ORIGINAL ARTICLE

Bactericidal and sporicidal performance of a polymer-encapsulated chlorine dioxide-coated surface

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Introduction

Micro-organisms are ubiquitous in our environment. Although many are harmless and even beneficial, some are well-known pathogens while others can elicit allergic responses in humans. Casual contact with surfaces and objects contaminated with infectious droplets (i.e. fomites) is an established route for transmission of many diseases (Pruss et al. 1999). Maintaining clean and pathogen-free surfaces is necessary in controlling the spread of infectious diseases. Metal surfaces containing silver, copper and brass possess intrinsic germicidal properties that can kill many pathogenic micro-organisms upon contact. They are commonly known as 'contact-killing' surfaces (Fang 1997; Block 2001). Similarly, chromium and nickel in stainless steel and zinc in galvanized iron are known to inhibit microbial growth (Fang 1997). Nanosilvers (Fu et al. 2006; Yu et al. 2007), photocatalytic TiO₂ (Sunada et al. 2003; Liu et al. 2007) and surface-tethered bactericides (e.g. quarternary ammonium compounds, phosphonium salts) (Isquith et al. 1972; Cen et al. 2003; Popa et al. 2003) are among the new generation of contact-killing, antimicrobial surface coatings developed in recent years. Nonmetals such as ceramics and enamels whose surfaces are physically nonadhesive and resist biofilm formation are also extensively used in sanitary settings. Microbes adhere poorly to ultra-hydrophobic surfaces and bacteria-repelling poly(ethylene glycol) (Desai et al. 1992). However, the antimicrobial properties of these materials diminish rapidly when the surface is fouled by dirt and contaminants, thus requiring frequent cleaning to maintain their effectiveness.

Keywords
antimicrobial coating, bactericidal, encapsulated ClO₂, polymer matrix, sporicidal.

Abstract

Aims: To investigate the physical characteristics and the bactericidal and sporicidal potential of a polymer-encapsulated ClO₂ coating.

Methods and Results: An antimicrobial coating based on polymer-encapsulated ClO₂ was developed. A low viscosity, water/oil/water double emulsion coating was formulated for easy on-site application. Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus were applied onto the coating to study the bactericidal capabilities of the coating. The bactericidal performance of the coating increased when the contact time with the tested bacteria increased. Over 99% of the E. coli, Ps. aeruginosa, B. subtilis were killed with a contact time of 30 min. Although endospores of B. subtilis are more resistant, about 75% of the spores were killed after 72 h on the coating. Moreover, a sustained release of gaseous ClO₂ was achieved to maintain about 90% removal of B. subtilis with a 10-min contact time during a 28-day study period. The coating also exhibits antiadhesive properties against bacteria.

Conclusions: A polymer-encapsulated ClO₂ coating with sustained release of ClO₂ and promising bactericidal and sporicidal features was tested for 28 days.

Significance and Impact of the Study: This study provides a new direction for developing polymer-encapsulated ClO₂ coatings that possess persistent bactericidal and sporicidal properties.
It is possible to store biocides (e.g. phenols, halogens) (Chung et al. 2003) and metals (e.g. silver ions) (Kluhe et al. 2000) in bulk materials and coatings for slow and constant release into the environment to provide sustained ‘release-killing’ until the biocide content is exhausted. For example, a chlorine dioxide (ClO₂) biocide is generated from the reaction between stored sodium chlorite salt and acids released from a polymer matrix by either hydrolysis or photolysis reactions (Callerame 1989; Wellinghoff 1997). The latest developments in new antimicrobial coatings are oriented towards multiple mechanistic approaches for surface disinfection. Cohen’s group (Li et al. 2004) employed layer-by-layer, self-assembly methods to produce antibacterial coatings capable of both release-killing and contact-killing based on stored silver salts and surface-grafted quarternary ammonium. Ho et al. (2004) used a polymer film to immobilize nanosilver to achieve both contact- and release-killing effects, while a grafted layer of polyethylene glycol repelled the adhesion of bacteria.

