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Modulating *Vibrio cholerae* Quorum-Sensing-Controlled Communication Using Autoinducer-Loaded Nanoparticles

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Abstract

The rise of bacterial antibiotic resistance has created a demand for alternatives to traditional antibiotics. Attractive possibilities include pro- and anti-quorum sensing therapies that function by modulating bacterial chemical communication circuits. We report the use of Flash NanoPrecipitation to deliver the *Vibrio cholerae* quorum-sensing signal CAI-1 ((S)-3-hydroxytridecan-4-one) in a water dispersible form as nanoparticles. The particles activate *V. cholerae* quorum-sensing responses five orders of magnitude higher than does the identically administered free CAI-1, and are diffusive across *in vivo* delivery barriers such as intestinal mucus. This work highlights the promise of combining quorum-sensing strategies with drug delivery approaches for the development of next-generation medicines.

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ASSOCIATED CONTENT

Supporting Information**.**

Detailed experimental procedures for nanoparticle formation, stability characterization, CAI-1 activity determination, biofilm inhibition assays, two supplemental tables, and seven supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

Keywords

Nanoparticle; drug-delivery; quorum sensing; CAI-1; *Vibrio cholerae*; autoinducer

The rise of bacterial antibiotic resistance is among the greatest public health problems in modern times.[1-6] An overarching goal is to develop drugs with novel mechanisms of action that effectively combat pathogens, and simultaneously introduce minimal selective pressure for the development of resistance.[7, 8] Quorum-sensing based medicines are a promising class of therapeutics that has the potential to meet these criteria. In contrast to traditional antibiotics that inhibit growth of or kill bacteria, quorum-sensing therapeutics function by modulating the bacterial communication pathways that control virulence cascades, thereby disabling pathogens.[9-11] Molecules that alter behavior rather than function by bacteriostatic or bactericidal mechanisms may reduce the selective pressure for resistance development. Quorum sensing is a cell-cell communication process that controls bacterial collective behaviors. The process relies on the production, release, and subsequent group-wide detection of small molecule signals called autoinducers.[12] There are two promising scenarios: quorum-sensing agonists that mimic autoinducers and promote non-virulent behaviors could be used against bacteria that naturally repress virulence traits via quorum sensing, and quorum sensing antagonists that would function by inhibiting bacterial communication could be used against bacteria that employ quorum sensing to initiate or sustain virulence.

The *Vibrio cholerae* quorum-sensing autoinducer called CAI-1 is a promising candidate to explore the delivery of a quorum-sensing agonist as a treatment for the disease cholera.[13-15] *V. cholerae* infects the small intestine causing acute diarrhea, which, if untreated, causes death in 50-70% of cases.[16, 17] Approximately 3-5 million people are affected by cholera each year.[18] At low cell density and in the absence of autoinducers, *V. cholerae* produces biofilms and expresses its repertoire of virulence factors.[19, 20] Presumably, this is the state in which *V. cholerae* exists upon initial infection of the human host. In response to the growth-dependent accumulation of quorum-sensing autoinducers, *V. cholerae* transitions into a non-biofilm-forming and non-virulent state. What is important for this work is that the accumulation of the CAI-1 autoinducer triggers a phosphorelay cascade that leads to decreased virulence factor production, repressed biofilm formation, and the production of proteases that detach *V. cholerae* from the intestine (Figure 1, S1), as confirmed with previous genotyping experiments.[21-23] Thus, the CAI-1 autoinducer is an interesting molecule to investigate for potential use as a quorum-sensing treatment for cholera, wherein its mode of action would be the promotion of the non-virulent state. For example, mice colonized with commensal *E. coli* engineered to produce CAI-1 in the small intestine demonstrate immunity against *V. cholerae* infection.[24] However, these cell-based delivery platforms require prophylactic administration of live and engineered organisms, which may pose challenges to translation. An orally delivered CAI-1 therapeutic would be preferable.

To be a successful oral therapy, active CAI-1 must reach *V. cholerae* residing in tubular epithelial invaginations (called crypts) in the upper small intestine.[25, 26] Obstacles to drug delivery include the inherent hydrophobicity of CAI-1, and the presence of unstirred water

