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A Thin Film Detection/Response System for Pathogenic Bacteria

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Abstract: This paper describes the modification of nonwoven fabric such that it responds by releasing an encapsulated antimicrobial from within an attached vesicle in response to two species of pathogenic bacteria (*Staphylococcus aureus* MSSA 476 and *Pseudomonas aeruginosa* PAO1), but does not respond to nonpathogenic *Escherichia coli* DH5α. This concept is based on the generalization that a majority of pathogenic bacteria secrete virulence factors such as toxins and lipases that actively damage cell membranes, typically observed as tissue damage around infected wounds, while nonpathogenic bacteria do not (or not at high concentration). The eventual aim of this work is to produce responsive dressings which release antimicrobials and change color only on infected wounds. This paper details preliminary approaches to achieving this goal, including vesicle−bacteria studies in aqueous suspension, and fluorescence imaging of fluorescein containing vesicles lysed by *S. aureus* and *P. aeruginosa*, but not by *E. coli*.

Introduction

The re-emergence of bacterial infection as a clinical problem in the late 20th century, after it was reportedly defeated by the widespread use of antibiotics from the 1950s, has initiated a growing interest in developing systems that either kill bacteria, or prevent bacterial attachment and growth. Many bacteria can attach to surfaces, where they colonize and form complex communities (termed "biofilms") that are notoriously resistant to antibiotics. This has led to the development of antimicrobial coatings containing agents such as silver or quaternary ammonium cations (QACs) that prevent/limit bacterial attachment and biofilm formation.[1] Such systems, while functional, are in essence passive as they do not respond to their local environment, and operate whether bacteria are present or not.

The rationale behind the experiments reported here, considered at a very elementary level, asks what it is that makes some bacteria pathogenic, and some not? Although this is a complex question, as an initial simplification, it is perhaps reasonable to classify common pathogenic bacteria as those that can colonize healthy tissue and damage cells by secreting enzymes and/or toxins. Pathogenic bacteria produce a large variety of toxins and virulence factors that affect host tissue in different ways. Some toxins interfere with intracellular signaling pathways in the host cells, which can have many adverse affects on function. For example, cholera toxin *Ctx* produced by *Vibrio cholerae* binds to the gut epithelium and enters the host cell via endocytosis leading to efflux of various electrolytes from the cell and causing rapid, severe dehydration.[2] Other bacterial toxins actively damage host cell membranes, either by enzymatic action (e.g., phopholipase A_2) or by forming pores (e.g., α-hemolysin and leukocidin), ultimately resulting in cell death by necrosis or apoptosis. The mode of action and structure−function relationship of membrane lipases and pore-forming toxins has been of interest for many years.[3,4] The type and concentration of toxins produced by pathogenic bacteria depends upon the particular species and strain (i.e., bacterial genotype) and the environmental conditions (nutrient supply, temperature, pH, etc.). A good example is *Escherichia coli*. While several strains of *E. coli* are highly pathogenic, including *E. coli* O157:H7, many nonpathogenic/commensal *E. coli* strains colonize the human gut as part of the normal gut microflora. In fact, some strains are beneficial, such as *E. coli* Nissle 1917 which has utility as a therapeutic probiotic agent.

Two of the most important bacteria in nosocomial infections are *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These species possess a large armory of virulence factors, including adhesins, which enable attachment to host tissues; enzymes, such as hyaluronidase, proteases and lipases, which degrade tissue components to allow deep tissue penetration; pore-forming toxins, such as α-hemolysin and leukocidin; and membrane proteins such as coagulase and Protein A that act to evade or suppress the host immune system. The two pathogenic strains considered in this paper are *S. aureus* MSSA 476 and *P. aeruginosa* PAO1. The genome sequence for both organisms has been published,[5,6] and genome mining for putative virulence factors reveals genes encoding toxins and membrane-damaging agents. For example, the *S. aureus* MSSA 476 genome carries