Chlorine dioxide is a fast-acting biocide that is effective against a broad spectrum of micro-organisms including, bacteria, fungi, spores, mold and viruses (Huang et al. 1997; Young and Setlow 2003; Sy et al. 2005; Simonet and Gantzzer 2006). Released as a gas, the antimicrobial action of ClO₂ is less affected by dirt and surface fouling. The gaseous ClO₂ was originally used for large-scale disinfection by bottling companies and as a replacement for the more toxic chlorine in drinking water treatment. The successful stabilization of ClO₂ in liquid form has resulted in broad applications, including the use of a ClO₂ fumigant to disinfect the air ducts in the US Capitol following the Anthrax episode in 2001 (Gordon and Rosenblatt 2005). ClO₂ is safe compared with other oxidizing disinfectants (e.g. chlorine and ozone) and is approved by US FDA for disinfection of poultry and beef products, fruits and vegetables.

The aim of this work was to develop a polymer micro-encapsulating liquid ClO₂ coating that could provide long-term surface disinfection through the sustained release of gaseous ClO₂ from encapsulated liquid ClO₂. This study describes: (i) the physical characteristics of the polymer-encapsulated ClO₂ coating, (ii) the bactericidal performance of the coating against Gram-positive and Gram-negative bacteria, (iii) the sporidical performance of the coating against the endospores of the B. subtilis and (iv) the potential antiadhesive property of the coating against bacteria.

Materials and methods

Preparation and coating of polymer-encapsulated ClO₂

The basic rules for preparing stable water-in-oil-in-water (w/o/w) emulsions described by Ficheux et al. (1998) were followed. The stabilized ClO₂ aqueous solution (United Laboratories Inc., IL, USA) was encapsulated by a water-in-oil-in-water (w/o/w) emulsion method (Pays et al. 2002), in which 25 ml of 1% (w/v) ClO₂ was suspended in lemon oil (Dreamworld, Guangzhou, China) and 25 ml of 5% (w/v) Pluronic P123 surfactant solution (EO₁₀₆PO₇₀EO₁₀₆, MW 5750 g mol⁻¹, with a critical micelle concentration 0.03 wt% at 25°C, BASF) was added by gentle stirring. The resulting emulsion was then added to an aqueous suspension of Pluronic F127 (EO₁₀₆PO₇₀EO₁₀₆, MW 12600 g mol⁻¹, critical micelle concentration 0.7 wt% at 25°C, BASF) obtained by dissolving 2.5 g of F127 in 50 ml of deionized water. This formulation had a storage capacity of 18% (w/w) or 180 mg ClO₂ per gram of solid. The emulsion was stored at 4°C before use, and as the antimicrobial coating was not immediately apparent to the eye, lemon oil was added to provide an olfactory cue.

Glass was chosen as the carrier substrate for the study. Rigorous screening was carried out because different components of the manufactured glass (e.g. ZnO and TiO₂) might have intrinsic bactercidal properties that could interfere with the measurements. Glass manufactured by Sail Brand, China was selected for its inertness and availability. The glass was cut into 2·5 × 2.5 cm² pieces and prepared according to the Association of Official Analytical Chemists (AOAC) guideline (2005a). Any defective coating surface was analysed by time-of-flight secondary ion mass spectrometry (ToF-SIMS) (Physical Electronics PHI 7200). A finely focused ion beam (10¹² ions per

Characterization of coated glass samples

The coated glass samples were examined under a JEOL 6300 scanning electron microscope (JEOL Ltd, Tokyo, Japan) at an acceleration voltage of 10–15 kV. The samples were mounted on aluminum stubs using a conducting carbon tape and sputter-coated with a thin layer of gold (c. 10 nm) to prevent sample charging. The scanning electron micrographs provided information about the uniformity of the coating and the morphology of the deposited polymer microcapsules. The composition of the coating surface was analysed by time-of-flight secondary ion mass spectrometry (ToF-SIMS) (Physical Electronics PHI 7200). A finely focused ion beam (10¹² ions per
The amount of ClO₂ that remained in the coating was gently shaking the tube in an incubator (Gallenkamp) at 37 ± 0°C for 3 days to obtain a high spore yield. One or two bacterial colonies were harvested from the plates and transferred to a 15-ml centrifuge tube containing 5 ml sterilized deionized water. The suspension was mixed well with a vortex. The spores were purified by centrifugation and water washing. Two millilitres of the suspension was transferred to an Eppendorf tube. The suspension was centrifuged at 10 000 g for 20 min at 4°C. The supernatant was decanted and 1 ml of cold sterilized deionized water (4°C) was added and the sample was resuspended at 4°C. An aliquot of the suspension was examined under a phase contrast microscope. The centrifugation and washing steps were repeated until more than 99% free spores were obtained as indicated by phase contrast microscopy. The purified spores were suspended in a phosphate-buffered saline (pH 7-4) solution and stored in the dark at 4°C for no more than 7 days. The concentration of the viable spores in the suspension was determined by the plate counting technique on TSA plates following serial dilution of an aliquot of the suspension.