and mucus layers that cover crypt openings.[27-30] As a highly hydrophobic molecule (cLogP of 4.6), CAI-1 is a sparingly water-soluble oil, limiting delivery and dispersal of CAI-1 at concentrations high enough for therapeutic activity. The unstirred water and mucus layers also present diffusion barriers that effectively block non-water soluble molecules and hydrophobic carriers from entering intestinal crypts.[31-33] Attempts to engineer quorum-sensing drugs with increased hydrophilicity to circumvent these limitations have resulted in molecules with decreased biological activity, and analogues developed with improved CAI-1 activity had actually increased hydrophobicity, making the compounds even more difficult to administer.[34, 35] Holistically, the development of new quorum sensing drugs without addressing the delivery of these compounds, such as through the mucus barrier and into crypts, can miss a major component of the challenge in addressing cholera infections however. The use of drug delivery nanocarriers, routinely employed in other fields of medicine, is a promising method for overcoming barriers to successful CAI-1 delivery to *V. cholerae* in intestinal crypts.[36-38] Specifically, drug nanocarriers coated with a dense water-soluble polyethylene glycol (PEG) surface layer can encapsulate hydrophobic compounds for dispersion to high concentrations in water, significantly increase drug surface area to volume ratios for improved dissolution kinetics, and facilitate drug transport through mucus layers.[39-41] For example, small 100 nm particles have 10^5 higher characteristic dissolution rates than otherwise identical centimeter sized tablets. Also, nanoparticles with sufficiently dense PEG coatings diffuse only 0-2 orders of magnitude slower in mucus than in water (depending on nanoparticle properties and PEG coverage), while non-PEG coated nanoparticles can exhibit up to 4-5 orders of magnitude slower diffusion in mucus than in water.[39] There has been some recent work in developing materials that can affect or detect quorum sensing behaviors, such as by entrapping peptides, proteins, and cells.[42-46] However, the application of nanoparticles for quorum-sensing-based drugs is new, and to the best of our knowledge, there has been no previous nanoparticle work aimed at the delivery of quorum-sensing compounds to gastrointestinal environments for the treatment of infectious diseases. The development of proof-of-concept quorum-sensing drug delivery platforms can advance the prospects toward the realization of quorum-sensing based therapeutics.

CAI-1 nanoparticles (CAI-1 NPs) were prepared using the kinetically-controlled, block-copolymer-directed precipitation process called Flash NanoPrecipitation (FNP), since it has been demonstrated for a range of hydrophobic therapeutics and generates dense PEG layers appropriate for mucus penetration (Figure 2).[47] In FNP, lipophilic compounds dissolved in water-miscible organic solvents are rapidly mixed against an aqueous antisolvent stream to promote high supersaturation, nucleation, and growth of the lipophilic core component.[48-50] Stabilizing block copolymers self-assemble on the surface of the lipophilic core to form nearly monodisperse particles that are stabilized against aggregation with a dense PEG surface brush.[51] FNP has been previously used to form a variety of therapeutically active, cell targeting, imaging-agent tagged, and cytocompatible nanoparticles.[52-55] Here we demonstrate the effectiveness of FNP to make water dispersible and bioactive CAI-1 NPs to address disease states associated with mucus films and biofilms, of which potential use in cholera treatment is an important example.

We first investigated whether CAI-1-loaded nanoparticles (CAI-1 NPs) decorated with a PEG surface could be formed with the FNP process using polystyrene-block-PEG (PS-b-PEG) as the stabilizer and vitamin E (VitE) as a co-core component. PS-b-PEG and VitE form stable and robust nanoparticles capable of encapsulating a variety of other drugs.[50] The role of the VitE is to enhance nucleation rates during particle formation. VitE is also significantly more hydrophobic than CAI-1, and thus, VitE provides a hydrophobic reservoir in the NP core to control CAI-1 release. Dynamic light scattering analyses showed that when flashed with PS-b-PEG as a stabilizer and VitE as a structural co-core, CAI-1 formed nanoparticles with an average diameter of 112 ± 1.7 nm and a polydispersity of 0.12 ± 0.01 (Figure 3A, S2; Table S1). These CAI-1 NPs when diluted into PBS were evenly dispersed, opalescent, and did not settle over time. Indeed, they appeared significantly different from pure CAI-1 in PBS, which were phase-separated and coalesced into macroscopic oil droplets (Figure 3B). PS-b-PEG and VitE flashed in the absence of CAI-1 assembled into smaller, unloaded nanoparticles (VitE NPs) with an average diameter of 65 ± 0.22 nm. Alternative methods to form nanoparticles with slower timescales of assembly, such as precipitation via solvent exchange across a dialysis membrane, resulted in large and polydisperse aggregates (Figure S3). These results demonstrate that the FNP process can be used to generate small, uniform, water-dispersible, colloidally stable, and CAI-1-containing nanoparticles.

