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Decontamination of surfaces by lysozyme encapsulated in reverse micelles

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Abstract

Cells and enzymes can be used to decontaminate soil, water supplies, personal equipment, weapons and hospital equipment that have been exposed to bacteria, toxins or viruses. One of the problems associated with the use of microorganisms and enzymes for decontamination purposes is that the presence of water is not acceptable for some applications such as electronic equipment. One way of circumventing this problem is to allow the enzyme to distribute between a water phase and an organic phase-containing surfactant and then use the encapsulated enzyme in reverse micelles directly into the device to be clean. Reverse micelles were used to deliver the enzyme (lysozyme) to the cell–surface interface. They serve as a way to increase the local concentration of lysozyme and decrease the amount of water delivered. Specifically, we explored the lysis by free lysozyme and lysozyme encapsulated in reverse micelles of *Klebsiella pneumoniae* and *Staphylococcus epidermidis* attached to steel, glass, and hydroxyapatite. These two bacteria have been selected because they are known to be pathogenic and because of their differences in cell wall structure. Lysozyme was added to the surfaces in either reverse micelles or as a free solution and was tested under conditions of stirring and no stirring. Stirring was implemented to study the interplay between mass transfer limitations and surface roughness. We have shown that free lysozyme or lysozyme encapsulated in reverse micelles is capable of decontaminating surfaces of different texture. Lysis of the cells is slower when the encapsulated enzyme is used but lysis is more complete.

Keywords: Reversed micelles; Enzyme kinetics; Scintillation counting; Dynamic light scattering; Lysozyme

1. Introduction

One of the problems associated with decontamination methods that use aqueous solutions is that water may damage costly equipment. A solution to this problem is to encapsulate the decontamination agent, for example, an enzyme, in reverse micelles, thus minimizing the use of water. Currently, reverse micelles are used in a number of biotechnological applications that include the enzymatic transformation of water insoluble substrates, the mimicking of biological structures, and in the understanding of basic problems of structural biochemistry [1]. In addition, because their inner water core dispersed in organic solvent is able to solubilize polar substances, they have been used to purify enzymes and minimize their denaturation [2].

The use of encapsulated enzymes for decontamination purposes can be view as a complex sequence of separation steps combined with a heterogeneous enzymatic reaction. In the first step, the enzyme needs to be sequestered by the reverse micelles. In this step, the enzyme is fractionated between the micelles core and the water phase. The second step consists of the delivery of the encapsulated enzymes to the contaminated surface. In this step, the enzyme partitions between the reverse micelles and the adsorbed (microbial) phase. Finally, the cells exposed to the enzyme are disrupted and their fragments distribute between the organic phase and the surface. Although the aim of the protocol is not the fractionation of any biological product, the partitioning of the enzyme into the reverse micelles and their partitioning between the reverse micelles and the surface are governed by the same principles that govern the distribution of proteins between two liquid phases.

The overall liquid–liquid extraction process by reverse micelles is performed, under vigorous stirring conditions, by forward extraction of the target protein from an aqueous solution to a reverse micellar organic phase followed by back-extraction, or stripping, of the target protein from the reverse micelles into a fresh aqueous phase [3]. The back-extraction is the phenomenon of concern when trying to deliver an enzyme encapsulated in reverse micelles onto

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a surface. The forward extraction is controlled primarily by electrostatic interactions between the charged protein and the micellar inner wall. However, it is also possible to perform forward extractions governed by hydrophobic interactions between the apolar regions of the molecule and the surfactant tail [4]. Transfer of the protein only occurs when the pH of the aqueous phase is such that the protein surface has a net charge opposite to that of the surfactant head groups and the ionic strength is low. On the contrary, to back-extract the protein, the working pH must be such that the protein has the same charge as the surfactant and the ionic strength must be high. The repulsive forces between equal sign charges on the protein and the surfactant diminish the micellar diameter causing the release of protein from the reverse micelles. The difference in ionic strength is necessary because it has been proven that low ionic strength favors protein transfer into reverse micelles and high ionic strength promotes its release [2]. Departure from these conditions causes a considerable decrease in the amount of enzyme recovered by back-extraction.

Conditions of high pH and high ionic strength are unlikely to be found in bacteria films. Thus, we expect a low delivery rate from the reverse micelles into the biofilm. However, a low delivery rate can be compensated by higher loads of the enzyme into the reverse micelles. If the preparation is sprayed as a thin film, the organic solvent will evaporate allowing the enzyme and the surfactant to reach the surface of the cells. The use of an encapsulated enzyme has some obvious advantages over the use of a free enzyme. Reverse micelles serve as a way to increase the local concentration of lysozyme and decrease the amount of water delivered. As stated earlier, large amounts of water can cause corrosion to the surfaces where contamination is likely to occur. It is also known that enzymes are more stable in the reverse micelles at room temperature; so, they can be prepared, stored, and ready for use immediately [3]. Irreversibly bind of the surfactant and the enzyme is one possible drawback since the enzyme may loose its activity.