Testing the bactericidal properties of the polymer-encapsulated ClO₂ coating

The tests followed the standard operating procedure of AOAC International (2005b,c) and were conducted in a sterilized biological safety cabinet (NuAire, Nu-425-400E) under ambient conditions (23 ± 2°C, 70% r.h.). To investigate the bactericidal performance of the coatings, a suspension of 100 µl of 10⁷ cell ml⁻¹ bacteria was spread evenly on the coated and uncoated (control) glass pieces for a specific contact time, which was 10, 20, 30 and 60 min, respectively. At the end of each designated contact time, the amount of viable bacteria that remained on the coating or control surface was enumerated. The tested glass pieces were firstly immersed into a culture tube containing 20 ml neutralizer for 30 min to stabilize and wash off the still surviving bacteria from the surface. The sterile neutralizer solution was freshly prepared by adding 1% (v/v) 0·1 mol l⁻¹ Na₂S₂O₃ to 600 ml of 0·85% (w/v) normal saline (NaCl, RDH) solution containing 0·1% (v/v) (final concentration) of polyoxyethylene sorbitan monooctenolate (TWEEN 80) followed by autoclaving at 121°C for 20 min. The glass pieces were then drip-dried and transferred to a second culture tube containing 20 ml of sterile nutrient broth (Nutrient broth No. 2, Oxoid) for 10 min to collect the residual bacteria, if any, on the surface after bathing in the neutralizer solution. A 100 µl aliquot of the neutralizer solution and nutrient broth collecting the
residual bacteria were spread onto separate TSA plates for viable culturing. The plates were incubated at 37 ± 0.1°C for 24 h. The viable bacteria were enumerated from the number of colonies formed and the concentration was calculated by normalizing with the volume (100 µl) applied. Three runs with five coating samples in each run were carried out for each contact time.

**Persistency study**

To investigate the persistency in the bactericidal performance of the coating, a batch of the coatings was prepared and left under ambient room conditions for a period of 28 days. Five coatings were randomly selected after 10 and 30 min and on day 1, 2, 3, 7, 14 and 28 after the coating preparation for the bactericidal test on *B. subtilis* with a standard contact time of 10 min.

**Testing the sporicidal properties of the polymer-encapsulated ClO₂ coating**

Endospores of *B. subtilis* (Carolina 15-4921A) were used as a model to study the sporicidal capability of the coating. The testing procedures were similar to those for vegetative cells according to the standard operating procedures of AOAC International (2003) to test sporicidal activity. In view of the resistant nature of the spores, longer contact times of the spores on the coatings were allowed from 0 to 2, 8, 24, 48 and 72 h. The initial spore concentration was 10⁵ spore ml⁻¹. The viability of the spores at the end of each contact time was enumerated by the plate-spreading method.

**Testing the antiadhesive properties of the polymer-encapsulant matrix**

The adhesion of *E. coli* K12 (Carolina 15-5065A) and *B. subtilis* (Carolina 15-4921A) on clean glass and glass coated with placebo encapsulants (polymer encapsulating sterilized distilled water) was examined. The coated glass was prepared by the same procedure described above, but the ClO₂ solution was replaced with sterilized, distilled water to remove any nonadherent bacteria (Veyries et al. 2000). Gram staining was performed on the coated and uncoated glass pieces and images taken by an optical microscope (magnification of 1000x) were recorded to reveal the qualitative degree of bacterial adhesion on the coated and uncoated surfaces.

