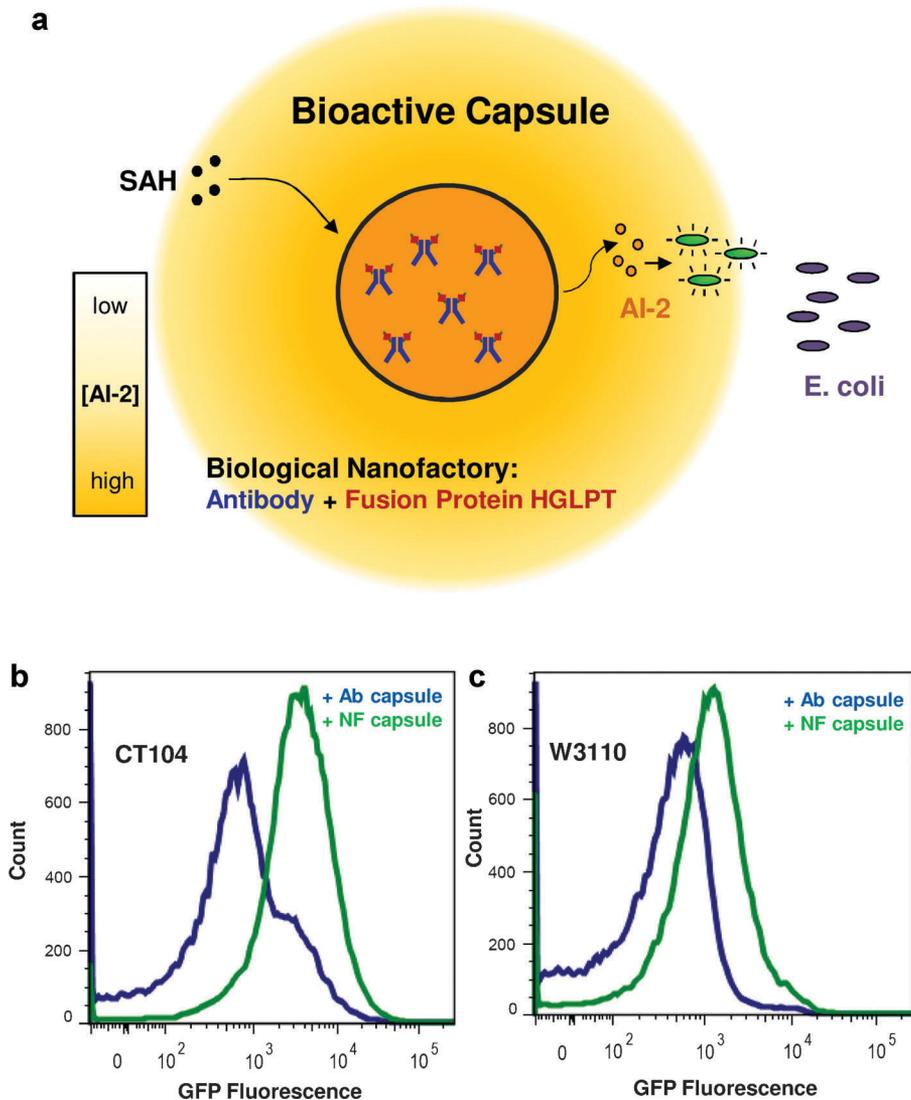


Figure 5. *E. coli* response to in-capsule synthesized AI-2 indicates stimulation and enhancement of a quorum sensing response. **a:** SAH diffuses into the capsule to be converted to AI-2, which diffuses out and is detected by *E. coli* that produce GFP in response to AI-2. Localization of the nanofactory inside the capsule necessarily creates an AI-2 gradient emanating from the capsule interior. AI-2 responsive expression of GFP in the presence of capsules containing antibody (“+Ab”) or nanofactory (“+NF”), in **(b)** CT104 *E. coli* (no native AI-2 production) and **(c)** W3110 *E. coli* (native AI-2 production), as measured by flow cytometry.