There has been an increased interest in decontamination methods for civilian and military sectors since the occurrence of the 9/11 episode. For hospitals, at a minimum, FDA regulations state that for devices that do not penetrate the skin or come in contact with normally sterile areas of the body, such as several types of endoscopes, must be disinfected, with an EPA-registered and FDA-cleared disinfectant. The disinfectant selected must be of the appropriate strength to kill the types of organisms that may contaminate the particular device. For many years, glutaraldehyde has been the disinfectant solution of choice within healthcare facilities. This non-corrosive solution does not damage tools such as endoscopes, and is highly resistant to neutralization by organic soil. However, a glutaraldehyde solution is toxic and poses various health risks to the people who use it. Instead, becoming more widely used, especially in endoscopy centers, are two disinfectant solutions known as Cidex OPA and Rapicide with are less toxic [5]. In order to reduce the common airborne infections in hospitals, various strategies such as improved maintenance and system cleaning, increased ventilation, “source control”, and ultraviolet germicidal irradiation are used. HVAC maintenance cleaning, often inaccurately described as “duct cleaning”, appears to have considerable value in reducing airborne microorganism concentrations whereas “source control”, which in this case refers to isolating infectious patients, is already in practice in virtually every hospital, and carries a substantial price. Individually housing patients in highly ventilated and filtered, segregated rooms is an expensive proposition [6].

Some recent investigations on containment and destruction of biologically contaminated objects can be found in the works of McDonald et al. [7], Hermann et al. [8] and Raber and McGuire [9]. McDonald et al. investigated the use of ultraviolet light and a chemical photosensitizer for disinfecting surfaces. Although their long-term goal is to apply their method to decontaminate surfaces, such as roads or airport runways, their report covers work performed mainly on solutions. In their method, a photosensitizer is sprayed on a surface or added to a solution and then the surface is illuminated with UV light, thus killing the bacteria. Hermman et al. have used a novel low temperature atmospheric pressure plasma source to produce chemically reactive fast-flow effluents that are capable of rapidly destroying a broad spectrum of chemical and biological warfare agents. The key to their approach is the use of an atmospheric pressure plasma jet, which produces short-lived reactive species and propels them onto the contaminated surface. Along similar lines, Farrar et al. [10] explored the effectiveness of two existing plasma sources in the destruction/killing of biological spores. They used two different technologies: a steam plasma torch and an arcjet thruster. Their objective was not to sterilize the entire sample surface but to significantly reduce the number of spores so that the health risk would be lowered for individuals exposed to cross-contaminated surfaces. More successful have been Raber and McGuire who developed a single reagent, which can be used in a commercial paint sprayer, effective against both chemical and biological warfare agents. The reagents name is “L-Gel” and is a mixture of a commercially available oxidizer known as “Oxone” and a silica gelling agent Cab-O-Sil EH-5. Their method explores acidic oxidation to facilitate hydrolysis of an agent at greater concentration and eliminates the need for a preliminary high pressure wash and the typical hydrolysis reaction which is the common approach used in chemical warfare decontamination of military areas. L-Gel is environmentally friendly, relatively non-corrosive, easy to apply, provides maximal contact times, and requires low maintenance. It is also readily available and relatively cheap.

In our approach, we investigated the use of a commercially available enzyme, lysozyme, both as a free solution and encapsulated in reverse micelles to decontaminate surfaces of different texture and hydrophobicity that have been contaminated by two different bacteria. The three substrate materials we chose (glass, stainless steel, and hydroxyapatite)

represent surfaces that are likely to be contaminated in wither civilian or military installations whereas the two bacteria strains we chose are *Klebsiella pneumoniae* and *Staphylococcus epidermidis*, which are common in hospital contamination.

Staphylococci are non-motile, Gram-positive cocci about 1 μm in diameter. They are spherical cells that form irregular clusters, like bunches of grapes. Klebsiella are rod-shaped (bacillus), non-motile, Gram-negative bacteria that have a large polysaccharide capsule and are aerobic. We have chosen one bacterium from each type because Gram-negative cells are supposed to be resistant to lysozyme attack whereas Gram-positive bacteria are susceptible to degradation by lysozyme.

S. epidermidis is a normal inhabitant of human skin and mucosal surfaces but may spread to the blood through breaks in mucosal or skin integrity. *K. pneumoniae* is typically found in the intestinal tract of humans but can also be found in soil, water, and grain. Both bacteria are commonly found in biofilms on catheters or other artificial appliances and have been connected to the existence of urinary tract infections. Moreover, *K. pneumoniae* is connected with causing sepsis in patients and is found most often when patients have been on antibiotics.

Lysozyme (peptidoglycan *N*-acetylmuramoylhydrolase) has a molecular weight of 14,300 and a p*I* of 11.35. It is active from pH 6.0 to 9.0 and over an ionic strength range from 0.01 to 0.1M, depending on the pH. The enzyme works by hydrolyzing the β(1 → 4) glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid in the polysaccharide backbone of peptidoglycan. The way in which lysozyme lyses bacteria is by hydrolyzing the peptidoglycan present in bacterial cell walls. It is commonly used in the lysis of microbial cells that have Gram-positive bacterial cell walls because they contain a large amount of peptidoglycan. Gram-negative bacteria are less prone because they contain less peptidoglycan and are further protected by their outer membrane. However, they can be made more susceptible to lysis by lysozyme with the addition of EDTA which chelates metal ions present in the outer membrane [11].