**Results**

**Polymer-encapsulated ClO₂ coating**

The water-in-oil-in-water double emulsion approach that encapsulates ClO₂ in the triblock copolymers Pluronic P123 and F127 results in a uniform, transparent and tactilely smooth coating as shown in Fig. 1a. The coating on the glass was allowed to dry under ambient conditions (i.e., 23 ± 2°C, 70% r.h.) for 24 h. Synresis or liquid bleeding from the polymer was not observed. A scanning electron micrograph of the coating was taken at 100x magnification and it is shown in Fig. 1b. It is apparent that the film displays uniform features that are similar to those of deposited microcapsules. TheToF-SIMS mapping (Fig. 1c) detected only organic molecular fragments originating from the polymer on the surface of the coating and none of the nonvolatile salts found in encapsulated ClO₂ solution, suggesting that the encapsulation of ClO₂ in the polymer matrix was successful.

**Bactericidal and sporicidal potential of the polymer-encapsulated coating**

The bactericidal potential of the polymer-encapsulated ClO₂ coating is summarized in Fig. 2. Different bacteria have different susceptibilities to the coating, yet, in general, the reduction profiles increase with the duration of the contact time of the tested bacteria on the coating. With a 10-min contact time, about 0-log, 0.6-log, 0.7-log and >2-log reduction of *Staph. aureus*, *Ps. aerugenos*, *B. subtilis*, and *E. coli* were recorded, respectively. When the contact time was increased to 30 min, the reduction further increased to 0.8-log, 3-log, 2.7-log and over 5-log reduction, correspondingly. Over 99-99% reduction of *Ps. Aerugenosa* and *E. coli*, *B. subtilis* and 90% reduction of *Staph. aureus* were achieved when the contact time increased to 60 min.

The sporicidal potential of the coating on the endospores of *B. subtilis* is summarized in Fig. 3. The plain glass surface (control) showed no sporicidal effect; on the contrary, the endospores on the glass started to germinate after 72 h as reflected in the increase in the viable count. However, with the polymer encapsulated ClO₂ coating, there is a persistent reduction in the viability of the endospores, from 0-log reduction to about 0-log reduction (75%) at 72 h.

**Release of encapsulated ClO₂ from the polymer matrix**

The persistence of antibacterial activities is shown in the sustained release of gaseous ClO₂ from the coated glass
surface and its sustained bactericidal potential (Fig. 4). Over the study period of 28 days (4 weeks), about 20% of the stored ClO₂, equivalent to \( c. \frac{1300}{g} \text{ClO}_2 \cdot \text{g}^{-1} \cdot \text{day}^{-1} \), were released (Fig. 4a). This amount is sufficient to reduce microbial growth. A parallel bactericidal test on *B. subtilis* showed that a sustained 0·8-log reduction was achieved after a 10-min contact time over the 28 day study period (Fig. 4b). This is equivalent to the disinfecting power of a 300 ppm ClO₂ solution. By extrapolating the rate of release from the 28-day study, we estimated that the stored ClO₂ in the reported preparation would last for at least 3 months with sustained bactericidal potential.

Antiadhesive property of the polymer coating

Members of the Pluronic family have been reported to exhibit antiadhesive properties against micro-organisms such as *Ps. aeruginosa, Staph. aureus* and *Staph. epidermidis* and are used as detergents in contact lens cleaning solutions (Portole’s *et al*. 1994). Water-containing emulsion microcapsules were prepared using the same procedure, with the ClO₂ solution replaced with sterilized, distilled water. This ‘placebo’ emulsion was coated on glass pieces and aliquots of *E. coli* and *B. subtilis* were added. It can be seen from the microscope images
(Fig. 5a,c) that the amount of E. coli and B. subtilis on the coated glass was significantly less (i.e. <10%) compared with that on the uncoated glass (Fig. 5b,d). This indicates that the polymers used for encapsulation prevented the adhesion of E. coli and B. subtilis on the glass surface.