media. As above, 150 μL samples of culture surrounding the capsules were taken and fixed in 2% paraformaldehyde until flow cytometry analysis (Fig. 5, panel c). We estimate that at the 6 h timepoint, 13% of cells were fluorescent in cultures exposed to “+Ab” capsules, while 50% of cells were fluorescent in cultures exposed to “+NF” capsules. We note that the initial level of fluorescent cells is lower in the wild-type strain due to its genetic background (Tsao et al., 2010). Importantly, the bioactive capsules were able to shift the wild-type bacterial population further towards the “on” QS state by increasing local AI-2 concentration beyond that which was established by native AI-2 production. By establishing communication with the population, the

capsule was able to *enhance and direct* wild-type bacterial response.

Conclusion

This study aimed to create a polysaccharide-based structure to demonstrate novel applications in interrogating biological communication. Our system utilized a liquid-filled alginate core surrounded by a chitosan shell that was crosslinked by TPP. Traditional applications for “carrier” structures have revolved heavily around delivery of target compounds or cells (Allen and Cullis, 2004; Lertsutthiwong

and Rojsitthisak, 2011). We, however, sought to develop a capsule that could actively interact and participate in bacterial communication and elicit a QS response by raising the local concentration of AI-2 using vicinal SAH. Biocatalytic capabilities were conferred to the capsule interior by encapsulating a biological nanofactory that can convert incoming SAH into AI-2. The capsule membrane was impermeable to large molecules, such as the nanofactory, but allowed free passage of small substrate and product molecules. Such a capsule can be used to elicit altered QS responses based on application time and location by producing signaling molecules. Thus, by “talking” to the internal microbial populations, the capsule can report on their conditions and health, and direct their collective behaviors in host-friendly manners. It may selectively identify and interfere with potentially pathogenic cells in complex environments, without the collateral damage incurred with traditional indiscriminate antimicrobials.

We envision this structure as a type of “cell” that can perform desired functions, built from bottom-up fabrication principles that function based on the conferred capabilities. That is, biopolymer-based structures are valuable candidates for such applications as they allow a fine-tuned approach to creating a bio-interactive entity with strictly defined *structural* and *functional* features, as opposed to purely biological systems with complex metabolic requirements. These designer “cells” allow for greater tunability and specificity for the desired output function, compared to their biological counterparts that must perform numerous other tasks for survival. These “cells” also pose no intrinsic threat due to their inability to replicate, and their biopolymer coating may act as an immune barrier.

Experimental

Solutions

Alginate solution, 1% (w/v), was made in distilled water by adding medium viscosity alginate from brown algae (Sigma, St. Louis, MO) and autoclaving for 15 min to complete dissolution. A 1% (w/v) solution of sodium tripolyphosphate (TPP, Sigma) was prepared in deionized water, and was then added to the alginate solution in 1:10 ratio to make a 1% alginate + 0.1% TPP (alginate-TPP) final solution. Chitosan solution, 1.6% (w/v), was prepared similarly to that described by Chen et al. (2002a), and then diluted to 1% (w/v) using deionized water. Briefly, chitosan from crab shells (Sigma) was dissolved to a concentration of 1.6% in deionized water and the pH was adjusted to 5–6. After overnight stirring, it was filtered to remove undissolved substances. Final chitosan concentration was verified by drying a known mass of liquid to obtain the mass of the chitosan. Outer storage solution for the capsules was prepared by dissolving NaCl (Sigma) in deionized water to a final concentration of 1.5 M.

Capsule Formation

Alginate-TPP solution was dropped into stirring chitosan with a 16 G1 needle (Becton Dickinson, Franklin Lakes, NJ) and maintained for 3 min, followed by a brief wash in deionized water. Capsules were incubated in 1 or 2.5 mL NaCl solution for storage and further experiments. Four capsules were obtained per 100 μL of alginate-TPP solution ($\sim 25 \mu\text{L}$ per capsule).