We demonstrate in this paper that lysozyme, either free or encapsulated in reversed micelles can be efficiently used for the decontamination of surfaces of various textures that have been contaminated by Gram-positive and Gram-negative bacteria. Under appropriate conditions, up to 98% of the deposits can be removed.

2. Experimental

2.1. Materials

Chicken egg white lysozyme, isooctane, and bis(2-ethylhexyl)sulfosuccinate sodium salt were purchased from Sigma (St. Louis, MO). Scintillation fluid and scintillation vials were purchased from ICN (Costa Mesa, CA). *K. pneumoniae* and *S. epidermidis*, were provided by Dr. David J. Westenberg (Biology Department, University of Missouri-Rolla). The hydroxyapatite discs were purchased from Clarkson Chromatography Products Inc. (South Williamsport, PA). The stainless steel washers were obtained from a local merchant and the glass beads were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Methods

Strains of *K. pneumoniae* and *S. epidermidis* were maintained by weekly transfers on brain heart infusion agar plates kept at 37 °C. Tubes containing 6 ml of brain heart infusion broth were inoculated with each strain and grown at 37 °C in an incubator/shaker for 15 h. An amount equal to 0.125 ml of each of the resulting cultures was used to inoculate vials containing 2.5 ml brain heart infusion broth and a piece of substrate that had been weighed and the mass recorded. The three substrates used in these experiments were glass beads, hydroxyapatite discs, and stainless steel discs. The samples were covered and incubated at 37 °C on an orbital mixer rotating at 20 rpm. Cell attachment was allowed to take place for 48 h with a change in medium at the 24 h mark. ^{3}H-thymidine was also added at the 24 h mark to radiolabel the bacteria in their latter exponential growth phase.

The reverse micelles were prepared according to the procedure of Kinugasa et al. [12]. Solutions of 0.05 M bis(2-ethylhexyl)sulfosuccinate sodium salt in isooctane and 10 mg/ml lysozyme in phosphate buffer solution, pH 7, containing 0.3 M KCl solution were prepared. Equal amounts of the two solutions were mixed on a stir plate on high speed for 1 h to allow extraction of the lysozyme into the reverse micelles. The dispersion was centrifuged for 30 min at 2000 × *g*. The top phase, which contains the enzyme encapsulated in the reverse micelles, was kept.

The size and size distribution of the reverse micelles were determined by dynamic light scattering (DLS). The DLS experiments were performed on a fiber-optic quasi-elastic light scattering (FOQELS) instrument from Brookhaven Instruments Corp (Brookhaven, NJ). The FOQELS uses an 800 nm solid state laser (100 mW) with a fixed scattering angle of 155° and a digital auto-correlator whose delay time intervals are not linearly spaced which allows broad distributions to be sampled properly. The sample was poured into a 4.5 ml rectangular plastic cuvette and placed inside a constant temperature holder which is capable to control the temperature in the range of 5–75 °C on steps of 0.1 °C. The temperature was fixed at 22 °C for all measurements.

The quantity measured in DLS is the auto-correlation function and is used to determine the diffusion coefficient of the scatters. The equivalent sphere diameter (ESD) or Stokes diameter of the scatters is obtained from the diffusivity assuming that the scatters are spherical. Particle size can be related to the diffusivity, *D*, for simple common shapes like a sphere, ellipsoid, cylinder or random coil. For a uniform

sphere, the Stokes–Einstein relation is:

$$D = \frac{k_B T}{3\pi\eta D_h} \quad (1)$$

where k_B is the Boltzmann's constant, T the temperature, η the viscosity of the liquid in which the particle is moving, and D_h the hydrodynamic diameter. When a distribution of sizes is present, the effective diameter can be obtained by averaging the intensity-weighted, the number-weighted, or the volume-weighted diameters. In FOQELS, a multi-modal size distribution (MSD) analysis is available through the non-negatively constrained least squares (NNLS) approach developed by Grabowski and Morrison [13].

The encapsulated enzyme was back-extracted into an aqueous solution to determine the activity of lysozyme recovered from the reverse micelles. Back-extraction was performed by mixing the reverse micelles with alkaline water, pH 11.5, containing 1.0 M KCl for 1 h. The mixture was again centrifuged and the water phase kept. The activity of back-extracted lysozyme was compared with the activity of untreated lysozyme. Enzymatic activity was determined by the assay described in Imoto and Yagishita [14] in which the hydrolyzes of sugars of glycol chitin by lysozyme is monitored at 420 nm.

Release of lysozyme from the reverse micelles was monitored by layering the micellar solution on top of water. An amount equal to 1.5 ml of deionized water or alkaline water was placed in a quartz cuvette. A micellar suspension was carefully layered on top of the water using a pipette. The two-phase system was left standing and the release of lysozyme from the micelles into the aqueous phase was monitored at 280 nm for up to 24 h using a Spectronic Genesysis spectrophotometer.