Discussion

The World Health Organization has reported that the most common route for transmission of infectious diseases is by indirect contact with surfaces contaminated by infectious droplets produced by an infected person’s coughing, sneezing or talking (Pruss et al. 1999). Bellamy et al. (1998) found amylase from saliva on close to 29% of exposed surfaces in domestic households. Human expiratory droplets and hence any inherent pathogens or embedded bacteria can settle on surfaces for days. Fomites, inanimate surfaces capable carrying germs, had been associated with contact transmission of viral, bacterial and fungal pathogens. Regular surface cleaning and disinfection are therefore important and recommended for breaking the chain of transmission. Chemical disinfectants are commonly employed for maintaining the cleanliness of surfaces.

The oxidizing biocide, ClO₂, is a common disinfectant. It is safe (McDonnel and Russell 1999) and effective against a broad spectrum of bacteria, spores and viruses over a wide pH range from 2 to 8 (Huang et al. 1997; Young and Setlow 2003; Sy et al. 2005; Simonet and Gantzer 2006). The disinfection ability of ClO₂ has also been reported for B. anthrax cells and spores (Canter et al. 2005), the severe acute respiratory syndrome-associated coronavirus (Wang et al. 2005) and the influenza A virus (H1N1) (Ogata and Shibata 2005). ClO₂ is a very reactive free radical molecule. Owing to its unique one-electron transfer reaction mechanism, it is also a highly selective oxidant (Gordon and Rosenblatt 2005). It attacks electron-rich centres in organic molecules (Gordon and Rosenblatt 2005) and kills micro-organisms through oxidizing their cell membranes (Berg et al. 1986) and denaturing their proteins (Ogata 2007). It breaks the inner membrane of spores preventing their proper germination (Young and Setlow 2003) and reacts with the viral envelope to cause irreparable damage and inactivation (Ogata and Shibata 2005). ClO₂ solution can be applied or sprayed directly onto the target surface for action. The disinfection ability, however, diminishes with time in terms of minutes as the ClO₂ vaporizes. In enabling a longer lasting disinfection performance, ClO₂ gas was generated from sodium chlorite salts stored in a polymer matrix through a reaction with an acid ( Callerame 1989; Wellinghoff 1997), and the acid was either stored with the salt or formed as byproduct of polymer decomposition. This process is inevitably slow as the generation of ClO₂ gas depends on the release or production of acid and its subsequent reaction with sodium chloride.

The polymer-encapsulated ClO₂ coating system described in this study has promising antibacterial potential. A stabilized ClO₂ solution was microencapsulated in the active triblock copolymers Pluronic P123 and F127 to form a stable water-in-oil-in-water (w/o/w) double
emulsion (Fig. 1). This process does not require any acid activation to generate the ClO₂ biocide, which eliminates the need for acid storage and subsequent reactions. The Pluronic family of triblock copolymers was selected because it is safe and widely used in medicines and cosmetics (Thies 1990; Morishita et al. 2001). Also, their responsiveness to different environmental cues such as temperature, pH and moisture has been amply demonstrated for controlled drug delivery (Tarcha 1991; Sosnik and Cohn 2004). The emulsion was readily sprayable and with the Pluronic polymers acting as a surfactant. The emulsion droplets deposited uniformly on surfaces as shown by the SEM picture in Fig. 1b. The chemical composition of the surface indicated that the ClO₂ was completely encapsulated in the polymer matrix instead of residing on the surface layer (Fig. 1c).

The experimental results in Figs 2 and 4 indicated that ClO₂ remained encapsulated after surface coating. A slow but sustained release of ClO₂ at about 1300 μg ClO₂ g⁻¹ day⁻¹ from a coating area of 1 mg cm⁻² loaded with 180 mg ClO₂ per gram of polymer was reported in this study. Assuming that there is a constant rate of ClO₂ release from the polymer, the coating prepared in this study can optimally last for 3 months. The P123 polymer does not favour a fast transport route via inverted micelles of encapsulates in the oil phase (Matsumoto and Kang 1989; Sela et al. 1995). Studies showed that molecules can migrate through the oil phase without affecting the double emulsion stability (Bibette et al. 1999). The ClO₂ encapsulated in this study is likely transported from the interior of the polymer to the peripheral surfaces via diffusion, resulting in a sustained release of ClO₂ over the 28-day study period.

