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The antibacterial properties of light-activated polydimethylsiloxane containing crystal violet

Ekrem Ozkan,[a] Elaine Allan[b] and Ivan P. Parkin*[a]

[a]*Materials Chemistry Research Centre, Department of Chemistry, University College London, 20 Gordon Street, London WC1H 0AJ, UK. E-mail: i.p.parkin@ucl.ac.uk*
[b]*Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, 256 Gray's Inn Road, London, WC1X 8LD, UK*

Abstract

Crystal violet was incorporated into polydimethylsiloxane (PDMS) by a swell-encapsulation-shrink method using chloroform as a swelling solvent. The polymer was characterized by IR, UV-vis and water contact angle measurements. The antimicrobial activity of the polymer was evaluated against *Escherichia coli and Staphylococcus aureus* under white light conditions comparable to that found in a healthcare environment. The crystal violet-incorporated polymer showed potent lethal photosensitization of both bacteria.

Introduction

Light-activated antimicrobial agents (LAAAs), also known as photosensitive dyes, are promising approaches to treat and circumvent infectious diseases. Many significant pathogenic bacteria show resistance to antibiotics and hence new methods to treat are being sought. While LAAAs show some antimicrobial activity in the dark, they are well known to produce free radicals and reactive oxygen species (ROS) under irradiation with white light or laser light which kill bacteria.[1, 2]

Antimicrobial photodynamic therapy is used to eradicate target cells by means of ROS.[3] In this therapy, doses of visible light can activate non-toxic agents and subsequently, types of ROS can be produced through two different reactions: electron transfer (type I) or energy transfer (type II) reactions. The ''type I'' reaction involves the transition of the agent from a low energy ground state to a higher energy triplet state, which is capable of reacting with biomolecules to generate free radicals (type I reaction), or with molecular oxygen to generate highly reactive singlet oxygen (type II reaction).[4] ROS produced upon irradiation of the agent can kill bacteria in the vicinity *via* multiple attack pathways, including the destruction of cellular membranes, intracellular proteins and DNA by oxidative damage, making it unlikely that the bacteria will develop resistance.[5-7] In addition, mammalian cells are more resistant to ROS than bacteria making it an attractive approach for treating infection.

LAAAs are generally inorganic or organic dyes such as crystal violet (CV), methylene blue (MB) and toluidine blue (TBO). It has been shown that such dyes can be incorporated into silicone by a simple ''swell-encapsulation-shrink'' technique and these

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materials show photo-activated antimicrobial activity.[7-10] For example, Naik *et al*. demonstrated that the MB incorporated polyurethane polymer was capable of reducing the numbers of *S. aureus* by up to 2.8 log when exposed to 24 h of white light illumination.[10]

In this paper, we report the incorporation of crystal violet into a curable silicone elastomer using a ''swell-encapsulation-shrink'' method. The silicone elastomer consists of a two-part curable silicone elastomer. The precursor, Slygard 184, can be cross-linked using its curing agent and the final material is polydimethylsiloxane (PDMS). This is an attractive elastomer because of its high flexibility, low cost, non-toxic nature, chemical inertness and ease of preparation. Furthermore, PDMS has an ability to absorb a range of aromatic materials.[11] PDMS is composed of a flexible (Si-O) backbone and a repeating ($Si(CH_3)_2O$) unit.[12] The objective of this work was to study the effectiveness of CV-incorporated PDMS in killing the Gram-negative bacterium, *Escherichia coli,* and the Gram-positive bacterium, *Staphylococcus aureus,* using white light.

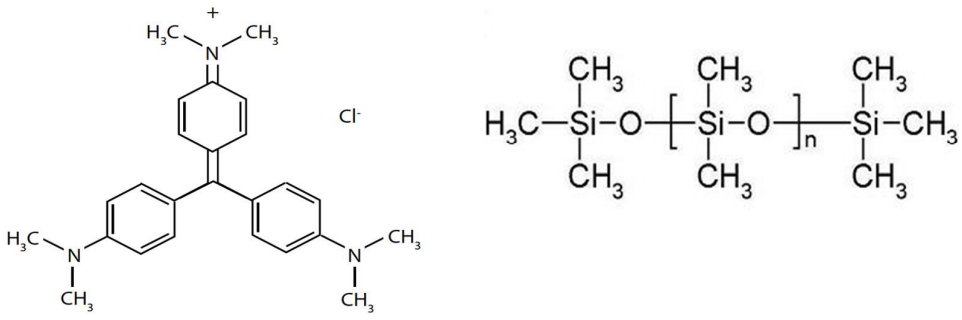

Figure 1: Chemical structures of (left) the photosensitizer dye, crystal violet, and the silicone elastomer polydimethylsiloxane.[13, 14]

