LETTER

Conjugated Polyelectrolyte Capsules: Light-Activated Antimicrobial Micro "Roach Motels"†

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ABSTRACT Microcapsules consisting of alternating layers of oppositely charged poly(phenylene ethynylene)-type conjugated polyelectrolytes (CPEs) were prepared via layer-by-layer deposition onto $MnCO_3$ template particles followed by dissolution of the template particles using an ethylenediaminetetraacetate solution. The resulting microcapsules exhibit bright-green fluorescence emission characteristics of the CPEs. Strong antimicrobial activity was observed upon mixing of polyelectrolyte capsules with *Cobetia marina* or *Pseudomonas aeruginosa* followed by white-light irradiation. It was demonstrated that the materials act as highly effective light-activated micro "Roach Motels" with greater than 95% kill after exposure to ~1 h of white light.

KEYWORDS: biocide • conjugated polymer • singlet oxygen • polyelectrolyte capsule • confocal microscopy

INTRODUCTION

Controlling pathogenic bacteria is a major global concern, from basic household maintenance to intentional release by terrorists. Although standard disinfection protocols work well on cells in suspension, those that are attached to surfaces, which account for the majority of bacteria in the world, are notoriously resistant to standard disinfection protocols (1). The magnitude of this problem is well illustrated in medical settings, in which bacteria attached to medical devices are associated with up to 1.4 million deaths per year (1). Among the organisms associated with nosocomial infections, the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is one of the most common, persistent, and lethal; the second leading cause of infection in intensive care units, this organism quickly acquires multiple drug resistance and lethality factors when introduced into the hospital setting (2). The strain *P. aeruginosa* PAO1 has served for many years as a nonpathogenic model for understanding mechanisms of *P. aeruginosa* adhesion (3) and is therefore also a good model organism for understanding disinfection.

New coatings that can capture, entrap, and kill pathogens, especially Gram-negative bacteria, have been the subject of several recent investigations (4−12). Especially interesting and attractive are antimicrobial coatings that can function catalytically and that can be used in a variety of environments. We have recently reported the use of cationic conjugated polyelectrolytes (CPEs) supported on colloids as antimicrobials that can entrap Gram-negative bacteria such as *P. aeruginosa* and *Cobetia marina* in a dark process at the colloid−suspension interface and subsequently kill the bacteria on irradiation with visible light (13). Here we report the remarkably enhanced light-activated biocidal activity of micron-sized polyelectrolyte hollow capsules consisting of alternating layers of a pair of anionic and cationic phenyleneethynylene-type CPEs. These novel photoactive polyelectrolyte capsules function in a manner reminiscent of the insect entrapping "Roach Motels" with respect to their ability to attract, concentrate, and kill bacteria trapped within and on the surface of the capsules.

RESULTS AND DISCUSSION

Microcapsules consisting of alternating layers of oppositely charged polyelectrolytes have been studied extensively in recent years (14, 15). Work in this area has explored the fabrication, structure, and function of the polyelectrolyte capsules, toward their application for compartmentalization and/or release of drugs or other agents under a controlled stimulus (15). Polyelectrolyte microcapsules are prepared via layer-by-layer (LbL) deposition of oppositely charged polyelectrolytes onto the surface of micron-sized sacrificial template particles. Dissolution of the template particles affords the hollow polyelectrolyte microcapsules. In the present investigation, we have adopted literature methods to fabricate novel polyelectrolyte capsules consisting of alternating layers of the phenylene ethynylene-type conjugated polycation and polyanion, **1a** and **1b**, respectively (Scheme 1) (16, 17). These CPE microcapsules are prepared via LbL deposition of four bilayers consisting of **1a** and **1b**

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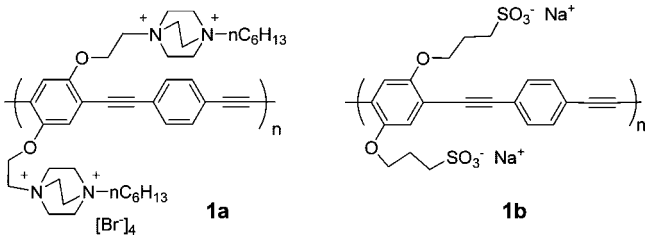
Scheme 1. Chemical Structures of CPEs



onto 5-μm-diameter $MnCO_3$ template particles. $MnCO_3$ particles were selected as the template because a convenient procedure is available that allows the fabrication of monodisperse samples with diameter in the micron size range (17). Following LbL deposition, the $MnCO_3$ template is dissolved by exposure of the particles to a solution of ethylenediaminetetraacetate (EDTA). The resulting polyelectrolyte capsules retain the photoactive properties characteristic of the poly(phenylene ethynylene) units; i.e., they are strongly fluorescent in the mid-visible, and as shown below, they display light-activated biocidal activity.