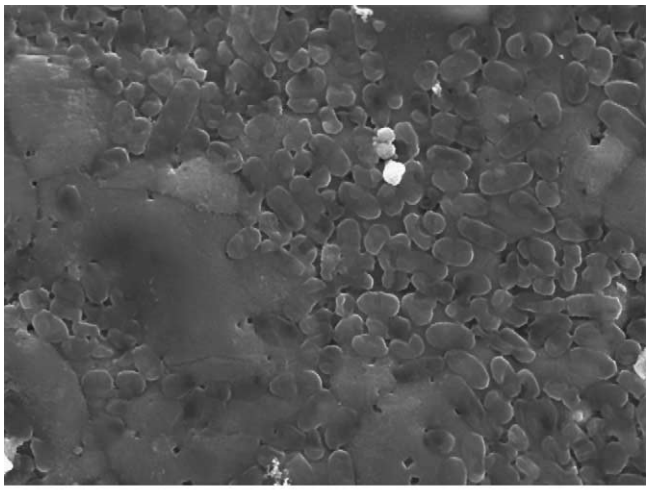
Lysis of bacteria attached to each substrate was studied under various conditions. After the 48 h cell attachment period, 0.5 ml of the media was removed and centrifuged for 5 min. The bacterial pellet was kept and dissolved in 0.3 ml of water and sonicated for 30 min. This was done to ensure that complete release of tritium from the bacteria was achieved. An amount equal to 0.3 ml of the pellet supernatant was also kept. The remaining media was removed and discarded from each vial and 1.0 ml of free lysozyme or of the encapsulated enzyme was added. Half of the samples were incubated with the enzyme without stirring whereas the other half was stirred on an orbital mixer at 20 rpm. Two different sets of decontamination experiments were performed; each corresponds to a different sampling time scale. In one set, samples were removed from the vials at 5 min intervals for a total of 15 min when free lysozyme was added or in 10 min intervals for a total of 30 min when reverse micelle encapsulated lysozyme was added. In the second set, samples were removed at 15 s, 1 min, and every minute after that for a total of 5 min with an additional sample taken at the 15 min mark under the conditions of no stirring or at 1, 2 min and every 2 min after that for a total of 10 min with one last sample taken at 20 min for an end point value when stirring was included. For the experiments performed at shorter lysozyme exposure times, stirring was performed using a vortex mixer because mixing with the orbital mixer was too strong. One hundred microliters samples were removed from each vial at regular intervals. After the targeted incubation time with the enzyme was reached, 15 min for free lysozyme and 20 min for the encapsulated lysozyme, the remaining lysozyme was removed, 0.3 ml of toluene was added to each substrate and then the samples were sonicated for 30 min. 0.3 ml of scintillation fluid was added to all samples (substrates, dissolved pellets, and supernatants) and then the samples were counted in a Beckman 7600 liquid scintillation counter. The results of lysis of the bacteria attached to each substrate are expressed as the percent of the deposit removed.

3. Results and discussion

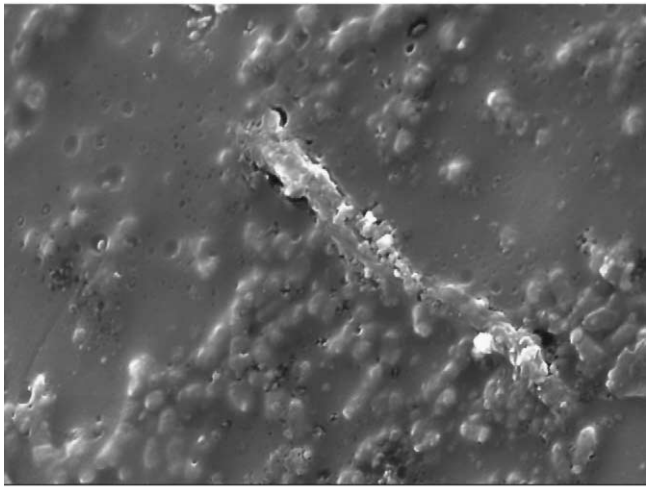
Before the extent of the decontamination of surfaces achieved by using the enzyme can be assessed, the attachment of the bacteria to each substrate needs to be confirmed. *K. pneumoniae* and *S. epidermidis* were allowed to attach to a piece of substrate (glass, steel, and hydroxyapatite) for 48 h. This growth period is sufficient because we are interested in the decontamination of sites that have been very recently contaminated or in sites that are commonly contaminated.

Verification of *K. pneumoniae* and *S. epidermidis* attachment to each substrate was done by visual inspection of the deposits using scanning electron microscopy. The complete procedure followed can be found in Bermudez and Forciniti [15]. In short, a Hitachi S570 scanning electron microscope was used with the resultant images recorded in Adobe Photoshop. All images were captured at 3500× and represent an area of about 16 μm^2 of the actual substrate. Fig. 1 is representative of *K. pneumoniae* and *S. epidermidis* attachment onto hydroxyapatite and glass in the allotted 48 h incubation period. The micrographs clearly indicate that cell attachment is occurring in the time allotted and to an extent sufficient for the investigation of their destruction or lysis by lysozyme. From these micrographs, we also see that hydroxyapatite has the most porous surface, resulting in the substrate with the highest surface area and hence cell attachment, and that glass and stainless steel look nearly the same. Fig. 1b shows that the colonies of *S. epidermidis* are covered by a slime-type of substance; this is typical for this microorganism.