For successful drug delivery, CAI-1 NPs must be appropriately stable. Premature nanoparticle breakup or dissolution could lead to CAI-1 release prior to reaching the small intestine, resulting in delivery failure. To characterize the stability of CAI-1 NPs, nanoparticle sizes were monitored over time when nanoparticles were incubated in various buffer conditions relevant to oral drug delivery. When incubated at 37°C for up to three days in phosphate buffered saline (PBS), CAI-1 NPs remained the same size (Figure 4A, S4). Importantly, this result suggests that CAI-1 NPs can be stored in aqueous solution without significant drug loss. Size changes also did not occur following dialysis against PBS with 10 kDa MWCO membranes, highlighting the stability of CAI-1 NPs even in excess aqueous buffer (Figure S5). For our intended purpose, it is crucial that CAI-1 NPs remain intact in the gastric acid encountered during transit from the stomach to the small intestine. To investigate CAI-1 NP stability in acidic environments mimicking that of the stomach, CAI-1 NPs were incubated in pH 2 buffer for three days at 37°C. CAI-1 NPs showed no change in size, suggesting that CAI-1 NPs are not degraded in acid (Figure 4B). CAI-1 NPs were also stable in LB broth, and thus they are not affected by the peptides and proteins present in tryptone or yeast extract (Figure 4C).

While limited CAI-1 release is beneficial for minimizing premature drug loss, excessively limited CAI-1 release when the nanoparticles reach the small intestine would lead to therapy failure. It is possible to have CAI-1 NP *in vitro* stability during storage prior to consumption (as only a minimal amount of CAI-1 is required to saturate the aqueous phase due to the hydrophobic nature of CAI-1), and also have CAI-1 release in *in vivo* conditions where a “sink” can draw out CAI-1. To test whether bile micelles present in the small intestine could act as “sinks” and suitable triggers for rapid CAI-1 solubilization and release, CAI-1 NP size was monitored over time when incubated in 2% bile salts. CAI-1 NP size decreased immediately following incubation in 2% bile, consistent with rapid CAI-1 transfer from the

nanoparticle core to bile micelles (Figure 4D). *In vivo*, bile micelles readily transport lipophilic materials across the mucus layers. VitE NPs lacking CAI-1 did not display similar size decreases (Figure S6), suggesting that it is the CAI-1 that is being released from the NPs rather than the VitE or the stabilizer.

CAI-1 NPs must be highly bioactive to make a successful therapy. To characterize the biological activity of CAI-1 NPs, we exploited a *V. cholerae* reporter strain (WN1102 Δ*cqsA* Δ*luxQ*/pBB1). *V. cholerae* WN1102 cannot produce its own CAI-1, but it responds to CAI-1 that is exogenously supplied. *V. cholerae* WN1102 harbors the luciferase operon that is exclusively induced in response to CAI-1. Thus, in the absence of CAI-1 *V. cholerae* WN1102 cells are dark, whereas in the presence of exogenously supplied CAI-1, *V. cholerae* WN1102 cells produce light. Importantly, the quorum-sensing signal transduction and genetic regulation pathway linking CAI-1 to the heterologous bioluminescence output is the same pathway that links CAI-1 to virulence factor production and biofilm formation in *V. cholerae*. However, the responses are opposite: when bioluminescence is *activated*, virulence factor expression and biofilm formation are *repressed*. Thus, high light production corresponds to low virulence factor production and low biofilm formation.

When concentrated CAI-1 dissolved in DMSO was added to cell cultures, cells activated a high level of bioluminescence (Figure 5A). However, while DMSO delivery can be used for *in vitro* research, it does not translate to clinically relevant *in vivo* oral delivery. When CAI-1 NPs suspended in PBS are added to the same final CAI-1 concentration as CAI-1 in DMSO is, cells also exhibited light production; indeed, with a similar dose-response profile. NPs deliver CAI-1 to cells as effectively as DMSO; however the NP form in water is suitable for oral delivery. When CAI-1 not in the NP form (the CAI-1 free in solution that is in equilibrium with bulk CAI-1, SI methods) was identically diluted and added, up to 100,000-fold less bioluminescence emission occurred. This result reflects the poor water solubility of CAI-1 and the fundamentally low levels of CAI that can be delivered using direct CAI-1 dispersion in an aqueous medium. Inert VitE NPs caused no induction of bioluminescence, showing that the high bioluminescence activity elicited by CAI-1 NPs was not due to the presence of the stabilizer or the co-core component. To verify that CAI-1 activity was from nanoparticles and not from CAI-1 that might have dissolved into solution during the FNP process, the activity of CAI-1 NPs separated from PBS by ultrafiltration or by dialysis was tested (Figure S7). Harvested nanoparticles possessed high CAI-1 activity, while nanoparticle-free ultrafiltration flow-through produced minimal activity. As controls we show that CAI-1 NPs do not affect cell growth: the growth rates of *V. cholerae* WN1102 supplemented with CAI-1 NPs in PBS, CAI-1 in DMSO, CAI-1 in PBS, and VitE NPs in PBS were all similar (Figure 5B), even at the maximum 50 μM CAI-1 concentration tested. Together, these results demonstrate that high levels of CAI-1 can be delivered from CAI-1 NPs in aqueous suspensions, eliminating the need for organic solvents when dealing with this hydrophobic molecule.