The materials present at various stages of the CPE microcapsule fabrication process were characterized by a variety of techniques, including dynamic light scattering (DLS), scanning electron microscopy (SEM), epifluorescence microscopy, and confocal laser scanning microscopy. First, the $MnCO_3$ template particles were prepared by using nanometer-sized particles to seed growth of monodisperse, micron-sized particles (17). In a typical run, $MnCO_3$ particles having a mean particle size of ~5.0 μm with a standard deviation of ~0.5 μm (DLS) were prepared. SEM images of the $MnCO_3$ template particles reveal their spherical shape and uniform size distribution. Next, four bilayers consisting of polycation **1a** and polyanion **1b** were deposited on the $MnCO_3$ template particles, beginning with **1b** and ending with **1a**, affording CPE-coated colloids featuring the cationic polymer at the surface. Given the positive charge of the capping polymer **1a**, we infer that the exterior of the polyelectrolyte capsule surface has a positive surface potential. The resulting CPE-coated particles were inspected by using epifluorescence and confocal fluorescence microscopy. For example, as shown in Figure 1b, the presence of the **1a**/**1b** coating on the surface of the $MnCO_3$ particles is signaled by the bright-green fluorescence that is characteristic of the CPEs (18). Bright-field confocal images (DIC mode) of the **1a**/**1b**-coated particles clearly show the core due to light refraction by the high-index $MnCO_3$. Finally, the **1a**/**1b** polyelectrolyte capsules were produced by treating a suspension of the coated particles with EDTA. Analysis of the resulting hollow capsules by using epifluorescence microscopy clearly shows the fluorescence from the conjugated polyelectrolyte layers (Figure 1c). Interestingly, the polyelectrolyte capsules appear to be flexible with creases in the capsule walls. As shown in Figure 1d, SEM images of dried **1a**/**1b** capsules reveal flat pancake-like structures, confirming that complete dissolution of the $MnCO_3$ template has occurred. Characterization of the capsules by confocal microscopy (data not shown) also confirms the absence of the $MnCO_3$ templates.

The bacterium used in this study, *P. aeruginosa* PAO1, was grown in a chemostat as described elsewhere (13). Interactions between *P. aeruginosa* and the hollow CPE capsules [hereafter referred to as micro "Roach Motels" (μRM)] were monitored by confocal fluorescence microscopy, and bacterial viability was determined by staining the bacteria with a 1:1 mixture of SYTO 60 and SYTOX Green stains obtained from Molecular Probes (www.invitrogen.com). These DNA stains penetrate the cells and produce red (~650 nm) and green (~530 nm) emission for live and dead bacteria, respectively. As noted above, the μRM also exhibit green fluorescence when irradiated with 400−450 nm light (Figure 1c). We have previously shown that 5 μm solid particles coated with the cationic conjugated polyelectrolyte **1a** by physisorption can capture several bacteria on their surface; capture of the bacteria by these particles is at least initially a reversible process (13). Slightly more efficient capture (and killing) of *P. aeruginosa* occurs when a cationic polymer having similar polymer repeat unit structure is grown covalently from the surface of 5 μm silica beads (13). Studies with electron microscopy indicate that the covalently grown polymer provides a rougher surface that may be better suited for trapping bacteria irreversibly. The surfaces of the μRM capsules formed from the combination of **1a** and **1b** appear much more corrugated than those studied previously (Figure 1), and we suspected that the μRM surfaces might be more effective at capturing bacteria than the solid colloids. We also felt that the open interior of the μRM might afford an irreversible capture of bacteria and much more efficient biocidal activity. As shown in Figure 2, mixing a suspension of *P. aeruginosa* with the μRM clearly results in effective capture of the bacteria before the capsules are irradiated. In Figure 2a, the live bacteria show an intense-red fluorescence while the single μRM exhibits a dull-green fluorescence; at least 14 live (and 1 or 2 dead) bacteria are associated both on the surface of the μRM and within the interior.