Because the main focus of our work consisted of using the enzyme encapsulated in reverse micelles, the size of the micelles as well as the activity of lysozyme trapped in the micelles needed to be determined. The activity of lysozyme in the reverse micelles was determined by the assay of Imoto and Yagishita [13]. Eighty percent of the activity of lysozyme was recovered after the enzyme was kept in the reverse micelles for 1 h. These values compared well with the values reported by Kinugasa et al. [12]. Of



(a)



(b)

Fig. 1. Series of micrographs showing different types of deposits of both microorganisms on the three substrates: (a) *K. pneumoniae* on hydroxyapatite, notice that the surface is about 50% covered by the bacteria; (b) *S. epidermidis* on glass, the bacteria seem to deposit in isolated clusters and to be covered by a slimy coating.

course, this test does not guarantee that the enzyme is going to be released into the biofilm. However, it does ensure that lysozyme was left active when inside the micelles.

The size of micelles and their size distribution were determined by DLS. Fig. 2 shows the histogram that represents the size distribution. The preparation is bimodal with two predominant species of sizes 10 and 31 nm. The smaller size represents 90% of the signal by volume.

We need to compare the results obtained by using the encapsulated enzyme with those obtained by using free lysozyme to evaluate the efficiency of the reverse micelles system to kill attached cells on the various substrates. Therefore, the efficiency of free lysozyme to lyse attached bacteria is discussed first. Our findings for bulk lysozyme are summarized in Fig. 3, which shows that the percentages of broken cells for each bacterium are similar for all

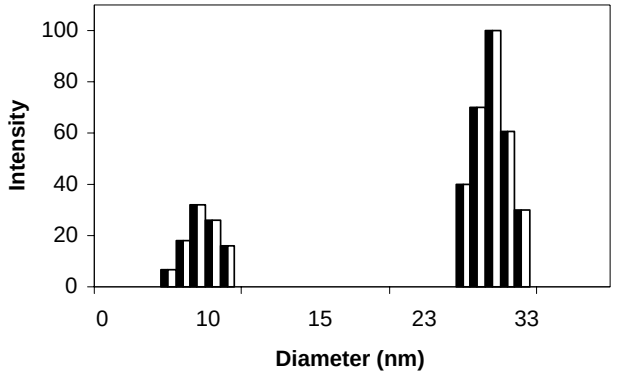
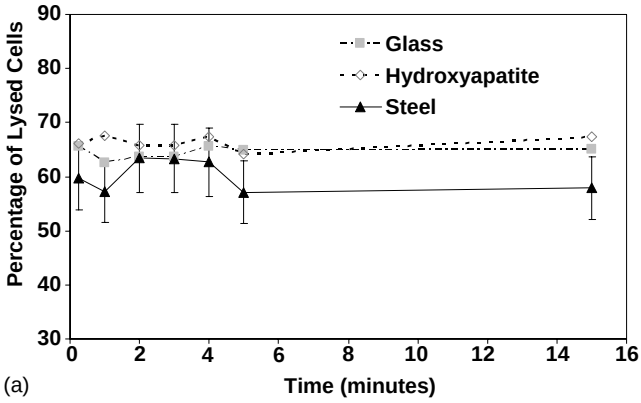
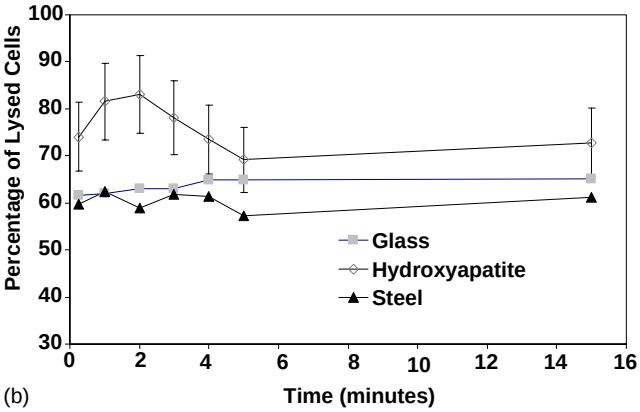


Fig. 2. Size and size distribution of the reverse micelles. The histogram shows the size distribution of a micellar suspension. The intensity-weighed histogram shows two main sizes centered at 10 and 30 nm. The *y*-axis is the intensity average of the scattered light. The *x*-axis is the absolute size of the scatters obtained by a multi-modal fitting of the time correlation function.

three substrates when no stirring is applied (∼65%), with the exception of *S. epidermidis* attached to hydroxyapatite (∼75%). The enzyme is able to disrupt *S. epidermidis* more efficiently when the bacteria are attached to hydroxyapatite than when the bacteria are attached to glass and steel. A similar trend is observed for *K. pneumonia*, but the



(a)



(b)

Fig. 3. Removal of bacteria using free lysozyme without stirring: (a) *K. pneumoniae*; (b) *S. epidermidis*. The lines are included solely to guide the reader's eyes.