Labeling Polysaccharides and Visualizing Capsule Structure

Chitosan was labeled with NHS-Rhodamine (Thermo Scientific, Rockford, IL, 46406) using standard labeling reaction protocols. Briefly, NHS-Rhodamine was dissolved in a mixture of dimethylformamide (DMF) and ethanol (1 part DMF:4 part ethanol) to a concentration of 2.5 mg mL^{-1} . Fifteen grams films of 1% (w/w) chitosan films were dried overnight in 45°C oven, and the dried films were neutralized with 1 M NaOH and washed with distilled water and 0.1 M PBS buffer (pH 7.4). For the labeling reaction, 20 μL of NHS-Rhodamine solution was added to each chitosan film with 35 mL PBS buffer, and incubated for 30 min. The films were rinsed with distilled water, dissolved in 0.1 M HCl and then precipitated out by adjusting the pH to 9 with 1 M NaOH. Precipitate was collected by centrifugation at 10,000 rpm for 5 min and washed with distilled water. The precipitated chitosan was dissolved in 0.1 M HCl and pH was adjusted to 5.6.

Alginate solutions were labeled with amine-modified fluorescent microspheres (Life Technologies, Grand Island, NY, F8765). To make a 0.1% solution of microspheres, 1 μL of fluorescent sphere suspension was mixed per 1 mL of alginate-TPP solution and vortexed for 1 min to allow homogenous distribution. Capsules were formed using the standard protocol developed above—dropping labeled alginate-TPP solution into stirring labeled chitosan.

Fluorescence microscopy was performed with MVX10 MacroView fluorescence stereomicroscope (Olympus, Center Valley, PA) equipped with a DP72 Camera. Images were taken with FITC and TRITC filter sets and were overlaid using the CellSens software to visualize both polysaccharides simultaneously. Confocal images were taken with Zeiss LSM-310 laser-scanning microscope using the TRITC and GFP filters to construct z-stack images. IMARIS software (Bitplane) was used to quantify the thickness of the chitosan shell from the confocal images.

Biomolecule Encapsulation

The biomolecule to be encapsulated was prepared at concentrations specified below in an alginate-TPP solution. The solution was then added dropwise to chitosan as described above for capsule formation, with the average volume of the resulting capsule being 25 μL .

Bovine Serum Albumin

AlexaFluor 488–conjugated bovine serum albumin (Invitrogen, Carlsbad, CA) was dissolved in the alginate–TPP solution to a concentration of 20 μM .

Antibody

Dylight 488-labeled AffiniPure Goat Anti-Mouse antibody (Jackson ImmunoResearch, West Grove, PA) was dissolved in 10 mM PBS without calcium and magnesium to a 50 μM stock concentration. Antibody encapsulation was performed similarly to BSA encapsulation by dissolving the protein in the alginate–TPP solution to a concentration of 0.5 or 1 μM . Capsule formation with 20 μM encapsulated antibody did not produce stable structures, thereby requiring use of lower antibody concentrations.

Nanofactory

The biological nanofactories were composed of the DyLight-488 labeled Goat Anti-Mouse antibody and the fusion protein HGLPT (dissolved in PBS). HGLPT was purified from BL21 ΔluxS *E. coli*, as described by Fernandes and Bentley (2009). Nanofactories were assembled by mixing the antibody and HGLPT in a 1:1 molar ratio to make a 25 μM nanofactory stock. This stock was dissolved in alginate–TPP solution to a concentration of 2.5 μM .