In our recent study with microspheres containing physisorbed **1a**, we found that the colloids entrap *C. marina* fairly effectively, and the entrapped bacteria are largely killed by 15 min exposure to visible light with a fiber-optic lamp (Fiber Lite model 190) (13). For *P. aeruginosa* with the same microspheres, there is some entrapment, but light-induced killing requires much longer exposures (up to 1 h) and even under these conditions there is only moderate killing. The surface-grafted conjugated polymer beads are somewhat more effective toward *P. aeruginosa*, but there is still much less killing than that for *C. marina*. In the present study, we find that the μRMs are much more effective at killing and capturing both *C. marina* and *P. aeruginosa*. In Figure 2b is shown a pair of μRMs that has been irradiated with the fiber-optic lamp for 15 min in a suspension containing *P. aeruginosa*. Under these conditions, there has been considerable photobleaching of the μRMs and their fluorescence is very weak. Significantly, most of the bacteria associated with the

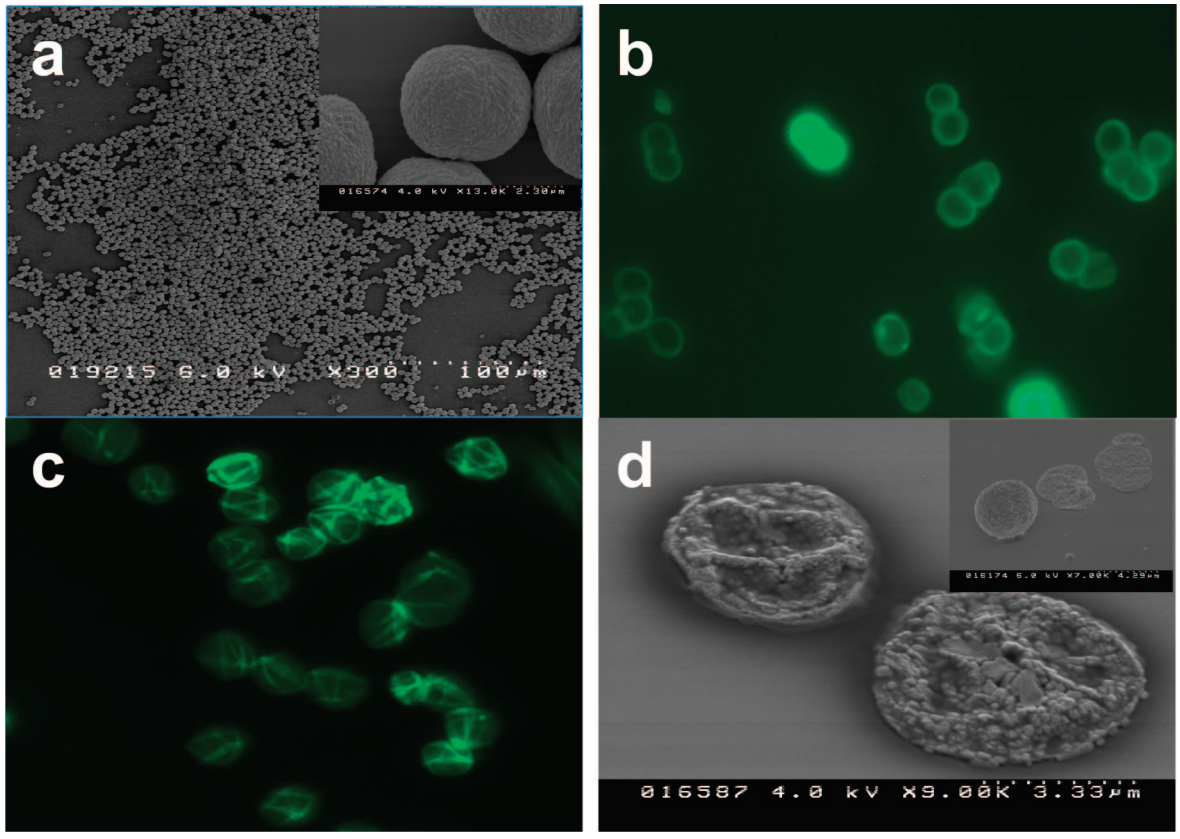


FIGURE 1. (a) SEM images of $MnCO_3$ microparticles (inset: expanded view). (b) Fluorescence microscope image of microparticles coated with four bilayers of CPEs. (c) Fluorescence microscope image of polyelectrolyte capsules. (d) SEM images of polyelectrolyte capsules (inset: large field of view).