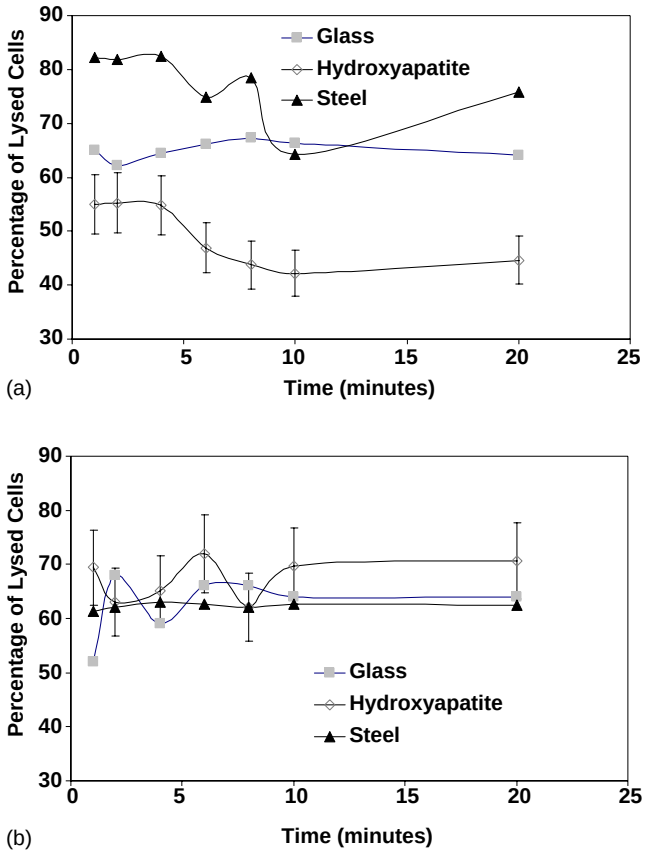


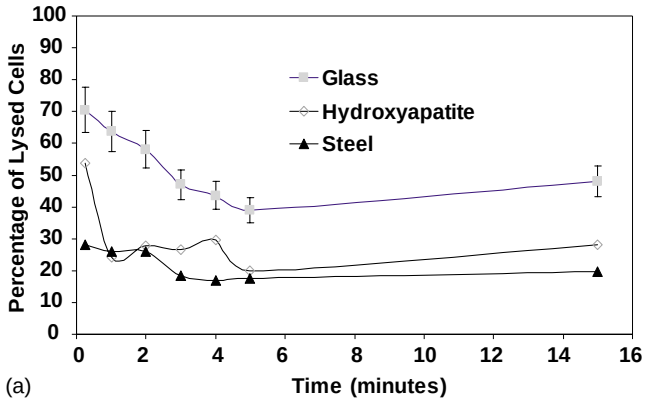
Fig. 4. Removal of bacteria using free lysozyme with stirring: (a) *K. pneumoniae*; (b) *S. epidermidis*. The lines are included solely to guide the reader's eyes.

differences between glass and hydroxyapatite, if any, are within the experimental error. It is also clear that more *S. epidermidis* than *K. pneumoniae* is removed from hydroxyapatite, which is expected because the former is a Gram-positive bacterium. These differences between the two microorganisms seem to disappear for the other two substrates, for which any differences between the two microorganisms are masked by the relatively large experimental errors. In spite of the short sampling times, no transient was detected. Because we are using a 48 h biofilm, some of the cells may be loosely attached and, therefore, they may be just removed (not broken) during sampling. This artifact will increase the apparent number of broken cells at short times.

There are obvious differences in the level of cell disruption upon stirring. These differences seem to be substrate and microorganism dependent. The extent of removal of *S. epidermidis* upon stirring (Fig. 4b) for glass and steel are similar (∼60%) whereas it is slightly higher for hydroxyapatite (∼70%). More noticeable differences between the different substrates are observed for *K. pneumoniae* (Fig. 4a). The removal for steel is ∼75% followed by glass (∼65%) and a very low removal rate for hydroxyapatite (∼50%). For *K. pneumoniae* on hydroxyapatite, we observed lower removal efficiency upon stirring, and we do not have an explanation for this observation. Still, the removal of *K. pneumoniae* from hydroxyapatite is lower than the removal of *S. epidermidis* from the same substrate. This is expected since the former is a Gram-negative bacterium. On the contrary, *S. epidermidis* seems to be more resistant to lysis when it is deposited on steel than on the other materials. Because the three substrates have similar surface energy; we expect lysozyme to reach each surface equally. Therefore, differences in the availability of the enzyme cannot be invoked to explain the observed trends. It is possible that the observed differences are due to different morphologies of the deposits and/or to a change in the resistance of the microorganism upon adhesion onto the surface; changes in the resistance of microorganisms to bactericides upon adhesion to surfaces is well documented. We can speculate, for example, that the *K. pneumoniae* deposits are more loosely attached to steel than to hydroxyapatite, facilitating the disruption by lysozyme in the former substrate since the action of the enzyme will be enhanced by shear stress.

It is well known that Gram-negative cells are more resistant to attack by lysozyme because of the presence of their additional outer membrane; however, in our investigations we have found evidence contrary to that observation. Although *K. pneumoniae* is a Gram-negative bacteria, lysis of both *S. epidermidis* and *K. pneumoniae* attached to either steel or glass is similar regardless if stirring is applied or not. This is odd since we would expect similar lysis levels only upon stirring because Gram-negative bacteria are more susceptible to shear [16]. SEM micrographs show that the overall amount of cell attachment to glass and steel is quite low. Therefore, if there are only a few bacteria attached, then the removal rate may appear to be higher than truly is. The data for hydroxyapatite is consistent with the fact that Gram-negative bacteria are more resistant to lysis. Overall, the free enzyme seems to be able to destroy only approximately 50% of Gram-positive or Gram-negative bacterial deposits.