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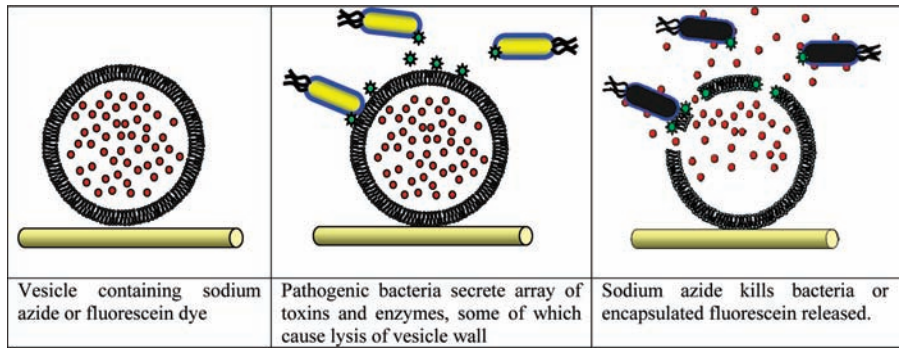

Figure 1. Schematic of responsive antimicrobial system, showing an immobilized Giant Unilamellar Vesicle (GUV) with encapsulated antimicrobial. Toxins secreted by pathogenic bacteria lyse the vesicle and release the antimicrobial, concomitantly killing the pathogenic bacteria.

several genes that encode the pore-forming toxins hemolysin and leukocidin and various lipases, while the *P. aeruginosa* PAO1 genome harbors genes for exotoxin A and hemolytic phospholipase.

There are a number of drug delivery approaches that utilize nanocapsule technology. In a recent paper, Griset et al. described the synthesis and testing of pH sensitive hydrogel nanoparticles which respond to their local environment.[7] At pH 7.4, the hydrogel nanoparticles were stable, whereas at pH 5, the polymer hydrolyzed and the particle swelled to up to six times its original diameter and in doing so released an encapsulated drug, paclitaxel, used as a chemotherapeutic agent against lung carcinomas. Mi et al. described a chitosan bilayer in a wound dressing that was reported to give a burst response, followed by slow release of the topical antimicrobial silver sulfadiazine.[8] This system has the potential advantage of combining the reported wound healing properties of chitin/chitosan with antibiotic action. However, it should be pointed out that there are reports that the presence of silver ions in wounds can actively reduce wound healing, and that silver is cytotoxic at concentrations required for it to be an effective antimicrobial.[9] A review of the potential for using polymer and lipid based carriers for prevention of biofilm formation is provided by Tamilvanan et al., though no mention was made of potential use in wound dressings, or the activation of such carriers by pathogenic bacteria.[10] Vesicles have been shown to be able to transfer drugs through skin (transdermally)[11] and to the lungs.[12] Attempts at using vesicles to deliver drugs within the bloodstream have had to combat a more difficult environment including various immune responses, both innate (e.g., complement and phagocytosis) and acquired (e.g., antibodies), that target foreign bodies.[13]

Plasma treatment of nonwoven fabrics has been explored by a number of researchers and industries. Plasma provides a simple and inexpensive methodology for changing the hydrophilicity of a fabric, for example, an oxygen plasma will make hydrophobic nonwoven polypropylene hydrophilic.[14] Recent work has also demonstrated that plasma processes can be used to modify nonwoven polypropylene with organo-silver compounds, and that these films can be used to kill bacteria including *P. aeruginosa* and *S. aureus*.[15] Pulse plasma deposition of maleic anhydride has been shown, under low power conditions, to produce an open film with retention of the anhydride ring structure.[16] This ring structure is sensitive to nucleophilic attack: leaving freshly polymerized films in water leads to hydrolysis and formation of carboxylic acids moieties, primary amines will rapidly couple, forming amide linkages.[17,18]

In this paper, we show that pathogenic bacteria can be used to be the agents of their own destruction by releasing toxins that rupture vesicles containing an antimicrobial agent. This is shown for two pathogenic species of bacteria, *P. aeruginosa* and *S. aureus*. A nonpathogenic strain of *E. coli*, which does not secrete toxins or membrane lysing agents, was used as a control system. Figure 1 outlines the experimental approach explored in this work. Pathogenic bacteria secrete membrane damaging toxins/enzymes that lyse the lipid membrane of Giant Unilamellar Vesicles (GUVs) that are either attached to a surface or floating in aqueous suspension. Epi-fluorescence microscopy was used to observe the release of fluorescent dye from surface attached vesicles. The release of sodium azide from both free-floating and surface-attached GUVs was determined by measuring the reduction in number of viable bacteria.