To assess the potential therapeutic relevance of CAI-1 NPs, we investigated the ability of CAI-1 NPs to inhibit biofilm formation. Biofilms are surface adhered, matrix-encased bacterial cells that display remarkable antibiotic resistance, are a leading cause of chronic infections, and are targets of much therapeutic interest.[56-59] When *V. cholerae* WN1102

cells were incubated with CAI-1 NPs under static growth conditions, biofilm production was inhibited in a dose-responsive manner that tracked with that of supplied CAI-1 (Figure 6). Specifically, at 50 μM CAI-1 concentration, CAI-1 NPs reduced biofilm production by 71 ± 2 % as assessed by crystal violet staining. *V. cholerae* WN1102 cells grown with CAI-1 supplied in PBS exhibited a biofilm reduction of 32 ± 4% at the equivalent CAI-1 dilution. VitE NPs were inert for biofilm inhibition, demonstrating that improved levels of biofilm inhibition caused by CAI-1 NPs are not a direct consequence of the presence of the stabilizer or co-core components.

The ability of CAI-1 NPs to diffuse through intestinal mucus is important for the *in vivo* delivery of CAI-1 across intestinal barriers to reach *V. cholerae* sites of infection. To evaluate the diffusivity of CAI-1 NPs in these settings, the particle movements of fluorescently labelled CAI-1 NPs were characterized in *ex vivo* tracking experiments. CAI-1 NPs exhibited Brownian diffusion in simulated intestinal fluid, as well as in intestinal mucus freshly harvested from mice (Fig. 7, SI Table S2). Importantly, CAI-1 NPs had only a 29 fold decrease in diffusivity while suspended in mucus, relative to CAI-1 NP diffusivity in intestinal fluid. The relatively unhindered movement of CAI-1 NPs in mucus is in sharp contrast to the movement of other unprotected nanoparticles in mucus, which can exhibit 4-5 orders of magnitude decreased diffusivity.[39] This confirms the high density-PEG surface coating previously measured, is the first exemplification of mucus penetration with FNP based nanoparticles, and highlights advantages of CAI-1 NPs in potential cholera treatments.[47]

Antibiotic resistance is a global public healthcare crisis that has created the need for alternatives to traditional antibiotics. Quorum-sensing research has revealed a variety of quorum-sensing agonists and antagonists that exhibit promise for development into antivirulence medicines to treat diseases via novel mechanisms of action. However, success relies on efficient delivery to target sites. Here, we present proof of concept experiments using Flash NanoPrecipitation to form PEG-coated, water-dispersible CAI-1 autoinducer nanocarriers that inhibit biofilm formation in *V. cholerae*. To the best of our knowledge, this work reports the first quorum-sensing-dependent drug delivery platform designed for use in the gastrointestinal environment for cholera treatment. Nanoparticle-encapsulated CAI-1 can induce five orders of magnitude higher quorum-sensing agonism outputs compared to identically delivered but non-encapsulated CAI-1 *in vitro*. Further studies are underway to optimize nanoparticle penetration through mucus layers in the GI tract, while preliminary results reveal that CAI-1 NPs are indeed diffusive in freshly isolated murine intestinal mucus. Additional studies are also underway to investigate CAI-1 delivery using biodegradable polymers; however, retention of the current PS-b-PEG based NPs in the GI tract mucus layer presents no significant toxicity concerns. This work demonstrates the benefits that nanotechnology can provide to quorum-sensing based therapeutics, and highlights the need for enhanced integration of quorum-sensing manipulation strategies and drug delivery approaches in the development of next generation antivirulence medicines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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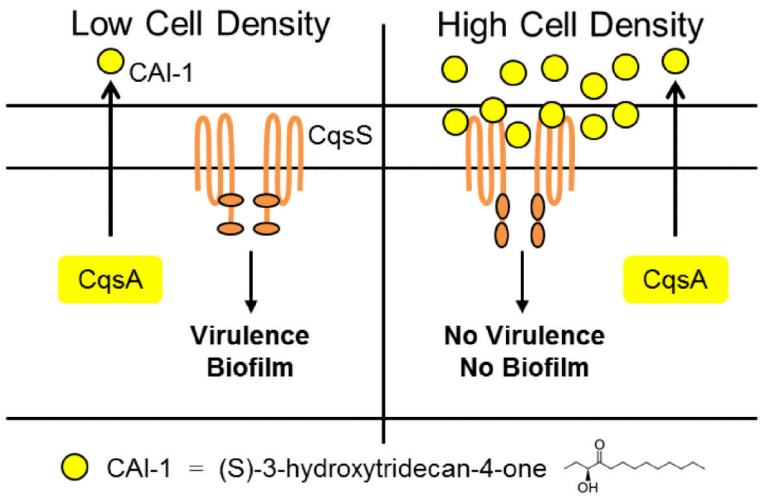

Figure 1.