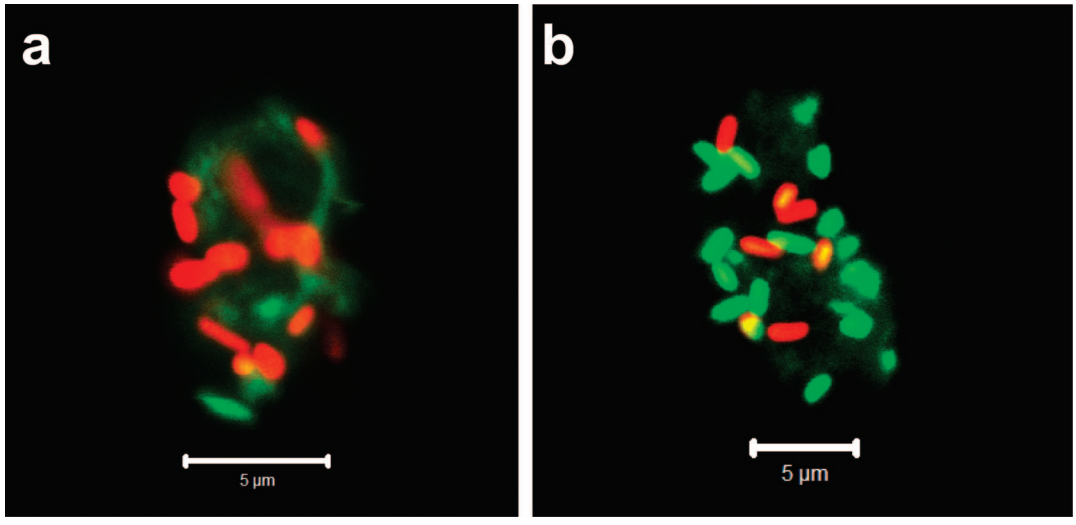


FIGURE 2. Composite confocal microscope images of μRMs with trapped *P. aeruginosa* (a) before and (b) after 15 min of irradiation with the fiber-optic lamp. Approximate live-to-dead ratios are (a) 7.0 and (b) 0.33.

μRMs exhibit green fluorescence, indicating that they have been killed by visible irradiation. (Under these conditions, irradiation of similar concentrations of physisorbed or covalently grafted polymer on solid colloids results in relatively little killing of *P. aeruginosa*.) Similar results are obtained when suspensions of μRM and *C. marina* are irradiated. Even more effective bacterial trapping and light-induced biocidal activity is observed for larger clusters of μRM, as shown in Figure 3. These clusters, typically containing 4−12 μRMs, form in the presence and absence of bacteria, as can be seen in the epifluorescence image in Figure 1c. Very prevalent are clusters of four μRMs that are approximately tetrahedral in shape; the cluster shown in Figure 3b appears to arise from fusion of two tetrahedral clusters. These μRM clusters are even more efficient in trapping bacteria than isolated μRMs. As shown in Figure 3a, one cluster exposed to a suspension of *P. aeruginosa* for 10 min in the dark has entrapped hundreds of bacteria, and almost all of the bacteria are live. As shown in Figure 3b, after irradiation with visible light for 1 h, all of *P. aeruginosa* associated with a μRM cluster both