Methods

Synthesis and Purification of Giant Unilamellar Vesicles (GUVs). GUVs were synthesized according to the work of Moscho et al.[19] A total of 41 mg of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 2 mg of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) (Avanti Polar Lipids), and 13 mg of cholesterol were added to a vial with 1 mL of chloroform. A 20 μL aliquot of this solution was added to a 50 mL round-bottomed flask containing 980 μL of chloroform and 150 μL of methanol.

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To a clean vessel, 100 mg of sodium azide (15 mg mL^{-1}) or fluorescein (final concentration 5 mmol dm^{-3}) was dissolved in 6.65 mL of Tris buffer pH 7.4. The stock azide or fluorescein mixture was then vortexed briefly and carefully added to the round bottomed flask containing the lipids. The resultant mixture was rotary-evaporated for 2 min at 40 °C. After the 2 min, the liquid in the round-bottom flask was observed to be a faint opalescent fluid which contained the GUVs, which were subsequently purified by passing through NAP-10 columns to remove nonencapsulated sodium azide or fluorescein.

Microbiology: MIC, Antimicrobial Vesicle Activity and Fluorescein Release. The minimum inhibitory concentration to kill 50% of the bacterial population (MIC_{50}) of sodium azide against the three bacterial strains, *S. aureus* (MSSA 476), *P. aeruginosa* (PAO1), and *E. coli* (DH5α) was determined as follows. Bacteria were grown overnight in 10 mL of LB, 37 °C, 220 rpm. A total of 100 μL of the overnight culture was subcultured in 10 mL of LB, 37 °C, 220 rpm and grown to exponential phase ($OD_{600} = 0.3-0.4$), and 100 μL was then inoculated into LB broth containing various concentrations of sodium azide. Cultures were then grown for 18 h, 37 °C, 220 rpm and bacterial growth was determined by measuring the optical density at 600 nm.

For the vesicle lysis/release measurement, 100 μL of bacteria at a concentration of ca. 10^9 cfu mL^{-1} in LB was added to a suspension of 900 μL of vesicles. Every 20 min, the bacteria concentration was determined by standard plating assays and colony counting. Vesicles containing encapsulated fluorescein were immobilized on a polystyrene Petri dish (vida infra) modified with a film of plasma deposited maleic anhydride. The GUVs were imaged using a Nikon eclipse TE2000-S epi-fluorescence microscope.

Plasma Deposition of Maleic Anhydride and Vesicle Attachment. The utility of plasma deposited maleic anhydride (pp-MA) for attachment of proteins, DNA, or other material has been detailed by others.[20] The plasma deposition system uses a copper coil to couple rf power via an inductance matching system to an evacuated tube terminated with stainless steel glass flanges that are connected to earth. The design of the reactor is similar to that of Short et al. and has been described elsewhere.[21] A home-built pulse generator allowed for either continuous (CW) or pulsed plasma treatment. The modulation is typically described by the duty cycle ($dc = t_{on}/(t_{on} + t_{off})$) which is generally in the order of milliseconds. Maleic anhydride powder was freeze-dried three times just prior to deposition to remove adsorbed water and then deposited using low power 1/40 at 50 W input power to afford the a high retention of anhydride and ring structure in the deposited film.