The coating also demonstrates consistent bactericidal potential. The ClO₂ released from the coating killed over 90% of the E. coli, Ps. aerugenosa, B. subtilis, and Staph. aureus and about 10% of B. subtilis endospores with a contact time of 30 min. The amount of B. subtilis killed was comparable with that killed after exposure to 300 ppm ClO₂ solution. The relative susceptibility of B. subtilis spores and the range of bacteria killed followed with the trend reported by Russell (2003). Gram-negative E. coli was most susceptible and endospores of B. subtilis were the least susceptible (Figs 2 and 3). Staphylococcus
aureus was the least susceptible to ClO₂ among the tested vegetative species. Staphylococcus aureus also exhibited similar low susceptibility to a hypochlorite (i.e. bleach) solution (data not shown). This may be related to the presence of carotenoid pigments in Staph. aureus that are antioxidative and provide the bacteria with some degree of protection from oxidizing biocides (Clauditz et al. 2006).

Despite the different susceptibilities of the bacteria to polymer-encapsulated ClO₂, the bactericidal and sporidical performance generally increased as the contact time was prolonged. This trend favours the use of the encapsulated coating as an antimicrobial surface. Under ambient conditions, droplets or fomites from whatever source will be deposited onto surfaces and remain on the site until they are transferred to other surfaces or objects after contact. This means that there is ample time for the ClO₂ to act on the species resting on the coating. We speculate that the bactericidal or sporidical activity is caused by multiple actions of the coating. Primarily, both dissolved and gaseous ClO₂ is released through diffusion from the ClO₂ encapsulated in the polymer matrix. In addition, the coating is rehydrated by droplets or fomites upon deposition. This rehydration destabilizes the outermost membrane film of the coating, resulting in its rupture and rapid flooding of the contact area with the encapsulated ClO₂. The release is sustained by the difference in the osmotic pressures between the exterior and interior water phases of the polymer formulation. The rehydration thus initiates the sporadic release of ClO₂ in addition to the usual release-killing mechanism employed by antimicrobial systems using ClO₂ (Callerame 1989; Wellinghoff 1997). This sporadic release potential gives the coating the advantage of self-disinfection of areas contaminated by droplets or fomites.

Formation of biofilms is detrimental to the coating surface. Phenotypic tolerance to oxidizing biocide can arise from biofilm formation via consumption of the biocide by the organic constituents of the biofilm (Chen and Stewart 1996), or by a ‘population-based’ resistance strategy (Cochran et al. 2000). No biofilm was observed in this reported polymeric matrix coating. The active biocide, ClO₂, used in the formulation prevents indiscriminate reactions and rapid consumption of ClO₂ by the biofilm because of rapid diffusion (Hosni et al. 2006) and greater reaction selectivity (Gordon and Rosenblat 2005). The absence of a biofilm may also be due to the anti-adhesive properties of the P123 and P127 polymers that prevent the adhesion both of the Gram-positive and Gram-negative bacteria (e.g. B. subtilis and E. coli) on its surface (Fig. 5). The chance of seeding a biofilm on the coating is also reduced. The antiadhesive property of the polymers is probably related to the anchorage of the hydrophobic core of the triblock polymers on the material surface, while its hydrophilic chains form sterically stabilized barriers against adhesion in the periphery (Bridgett et al. 1992). The antiadhesive property of the coating surface reduces the chance of the coating degeneration as a result of biofilm formation and allows the coating surface to be a self-maintaining bactericidal system.

In conclusion, a prototype of an antimicrobial coating was prepared with polymer-encapsulated ClO₂ through the double w/o/w emulsion system for general surface application. The coating is easily sprayable because of its low viscosity. A sustained release of gaseous ClO₂ at a maintenance level was demonstrated for a prolonged period (i.e. 28 days). A surface concentration of about 300 ppm ClO₂ was obtained to prevent the bacteria or endospores from attaching on the coating. Contamination by droplets or fomites triggers an increased release of biocide in the affected area, resulting in rapid disinfection. The polymer-encapsulated ClO₂ coating also exhibited promising bactericidal potential against a spectrum of bacterial species and sporidical potential against Bacillus endospores over a contact time of 60 min.

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