Figs. 5 and 6 show the results obtained when the attached cells are exposed to lysozyme encapsulated in reverse micelles. The percentage of lysed cells decays over time in the absence of stirring (Fig. 5); this is anomalous. All curves level off at approximately 30% removal after 4 min. Glass is better cleaned than hydroxyapatite or steel. Still, the removal of cells is much lower than for the free enzyme in the absence of stirring. The removal from glass is similar for both microorganisms whereas the removal from steel and hydroxyapatite is slightly higher for the Gram-positive bacteria. These experiments were repeated twice, using different starting cultures, each time resulting in the same trend. The only possible explanation for this observation is the following. Because the micellar suspension is added to the substrate with a pipette, the thin layer of water/cells that covers the biofilm is disturbed. This creates an emulsion in the isooctane phase; the phase from which we sample. Then, as time goes by, the emulsion settles resulting in an exponential decrease in the amount of radioactivity that is detected.



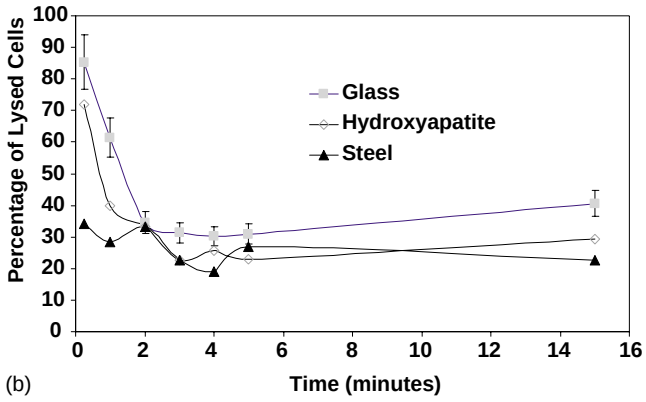
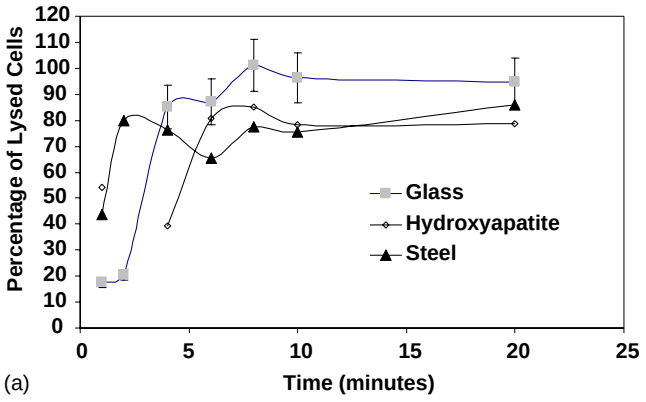


Fig. 5. Removal of bacteria using lysozyme encapsulated in reversed micelles under no stirring conditions: (a) *K. pneumoniae*; (b) *S. epidermidis*. The lines are included solely to guide the reader's eyes.



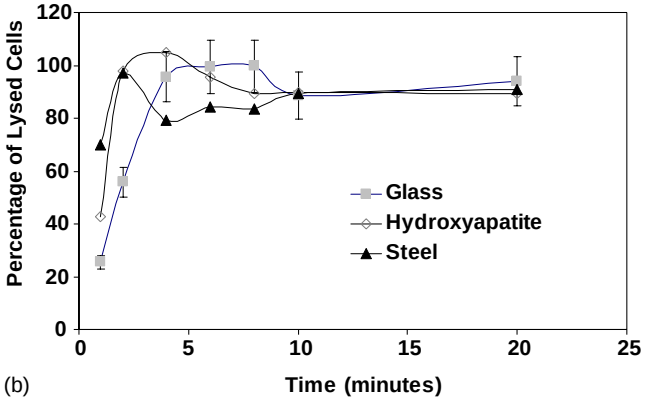


Fig. 6. Removal of bacteria using lysozyme encapsulated in reversed micelles under stirring conditions: (a) *K. pneumoniae*; (b) *S. epidermidis*. The lines are included solely to guide the reader's eyes.

In addition, at the end of the transient no further disruption of cells occurs because the interface between isooctane and the biofilm is sealed by surfactant; thus, no further action of the enzyme is possible. To confirm or reject this hypothesis, we conducted a different kind of experiment. We layered a micellar suspension on top of a macroscopic aqueous phase and monitored the amount of lysozyme released into the aqueous phase. The concentration of the enzyme in the water phase remains close to zero for about 1 h (Fig. 7). After that, the concentration of lysozyme in the water phase increases exponentially to reach a plateau after 7 h. Visual inspection of the cuvette shows a layer of surfactant formed at the isooctane–water interface (Fig. 8). This accumulation of surfactant at the interface sealed the surface after releasing a minor amount of lysozyme into the aqueous phase. The presence of this thin film did not allow the passage of any more lysozyme into the aqueous phase. We can only speculate whether or not lysis of the cells continues after the interface is sealed.