Freshly prepared pp-MA modified nonwoven polypropylene squares (2 × 2 cm) or polystyrene Petri dishes were removed from the reactor and immediately placed in 2 mL of the GUV suspension. The substrates were left for 1 h, then removed and rinsed three times in PBS buffer to remove most noncovalently bound vesicles. The GUV modified surfaces were then tested for their response to the three species of bacteria, with the sodium azide containing vesicles using the JIS 1902 antimicrobial assay and the Petri dishes modified with fluorescein encapsulated vesicles for microscopy. All subsequent measurements of modified surfaces were made within 24 h of conjugation, since these vesicles had not been optimized for stability and it was important to prevent the modified surfaces from drying. Stabilization of the vesicles to air is the subject of ongoing research.

Measurement of Antimicrobial Activity from Vesicle Modified Nonwoven. This assay is based on the Japanese Industry standard (JIS 1902) for determination of the antimicrobial efficacy of treated fabrics. Vesicle conjugated polypropylene nonwoven squares, 2 cm × 2 cm, were rinsed in Milli-Q water and then inoculated with 200 μL of an overnight bacterial culture, diluted to 10^5 cfu mL^{-1} in LB media. Untreated control samples of nonwoven were UV sterilized and treated in an identical way. The fabric squares were either incubated at 37 °C for 4 h, or were analyzed immediately to determine the concentration of adherent bacteria. In either case, the fabric square was rinsed in physiological saline (0.9% NaCl w/v), placed in 5 mL of physiological saline and vigorously vortexed for 5 × 5 s to remove the adherent bacteria. The saline/bacterial solution was then spread on LB agar at four different dilution factors and incubated at 37 °C for 18 h. Bacterial colonies, theoretically originating from a single bacterial cell termed "colony forming unit" (cfu), were counted and the original bacterial concentration determined.

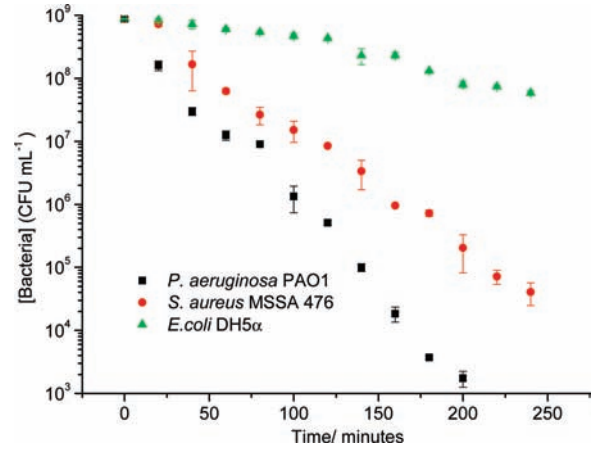

Figure 2. Reduction in the number of viable bacteria (cfu/mL) following exposure to vesicles containing sodium azide in aqueous suspension, over a 4 h period. The strains tested were the pathogenic *P. aeruginosa* PAO1 and *S. aureus* MSSA 476 and the nonpathogenic *E. coli* DH5α ($n = 3$).

Results and Discussion

Time Resolved Bacterial Killing by Vesicles in Suspension. Measurements of the bacterial mediated vesicle lysis (and subsequent self-destruction) were made by sampling and plating out the viable bacterial population every 20 min following exposure to vesicles containing sodium azide. The results, presented in Figure 2, clearly show a decrease in the concentration of viable *P. aeruginosa* and *S. aureus* over 4 h. A simple linear fit to the data in Figure 2 suggests that *P. aeruginosa* is being reduced in concentration at a rate of 0.12 cfu mL^{-1} min^{-1}, while the concentration of *S. aureus* is declining at a rate of 0.03 cfu mL^{-1} min^{-1}. The shape of the bacterial optical density-sodium azide concentration graphs (shown in the Supporting Information, Figure 2) gives a clue to this behavior. *P. aeruginosa* starts to respond (declines in concentration) to sodium azide at a concentration of 20 μg mL^{-1} and is almost completely inhibited at concentrations of 200 μg mL^{-1}; in contrast to *S. aureus*, which only becomes sensitive to sodium azide at 100 μg mL^{-1} and needs 500 μg mL^{-1} for complete inhibition. If sodium azide is being slowly released at a constant rate in both cases, the rate at which *P. aeruginosa* is inhibited will therefore be greater than *S. aureus* at low concentrations of sodium azide. This difference in rate appears to superficially correlate with the difference in MIC of the two bacteria, although this may be no more than an apparent correlation since the factors which affect the rate of concentration decrease will also depend on the expression levels of virulence factors (which lyse the vesicles) from the two bacterial species, which are not known.