Materials and Methods

Elastomer preparation

Silicone elastomers were prepared using viscous liquid polydimethylsiloxane (Dow Corning Corporation Ltd.) as a starting material; this was mixed with the crosslinking agent in a 10:1 ratio, and spread uniformly on to a glass petri dish. The polymer was then cured at 120 °C for 60 min. After cooling, the sheet was cut into smaller pieces (squares 1.0 x 1.0 cm).

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Preparation of polymers with embedded crystal violet

CV solutions were prepared at a concentration of 1000 ppm in chloroform (Sigma Aldrich). 1.0 cm^2 samples of polymer were placed into the CV solutions and left to swell in the dark for 72 h. Afterwards, the samples were washed and dried in the dark at room temperature for 24 h.

Materials Characterization

A Perkin-Elmer Lambda 950 UV-vis Spectrometer was used to measure the UV-vis absorption spectra analyses of the polymers within the range 400-800 nm. IR analysis of the polymers was performed within the range of 400-4000 cm^{-1} with an accumulation of 16 scans per sample using a Bruker Platinum ATR. Water droplet contact angles were measured using a First Ten angstroms 1000 device with a side mounted rapid fire camera fire casting 5 µL water droplet on the surface of each sample and 5 replicates on fresh samples were performed. The data was analyzed using FTA32 software.

Leaching test

The stability of the CV coated elastomer in solution was determined: CV coated sections (1 cm^2) were immersed in phosphate buffered saline (PBS) (2.5 ml, 37 °C) for 160 hours. The UV-Vis absorbance of the PBS (596 nm, Pharmacia Biotech Ultrospec 2000) was measured periodically to monitor leaching of the CV from the polymer into the surrounding solution. The concentration of the CV in solution was determined on the basis of its absorbance at 596 nm, compared to a calibration curve.

Bactericidal assay

Pure PDMS polymer (control) and CV coated polymer were used to determine the bactericidal activity of the materials against *E. coli* ATCC 25922 and *S. aureus* 8325-4. The bacteria were stored at -70 °C in Brain Heart Infusion broth (BHI, Oxoid) containing 20% (v/v) glycerol and propagated on either MacConkey agar (MAC, Oxoid Ltd.) in the case of *E. coli* or Mannitol Salt agar (MSA, Oxoid Ltd.) in the case of *S. aureus*, for a maximum of 2 subcultures at intervals of 2 weeks.

BHI broth (10 ml) was inoculated with 1 bacterial colony and cultured in air at 37 °C for 17 h with shaking at 200 rpm. The bacterial pellet was recovered by centrifugation (20 °C, 4000 x g, 5 min), washed in PBS (10 ml) and centrifuged again (20 °C, 4000 x g, 5 min) to recover the bacteria which were finally resuspended in PBS (10 ml). The washed bacterial suspension was diluted 1 in 1000 in PBS to give an inoculum of approximately 10^6 cfu/ml.

Duplicates of each polymer sample were inoculated with 25 µl of the inoculum and covered with a sterile cover slip (22 mm x 22 mm). The samples were then irradiated for 6 hour in the case of *E. coli* and for 2 hour in the case of *S. aureus* utilizing a white light source (General Electric 28 W Watt MiserTM T5 2D compact fluorescent lamp). The light intensity was arranged to emit an average light intensity of 10500 ± 250 lux at a distance of 16 cm from the samples. A further set of samples (in duplicate) was maintained in the dark for the duration of the irradiation time. Post irradiation, the inoculated samples and cover slips were placed into PBS in 50 ml plastic tubes and

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vortexed for 20 seconds. The neat suspension and ten-fold serial dilutions were plated on the appropriate agar, incubated aerobically overnight at 37 °C and the colonies enumerated to determine the number of surviving bacteria. The bacterial numbers in the inocula were also determined in each experiment by viable colony counting. Each experiment included two technical replicates and the experiment was reproduced three times. The Mann-Whitney U test was used to determine the statistical significance of the following comparisons: (i) the activity of the CV-treated polymer compared to the pure polymer when both were incubated in the dark and (ii) the activity of the irradiated CV-treated polymer compared to the same material incubated in the dark.