Simplified quorum-sensing pathway in *V. cholerae*. The CAI-1 autoinducer is produced by the CqsA enzyme. Released CAI-1 binds to the CqsS receptor, and alters CqsS phosphorelay activity. Left panel, at low cell density, and low CAI-1 concentration, signal transduction from CqsS results in virulence factor expression and biofilm formation. At high cell density, CAI-1 accumulates. Binding of CAI-1 to CqsS results in decreased virulence factor production and decreased biofilm production, allowing CAI-1 to be potentially used in antivirulence therapies for treating cholera. In the cartoon, CAI-1 is denoted by yellow circles. The structure of the molecule is shown below the cartoon for reference.

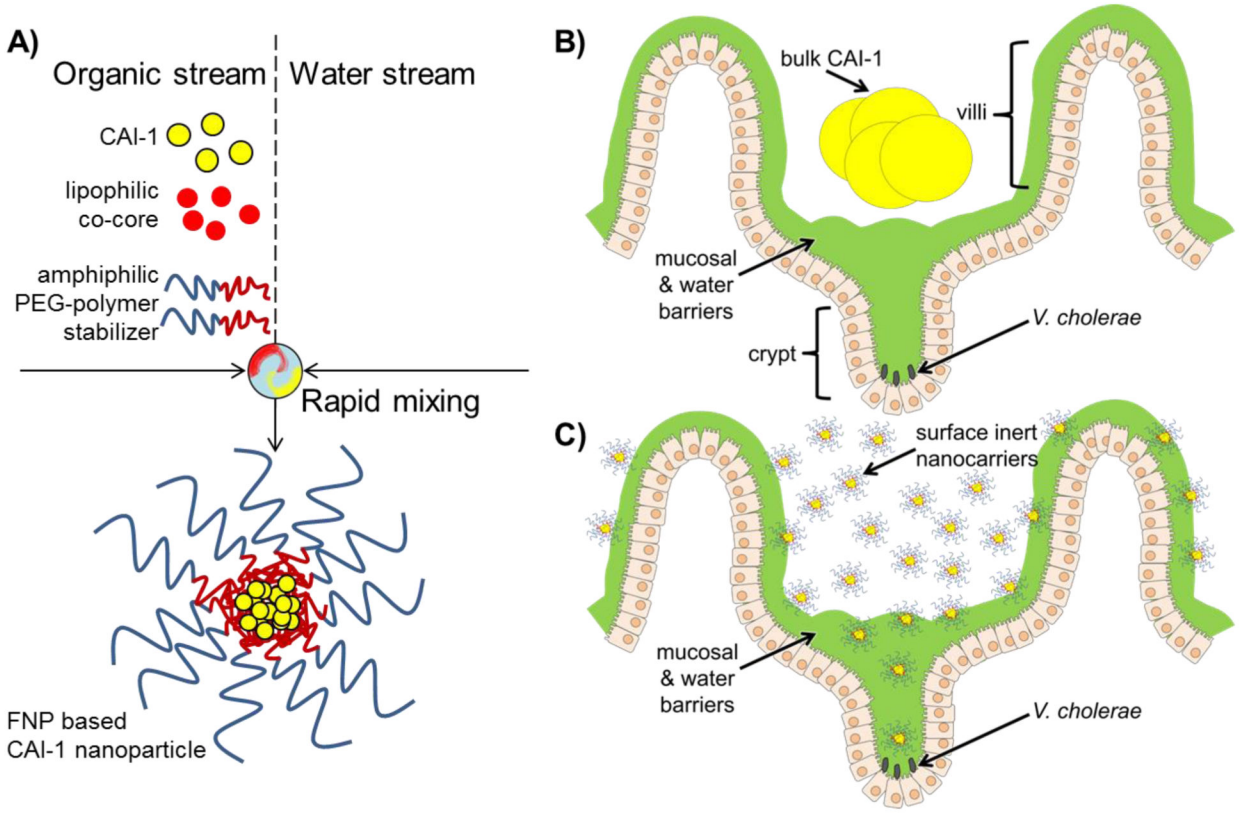

Figure 2.

Flash NanoPrecipitation and drug delivery of CAI-1. (A) CAI-1 nanoparticles decorated with a polyethylene glycol surface (PEG) can be formed by rapidly precipitating CAI-1 in the presence of amphiphilic PEG diblock copolymers and lipophilic co-core materials. PEG diblock copolymers self-assemble with precipitating CAI-1, and form nanoparticles that are stabilized with a dense PEG brush surface. The PEG layer allows CAI-1 nanoparticles to be dispersed in water at high concentrations, and endows nanoparticles with an inert hydrophilic surface, that can potentially improve orally administered CAI-1 delivery. (B) Hydrophobic bulk CAI-1 cannot penetrate the mucus that covers crypts in the small intestine where *V. cholerae* reside during infection. (C) Delivery of CAI-1 in small PEG covered nanoparticles can allow CAI-1 to directly penetrate mucus layers, or get rapidly solubilized into bile micelle carriers that can also penetrate intestinal barriers.