In contrast, there was a much lower decrease in the number of *E. coli*, despite this strain being the most sensitive to sodium

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Table 1. The Minimum Inhibitory Concentration (MIC_{50}) of Sodium Azide against the Strains of Bacteria Used in This Study ($n = 3$)

bacteria species	MIC_{50}
P. aeruginosa PAO1	44 μg mL^{-1}
S. aureus MSSA 476	186 μg mL^{-1}
E. coli DH5α	34 μg mL^{-1}

azide (Table 1). We can therefore conclude that the pathogenic bacteria are rapidly killed/inhibited by sodium azide released from the vesicles, which are specifically lysed by membrane-damaging exotoxins produced by the pathogenic strains.

Attachment of Vesicles to pp-MA Nonwoven Polypropylene. The utility of vesicles as anti-infective agents in wound dressings requires that they can be attached to fabric. For this part of the study, nonwoven polypropylene was coated in a plasma with a thin film of maleic anhydride under 1 ms/40 ms pulse conditions, to ensure a high degree of ring retention. The anhydride ring is sensitive to nucleophilic attack by primary amines.

Analysis of Attached Vesicles. The FTIR spectra (Supporting Information, Figure 1) shows polypropylene nonwoven, maleic anhydride ring retention following pp-MA deposition, and effect of adding vesicles to pp-MA modified nonwoven. The polypropylene shows the expected C−H bend features. The pp-MA modified polypropylene exhibits the expected anhydride group C═O absorption at 1780 and 1850 cm^{-1}. Attachment of the lipid vesicles to the pp-MA nonwoven shows a strong, broad adsorption at 1630 cm^{-1}, resulting primarily from amide C═O in the reacted MA and from ester C═O in the fatty acid chains of the phospholipids.

Bacterial Control by Attached Vesicles on pp-MA Modified Polypropylene. Having successfully immobilized vesicles on the pp-MA modified nonwoven, the next part of the investigation looked at studying the antimicrobial performance of the modified fabrics against the two pathogenic bacteria (*P. aeruginosa* PAO1 and *S. aureus* MSSA 476) and a nonpathogenic control (*E. coli* DH5α). The control gives important information on whether the fundamental hypothesis is correct: pathogenic bacteria lyse the vesicles and ensure their self-destruction, but nonpathogenic bacteria are largely unaffected.

The antimicrobial performance of the sodium azide containing vesicles attached to fabrics was tested using the methodology described earlier. The results in Figure 3 show that the vesicle modified nonwoven fabric completely inhibits both pathogenic species of bacteria (*P. aeruginosa* and *S. aureus*), while the nonpathogenic *E. coli* (which does not secrete membrane damaging toxins) colonized the vesicle-modified fabric to almost the same extent as the nonmodified control. It is likely that some passive leakage of sodium azide limited the growth of *E. coli* to an extent. The *E. coli* control is therefore useful as a measure of the 'leakiness' of the vesicles containing sodium azide. This is being used in current and future research into improving the stability and reducing passive leakage from attached vesicles.