Monitoring for Biomolecule Diffusion From Capsules

Capsules were monitored for permeability with respect to the contained biomolecule. Capsules were individually incubated in 1 or 2.5 mL NaCl solutions at room temperature. Biomolecule diffusion out of the capsule was monitored by measuring fluorescence of 50 μL samples of the surrounding solution periodically using the SpectraMax M2e plate reader (Molecular Devices, Sunnyvale, CA). After the last time point, the relative quantity of biomolecule retained by the capsule was ascertained by taking additional fluorescence measurements. In this case, the NaCl solution was replaced and the capsule was punctured, enabling any contained molecule to be released into the fresh NaCl solution. Fluorescence measurements by the plate reader were used to detect molecular presence in the solution resulting from capsule rupture.

Addition of SAH Substrate to Nanofactory Environment

In all experiments involving AI-2 synthesis, S-adenosylhomocysteine (SAH) was prepared at a stock concentration of 10 mM in PBS and diluted to 1 mM by pipetting 100 μL of stock solution per 1 mL NaCl solution used for capsule incubation or puncture. SAH is the substrate for the enzyme sequence comprising the nanofactory.

To test for enzymatic conversion of SAH after the encapsulation process of the nanofactory, capsules were placed in 1 mL saline solution for a set incubation period. The surrounding solution was removed and supplemented with 1 mM SAH. Furthermore, after removing the original NaCl solution surrounding the capsules, fresh 1 mL NaCl was added and capsules were punctured to release retained nanofactory. This solution was then supplemented with 1 mM SAH. Alternatively, to specifically verify enzymatic activity of the nanofactory *while contained* within the capsule, capsules prepared with encapsulated nanofactories were directly placed (without puncture) into 1 mL NaCl solution supplemented with 1 mM SAH. All solutions were incubated at 37°C for 2 h to allow AI-2 synthesis, and then stored at –20°C until analysis.

Measuring Nanofactory Activity by *V. harveyi* AI-2 Bioluminescence Assay

AI-2, the enzymatic product, was detected in samples by inducing the luminescent reporter *V. harveyi* strain, BB170. NaCl solutions obtained from outside the capsules and those containing ruptured capsules to which SAH was added, were assayed using the protocol outlined by Surette and Bassler (1998). Briefly, BB170 was inoculated in AB medium, and grown for 16 h at 30°C while shaking. The culture was then diluted 1:5,000 in fresh AB media and 180 μL aliquots were prepared in 5 mL culture tubes which contained 20 μL of the sample to be analyzed. Tubes were shaken at 250 rpm in 30°C for 4 h, and luminescence measurements were taken with a luminometer (EG&G Berthold, Gaithersburg, MD).

Measuring Nanofactory Activity by Co-Culturing Cells and Capsules

W3110 (pCT5-amp^r + pET-GFPuv-kan^r) (Tsao et al., 2010), and CT104 (ΔluxS , pCT6-amp^r + pET-EGFP-kan^r) strains were used as reporters of AI-2. Plasmid constructs are described by Tsao et al. (2010). All *E. coli* strains were grown in LB medium supplemented with kanamycin and ampicillin (50 $\mu\text{g mL}^{-1}$ per antibiotic) at 37°C overnight. They were reinoculated at 3% of an overnight culture in LB + kanamycin + ampicillin. Cultures were grown at 37°C and 250 rpm until an optical density (at 600 nm) of 0.4 was reached. Subsequently, cultures were centrifuged at 3,000g for 7 min and resuspended in the following solution: 1.5 M NaCl + 10% LB (v/v) + kanamycin + ampicillin (50 $\mu\text{g mL}^{-1}$ per antibiotic) + 1 mM SAH (NaCl/LB). The resuspension was divided into 1 mL aliquots; one capsule per tube was added and cultures were returned to a 37°C incubator without shaking. Capsules contained either an antibody only (as a negative control) or the nanofactory (prepared in a manner described earlier). One hundred fifty microliters of samples were recovered from each culture and fixed in 2% paraformaldehyde (in PBS). Samples were analyzed by flow cytometry (FACSCanto II, BD Biosciences, San Jose, CA) for

fluorescence expression and distribution profiles were prepared using the FlowJo software (TreeStar).

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