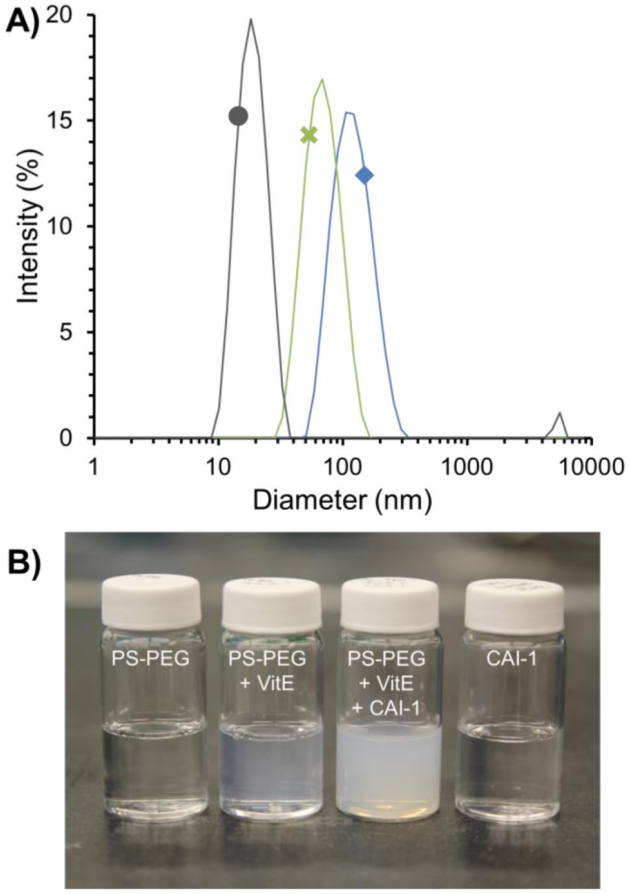

Figure 3.

Flash NanoPrecipitation of CAI-1 nanoparticles. (A) Dynamic light scattering size distributions of CAI-1 NPs flashed with stabilizer, co-core, and CAI-1 (◆); of VitE NPs flashed with stabilizer and co-core (×); and of empty micelles flashed with only stabilizer (●). (B) Visual appearance of stabilizer micelles, VitE NPs, CAI-1 NPs, and CAI-1 in PBS. The CAI-1 in PBS is an oil that floats as a liquid drop on the water surface.