Fluorescence Microscopy of Vesicle Lysis by Bacteria. Fluorescence microscopy was used to provide further confirmation of the hypothesis that the pathogenic bacterial strains lyse vesicles, while the nonpathogenic strain does not. The advantage of GUVs is that they can be easily visualized, especially if containing a water-soluble dye such as fluorescein. The results shown in Figure 4 clearly show individual immobilized vesicles

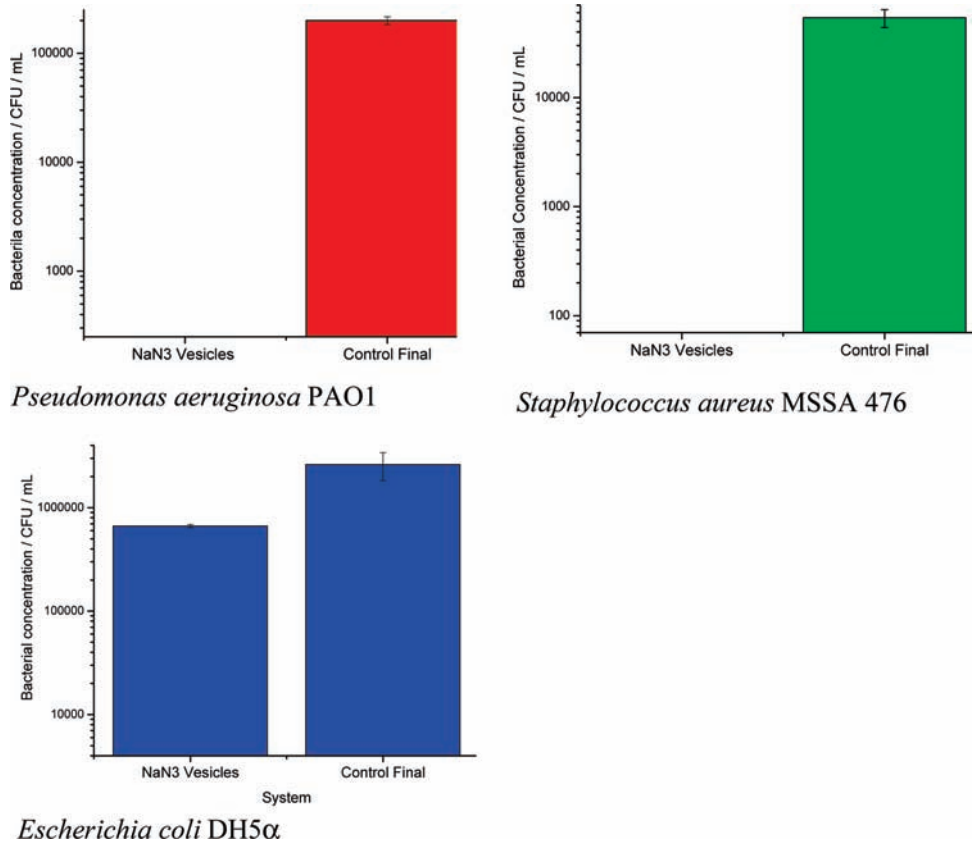

Figure 3. Persistence and growth of two pathogenic (*S. aureus* MSSA 476 and *P. aeruginosa* PAO1) and one nonpathogenic (*E. coli* DH5α) strains of bacteria on fabric modified with vesicles containing sodium azide (NaN_3 vesicles) in comparison to nonmodified, UV sterilized nonwoven (control), over a 4 h period. The pathogenic bacteria secrete membrane damaging toxins which lyse the vesicles, releasing sodium azide at a concentration that inhibits bacterial growth, with total clearing of *S. aureus* and 2 cfu mL^{-1} of *P. aeruginosa* being measured after 4 h.