The problems associated with having an adequate supply of the enzyme on the biofilm disappear upon stirring, where the disruption efficiencies for both microorganisms on each of the substrates are higher than 80%. Fig. 6 indicates that transients exist and they seem to depend on the kind of microorganism and on the type of substrate. Disruption occurs almost immediately for *S. epidermidis* on steel whereas the disruption increases from 40 to 80% for *K. pneumoniae* in the first few minutes. A transient is observed for both microorganisms on glass. The disruption increases from 20 to 100% in just less than 5 min for *K. pneumoniae* and it increases from 25 to 95% in nearly the same time for *S. epidermidis*. The disruption of *S. epidermidis* on hydroxyapatite increases from 40 to 85% in about 5 min whereas the amount of *K. pneumoniae* cells disrupted increases from 40 to 80% in approximately 6 min.

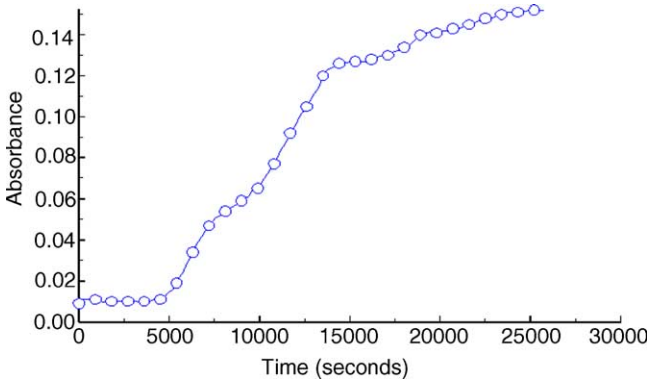


Fig. 7. Release of lysozyme from reverse micelles into alkaline water. The absorbance at 280 nm was monitor in the aqueous phase.

Fig. 8. The picture shows the layer of surfactant that accumulates at the octane–water interface when back-extraction of the encapsulated enzyme in the absence of stirring is attempted.

Lysis of the cells is slower when the encapsulated enzyme is used but it is more complete than when the free enzyme is used. Stirring seems to be necessary for complete disruption when the encapsulated enzyme is used. The final removal percentage for *S. epidermidis* is the same independently of the substrate. On the contrary, *K pneumoniae* is practically completely removed from glass whereas the removal from hydroxyapatite reaches 70%. The slower kinetics is observed for hydroaxyapatite. The fact that the reverse micelles by themselves have only minor effects on the amount of disrupted cells and that no transient is observed suggested that there is a synergetic effect of the enzyme, the surfactant and maybe the organic solvent. We argue that the presence of the surfactant may increase the fluidity of the membranes facilitating the enzymatic action. It is also possible, that the enzyme is partially denatured or that some of the surfactant remains attach to the protein, conferring it a more hydrophobic character. It has been proposed to attach hydrophobic groups to lysozyme to better its bactericide action against both Gram-positive and Gram-negative bacteria. For example, Ito et al. [17] added polyproline, a hydrophobic peptide, to lysozyme and they found an improved bactericidal activity against both Gram-negative and Gram-positive bacteria. They argue that the increased hydrophobicity of the mutant enzyme may induce interactions of lysozyme with the outer membrane of *E. coli* and subsequent penetration of the enzyme into the inner membrane, resulting in the increase of bactericidal activity. It has even been proposed to denature the protein by heating to improve its killing actions. For example, Ibrahim et al. [18] used partially unfolded lysozyme at neutral pH to kill and agglutinate Gram-negative and Gram-positive bacteria. They found that inactive lysozyme (heated at 80 °C and pH 7.0 or at pH 6.0 over 90 °C) exhibited strong bactericidal activity against Gram-negative and Gram-positive bacteria, suggesting a bactericidal action independent of catalytic function. Thus, their results introduce an interesting finding that partial unfolding of lysozyme with the proper acquisition of the hydrophobic pocket to the surface can switch its antimicrobial activity to include Gram-negative bacteria without a detrimental effect on the inherent bactericidal effect against Gram-positive bacteria. Their data suggest that the unique antimicrobial action of unfolded lysozyme can be attributed to membrane binding and subsequent perturbation of membrane functions.

Because of the positive effect of stirring when reverse micelles were used, we explored the effect on removal of a more aggressive stirring protocol. The vials were place in a rotary shaker for various periods of times. Vigorous stirring increased the overall number of lysed cells and both *K. pneumoniae* and *S. epidermidis* show high levels of lysis. The total amount removed is close to 100% independently of the substrate and type of microorganism. For example, the amount of *S. epidermidis* removed from hydroxyapatite increases from ∼10% after 10 min of exposure to almost 100% after 30 min. The fact that both bacteria show similar lysis patterns is odd because *K. pneumoniae* is a Gram-negative bacterium. We may argue, however, that their higher susceptibility to mechanical shear compensates for the fact that they are more resilient to lysis by lysozyme.

4. Conclusions

We have shown that lysozyme encapsulated in reverse micelles is just as effective as free lysozyme in the decontamination of surfaces. In our investigations, the percentage of cells lysed by lysozyme in reverse micelles compares well to that of free lysozyme and in some cases was more effective. This is important for the decontamination of surfaces that may corrode by continual exposure to water. Our method eliminates the need to have the enzyme in an aqueous solution.

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