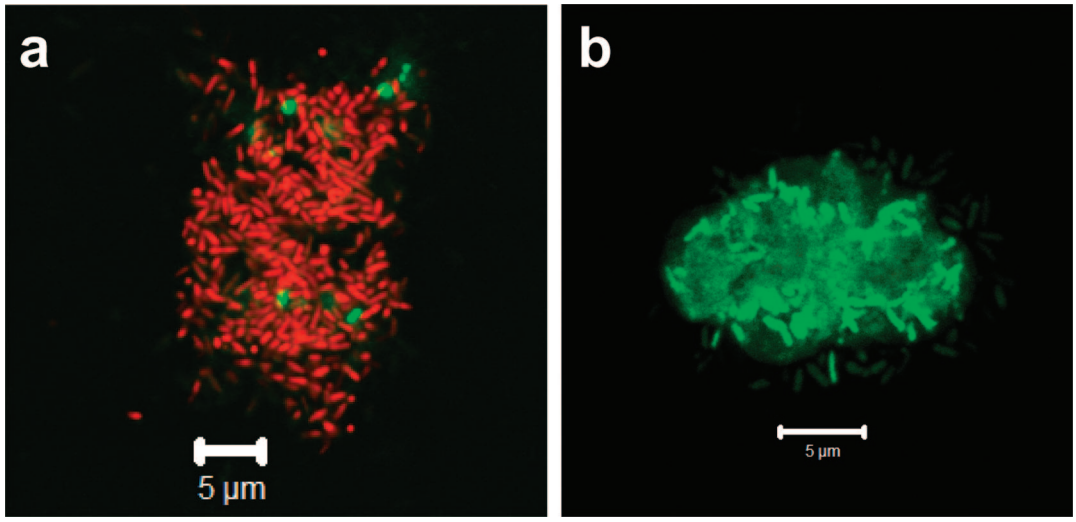


FIGURE 3. (a) Confocal microscope image of a μRM cluster 10 min after introduction into a solution of *P. aeruginosa* (107/mL) kept in the dark. (b) Interior image of a μRM cluster, showing bacteria entrapped within the cluster and killed after 1 h of exposure to white light (the entire Z-stack is shown in Supporting Information Figure S1).

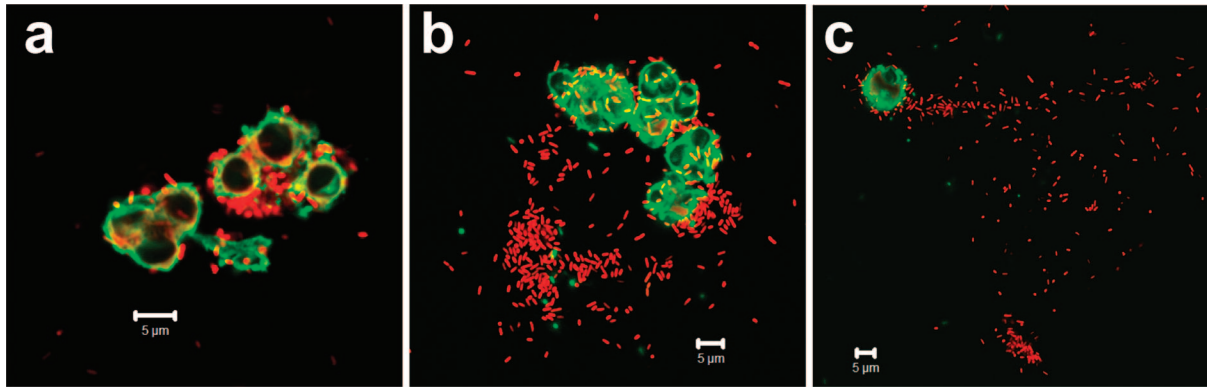


FIGURE 4. Various forms of exuded filaments or sheets with entrapped bacteria.

within and on the surface have been killed. The extent of killing of *P. aeruginosa* by irradiation of the μRM is remarkable.

We have recently shown that CPEs such as **1a** and **1b** are effective sensitizers of singlet oxygen in both aqueous and organic solutions (13). We have also determined that the light-induced antimicrobial action of colloids containing **1a** and similar cationic CPEs requires the presence of oxygen, as found in similar systems (19). This has led us to conclude that the light-activated antimicrobial activity results from the generation of singlet oxygen and likely successor reactive oxygen intermediates. We suspect that the same mechanism for the light-activated activity is operative for the μRM. What likely accounts for the more rapid and effective killing for the μRM is their greater efficiency in entrapping the bacteria irreversibly, or for longer periods of time, compared to the solid colloids. As we have discussed above, the μRM should have a positive surface potential, yet also regions of negative charge density as well as hydrophobic regions. In other work, we have noted that bacteria, including those studied here, attach in greater numbers to surfaces that contain both positively charged and hydrophobic groups. The heterogeneity of the materials comprising the μRM may thus contribute to their ability to attract and bind to bacteria. Another factor that may contribute to the irreversible nature of the binding is the potential for the material surface to reconstruct upon interaction with the bacterium.

A final remarkable effect that is observed is the time evolution of the μRM/bacteria suspensions. As documented in a series of composite confocal images shown in Figure 4, over the course of several hours after being mixed with bacteria, the μRM exude fibrillar structures (Figure 4a) that appear to be especially effective in capturing bacteria. In addition to the fibrils, sheetlike structures that are very difficult to image with the confocal microscope (due to rapid photobleaching) are present, and these structures are also very effective at capturing bacteria (see Figures 4b,c). Although the exact nature of these structures is not known at the present time, we believe that the material is likely a hydrogel consisting of the mixed CPEs.