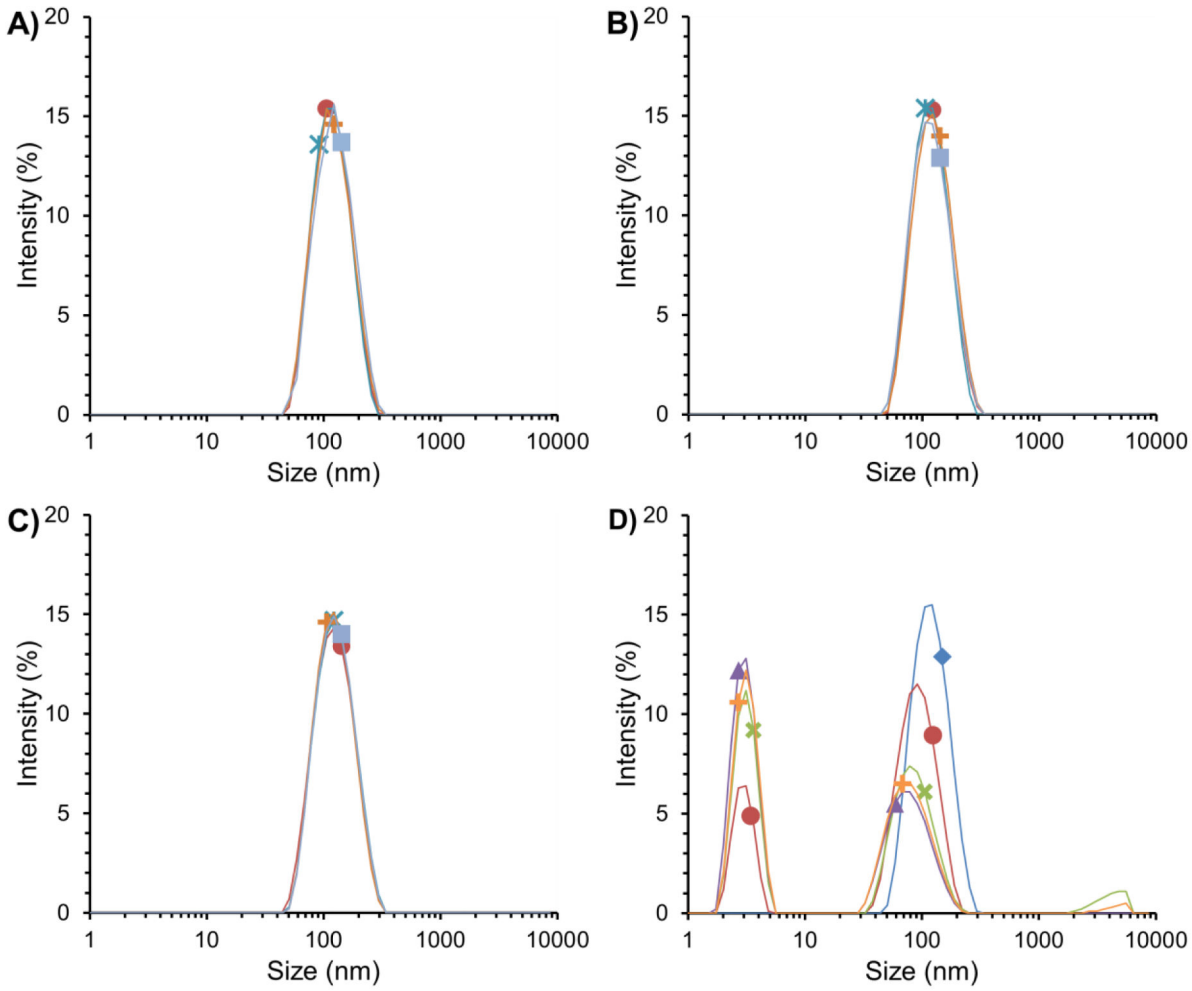

Figure 4.

Stability of CAI-1 nanoparticles. Dynamic light scattering size distributions of CAI-1 NPs following incubation in (A) PBS, (B) pH 2 PBS, and (C) LB medium at three minutes (●), one day (■), two days (✱), and three days (+). (D) Size distributions of CAI-1 NPs prior to incubation (◆) and following incubation in 2% bile salts at 37°C at three minutes (●), thirty minutes (×), three hours (▲), and three days (+).

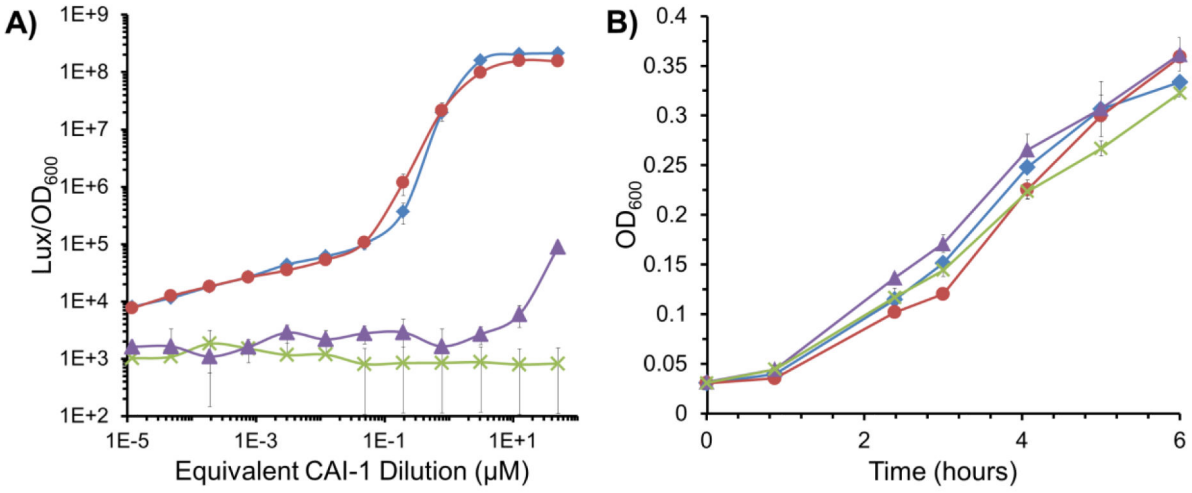

Figure 5.

Bioactivity of CAI-1 nanoparticles. (A) Bioluminescence response of *V. cholerae* WN1102 supplemented with different amounts of CAI-1 NPs in PBS (◆), CAI-1 in DMSO (●), CAI-1 in PBS (▲), or VitE NPs in PBS (×). Concentrated CAI-1 NPs in PBS and CAI-1 in DMSO were added to cell cultures to the final concentrations indicated. CAI-1 in PBS and VitE NPs in PBS were equivalently diluted and added to cell cultures. (B) Growth of *V. cholerae* ultures at 37°C. CAI-1 NPs in PBS and CAI-1 in DMSO were added to cell cultures to a final CAI-1 concentration of 50 μM. CAI-1 in PBS, and VitE NPs in PBS were equivalently diluted and added to cell cultures. In both panels, values are averages and error bars are standard deviations of three separate measurements.