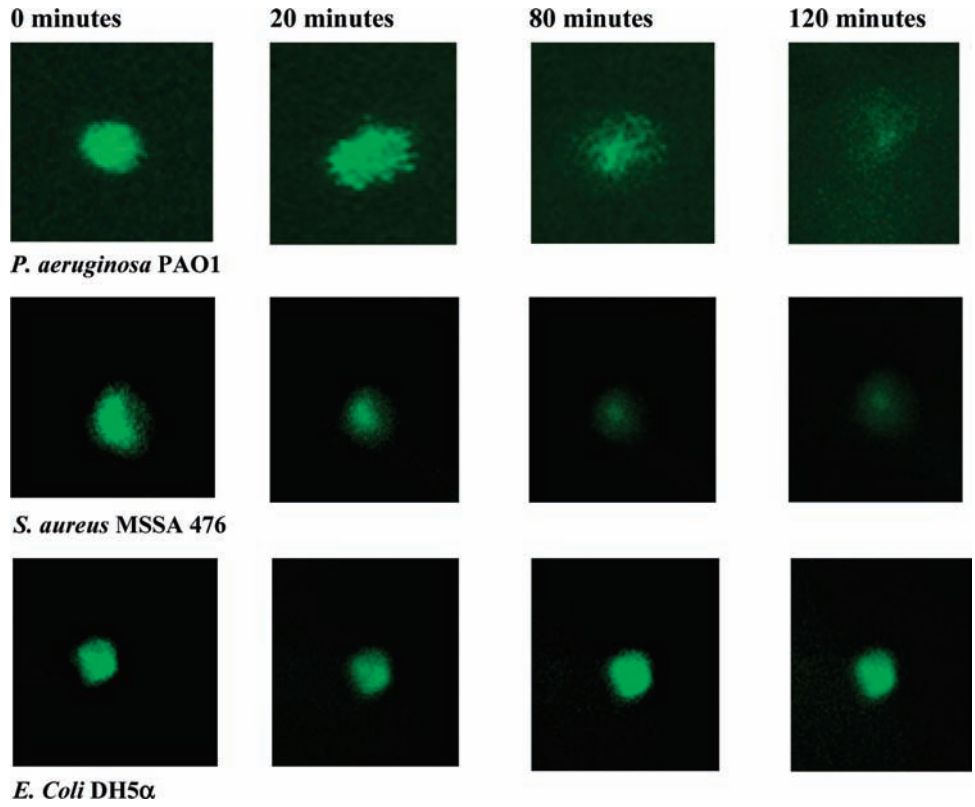

Figure 4. Fluorescence imaging of surface attached vesicles exposed to the three species of bacteria. Box width 60 μm.

on pp-MA modified Petri dishes, and subsequent lysis on addition of *P. aeruginosa* and *S. aureus*, but not following addition of *E. coli*. There were interesting qualitative difference in the images of the vesicles being lysed by *P. aeruginosa* compared with *S. aureus*. *P. aeruginosa* appears to have caused the vesicle to initially swell, before loosing its encapsulated dye, possibly within smaller lipid structures; the vesicles exposed to *S. aureus* gradually lost fluorescence intensity, but in a way that is suggestive of slow release of dye rather than swelling. This difference could correspond to differences in the mode of action of the different virulence factors secreted by *P. aeruginosa* compared with *S. aureus*.

Discussion and Conclusions

In this paper, we have demonstrated that a simple vesicle system can be used as a 'Trojan horse' for controlling bacterial growth and infection. The ultimate aim of this work is to attempt to engineer a 'smart' wound dressing system that only releases an encapsulated antimicrobial agent in the presence of pathogenic bacteria, without responding to commensal/harmless bacteria. This is particularly desirable given the importance of the normal microflora in providing a natural defense against infection.[22] Furthermore, by ensuring antibiotics are released only in response to pathogenic bacteria, this will minimize the evolutionary pressure for the selection of antibiotic resistance and prolong the efficacy and shelf life of the encapsulated antimicrobial.[23] It is intended that a simple colorimetric or fluorometric response could also be built into the device, giving patients or clinicians early warning of wound infection. Important problems regarding vesicle stability, tuning of response, manufacturability, and immunological response remain to be solved. It is unlikely, for example, that GUVs would be used in a final product, though part polymerized acetylene fatty acid/phospholipid vesicles may find utility, this being the subject of current research.

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Supporting Information Available: FTIR data for the plasma deposition and subsequent attachment of vesicles on polypropylene nonwoven; MIC graphs for sodium azide against the test species/strains of bacteria; data from figure 3 in tabular form. This material is available free of charge via the Internet at http://pubs.acs.org.

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