Results and discussion

Synthesis

Dye-impregnated polymer was prepared using a swell-encapsulation-shrink method with 1000 ppm of CV, Fig 2. Polydimethylsiloxane squares (1 cm x 1 cm) were placed in a chloroform solution saturated with CV for 72 h in the dark. They were subsequently removed from the solution, washed and air-dried (24 h) so that the solvent evaporated. This method generated a purple-colored silicone that had shrunk to its original size and contained physically encapsulated CV. Chloroform was used because it not only solubilizes the dye, but it also swells the samples to enable CV incorporation.[15] Furthermore, as shown in the cross sectional photograph in Fig. 2(b), the process involved dye diffusion through the bulk of the elastomer.

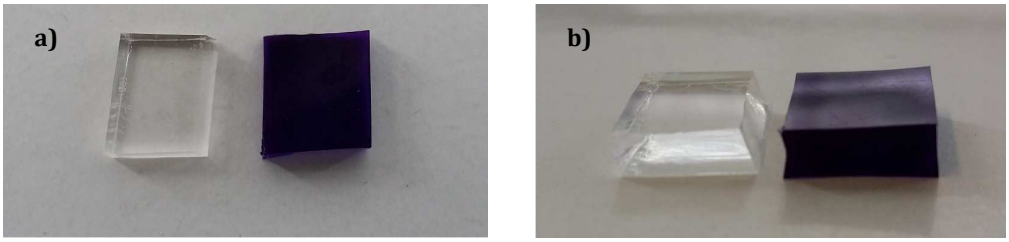

Figure 2: Images of PDMS samples a) before -on the left- and after the swell-encapsulation-shrink process in chloroform with 1000 ppm of crystal violet –on the right. (b) A cross section of the polymers prepared by immersion in 1000 ppm crystal violet dipping solution for 72 h.

Characterization

The IR spectrum of the samples was obtained by ATR. The spectra (data not shown) detected no significant change across the sample range after embedding CV in the polymer matrix. The results demonstrated that the swell-encapsulation-shrink technique had no influence on the physical and chemical structure of the silicones. IR spectra only gave peaks related to the host polymer matrix.

The UV-vis absorbance spectra of silicone samples were measured within the range 400-800 nm (Fig. 3). While pure PDMS does not show any absorbance in the visible region, when immobilized in 100 ppm CV solution, the main absorption peak of CV-encapsulated silicone is at $\lambda \approx 590$ nm, with a shoulder peak of weaker intensity at $\lambda \approx 533$ nm. The sample prepared with 1000 ppm crystal violet reached signal saturation resulting in absorption with more intense peaks (data not shown).

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The light source used in this work was a General Electric 28 W Watt MiserTM T5 2D compact fluorescent lamp with a color temperature of 3500 K, which emits light across the visible spectrum.[16] This light source was employed since it has the same characteristics as fluorescent lights utilized in hospitals in the United Kingdom.[17] The spectral profile of the lamp is composed of approximately 405, 435, 495, 545, 588 and 610 nm (Fig. 4). It can be seen that the absorbance of the crystal violet silicone includes four of the main emission peaks of the light source.

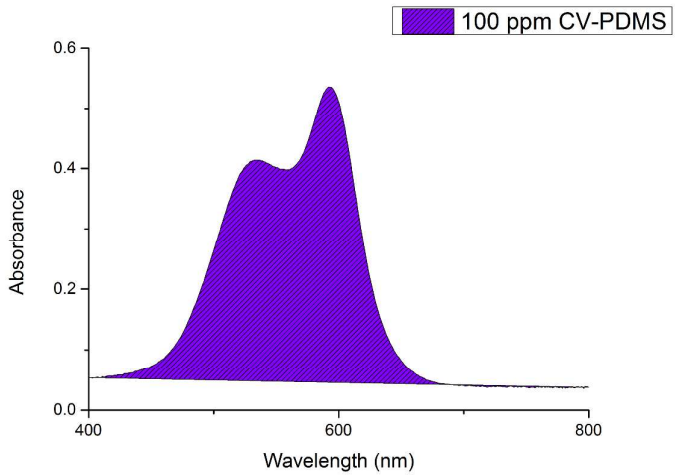

Figure 3: UV-vis absorbance spectra of CV-coated silicon polymer at a concentration of 100 ppm