In summary we report the preparation of novel polyelectrolyte microcapsules consisting of the photoactive CPEs **1a** and **1b**. The microcapsules display strong green fluorescence, indicating that the constituent conjugated polymers retain their photophysical properties in the capsule structure. Experiments involving mixing of suspensions of polyelectrolyte capsules with *C. marina* and *P. aeruginosa* demonstrate that the materials act as highly effective light-activated μRM. In particular, we demonstrate using live/dead assays

greater than 95% kill after exposure to ~1 h of white light. The remarkable activity of the μRM is clearly due in part to the morphology of the capsule structures, which leads to very effective capture and entrapment of the bacteria by the photoactive polymer material. In ongoing work, we seek to explore in more detail the mechanism and efficacy of these novel materials in potential antimicrobial applications.

EXPERIMENTAL SECTION

$MnCO_3$ microparticles were synthesized by modification of a literature procedure (17). In separate containers, aqueous solutions of $MnSO_4$ (1 L, 6 mM) and NH_4HCO_3 (1 L, 60 mM) were heated to 50 °C. A nanoseed solution was prepared by mixing 4 mg of NH_4HCO_3 and 0.1 mg of $MnSO_4$ in 20 mL of deionized water and stirring the solution for 10 min. Prior to the addition of the nanoseed solution, 5 mL of isopropyl alcohol (IPA) was added to each of the warm salt solutions to give a final concentration of 0.5%. Immediately following the addition of IPA, a 10 mL aliquot of the nanoseed solution was added to the $MnSO_4$ solution. The solution was vigorously stirred and the NH_4HCO_3 solution was quickly poured into the $MnSO_4$ solution with stirring. The mixture instantly turned cloudy, stirring was continued for 2 min, and then the solution was allowed to stand for 35 min. Excess aqueous solution was decanted from the precipitated $MnCO_3$ microparticles, which were then isolated by centrifugation, followed by washing three times with deionized water. A typical preparation afforded ~150 mg of $MnCO_3$ microparticles with 5 μm median diameter.

The **1a**/**1b**-coated $MnCO_3$ microparticles were prepared by a modified literature procedure (14−17, 20). $MnCO_3$ particles (155 mg) were dispersed in 11.75 mL of deionized water assisted by stirring and sonication (~10 s). A 3.25 mL aliquot of a solution of **1b** (4.61 mM) and 430 mg of NaCl was added to the $MnCO_3$ particle suspension, and the mixture was stirred for 15 min. The **1b**-coated particles were collected by centrifugation and washed three times with an aqueous NaCl solution (0.2 M). The particles were redispersed in 3.5 mL of deionized water, assisted by stirring and sonication, and the same procedure was repeated using an 11.5 mL aliquot solution of **1a** (1.3 mM). The overall process was repeated three times more to afford particles coated with four bilayers. Following CPE deposition onto the particles, the $MnCO_3$ core was dissolved by using a modified literature procedure (14, 16). An aqueous suspension of the CPE-coated particles (1.5 mL) was placed into a 3 mL centrifuge tube, and 1.5 mL of a 0.1 M EDTA solution was added. The mixture was shaken occasionally during a 2 h period to keep the particles and capsules suspended. The resulting CPE microcapsules were washed twice with deionized water and collected via centrifugation at 10 000*g* for 15 min.

The bacteria were grown in a chemostat, extracted, washed, and resuspended in a NaCl buffer. The CPE colloids were then added to the bacterial suspension and subsequently exposed to experimental conditions (13). Live/dead assays were performed using SYTO 60 and SYTOX Green stains obtained from Molecular Probes (www.invitrogen.com). These DNA stains produce red (~650 nm) and green (~530 nm) emission for live and dead bacteria, respectively. Upon completion of the experimental treatment, the stains were added as a 1:1 mixture to the bacterial samples (2 μL of mixed stains per 1 mL of suspension) and incubated for 15 min in a 0.85% NaCl solution. Cells were then examined under a 40× oil objective on a Zeiss LSM 510 Meta confocal laser scanning microscope and the number of live cells (i.e., those fluorescing red only) and dead cells (i.e., those fluorescing green) compared.

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Supporting Information Available: Confocal microscope Z-stack image set for Figure 3b. This material is available free of charge via the Internet at http://pubs.acs.org.

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