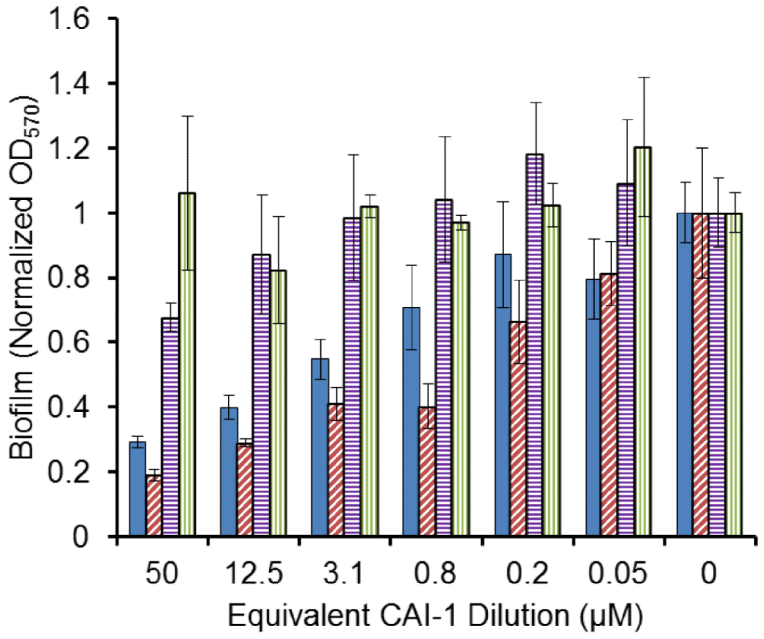

Figure 6.

Biofilm inhibition using CAI-1 NPs. Crystal violet colorimetric analysis of adherent cells when *V. cholerae* was grown with supplemented CAI-1 NPs in PBS (■), CAI-1 in DMSO (▨), CAI-1 in PBS (▤), or VitE NPs in PBS(▥). Values are averages and error bars are standard deviations of four separate measurements.

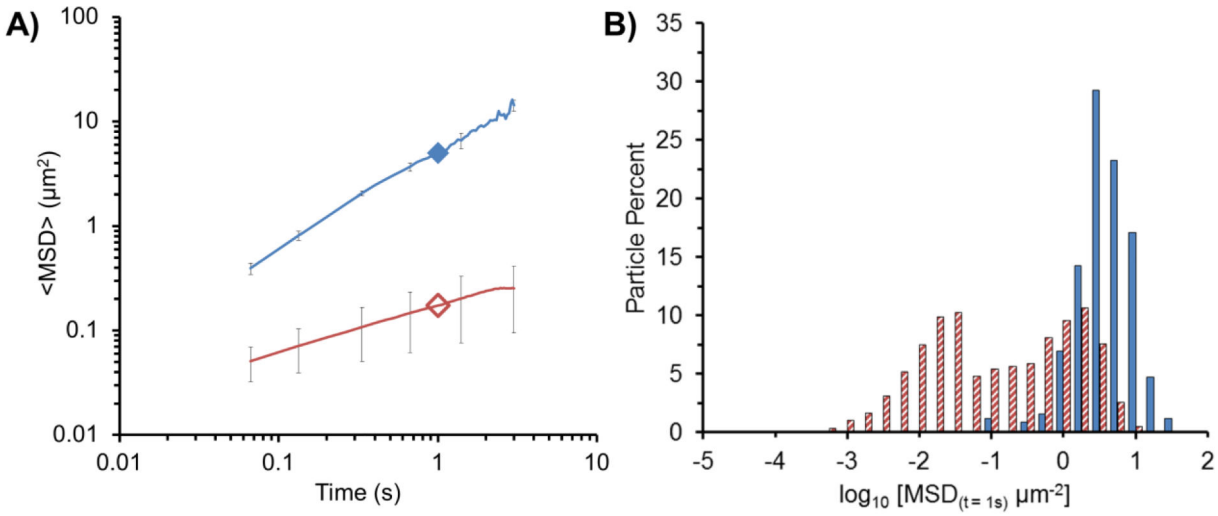

Figure 7.

Nanoparticle transport in murine small intestinal mucus. (A) Ensemble-average mean square displacement (<MSD>) of Ettp5-labeled CAI-1 NPs over time in simulated intestinal fluid (◆) and in mucus coating from freshly excised murine small intestine tissue (◇). (B) Distributions of the logarithms of individual particle MSD at a time scale of 1 s for Ettp5-labeled CAI-1 NPs in simulated intestinal fluid (■) and in mucus coating from freshly excised murine small intestine tissue (▨).