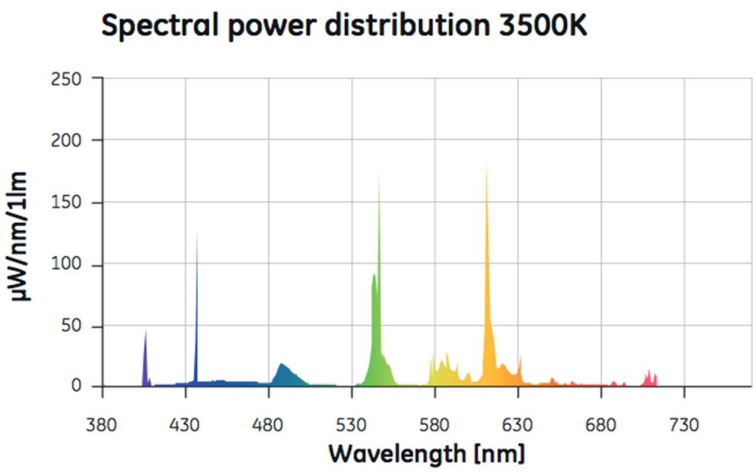

Figure 4: Spectral Power Distribution of General Electric 28W MIser™ 2D lamp.[16]

The contact angles of water on both the untreated elastomer and the treated elastomer were measured (Table 1). The table shows that the surface of untreated PDMS was hydrophobic itself with a water contact angle of 99.1°. In addition, a slight increase in the wetting properties of the untreated elastomer was seen when contrasted with the treated elastomer after the swell-encapsulation-shrink process. However, this was within the error of the experiment.

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Table 1: Average contact angle measurements (°) ± standard deviation of PDMS samples.

Samples	Water contact angle (⁰)
Untreated	99.1 ± 1.7
CV-treated	101.1 ± 2.2

Hydrophobicity is an important key factor in preventing bacterial adhesion. Previous study has shown that superhydrophobic PDMS surfaces reduced the attachment of *E. coli* and *S. aureus,*[18] suggesting its potential use as an antimicrobial surface.

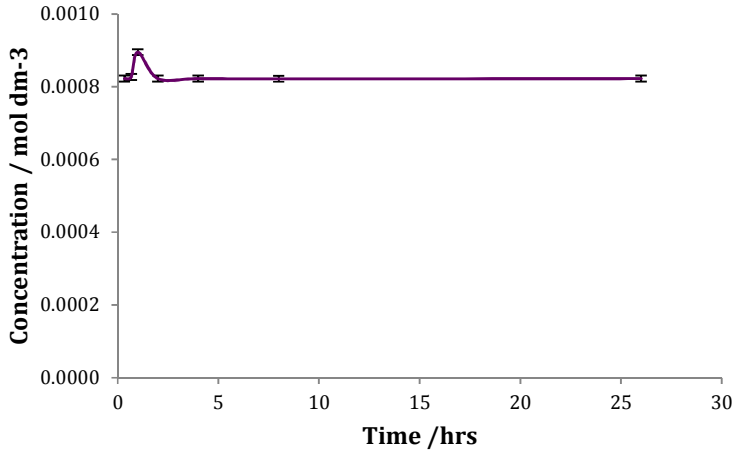

Figure 5: Leaching of crystal violet (mol dm^{-3}) from a CV-coated silicon polymer into PBS solution at 37 °C, was measured as function of time (hours).

In order to examine whether there had been any leaching of the CV-incorporated polymer, the polymer was placed in PBS and leaching measured spectroscopically as a function of time (Fig. 5). The data demonstrates that the sample released some CV into solution upon immersion (the first measurement of the solution was taken after 20 min from the sample immersion), even though the leaching of dye from the polymer plateaued quickly with time. Over a period of more than 160 h, the leaching of CV into the solution was ≈ 0.0008 mol dm^{-3} (within experimental error). We attribute this leaching to surface bound CV that was not properly embedded in the polymer. In clinical use this initial leaching could be avoided by simply washing or wiping the surface with a damp cloth.

Bactericidal activity

The antibacterial activities of both untreated and embedded silicon polymers were assessed against two common hospital-associated pathogens: the Gram-negative bacterium, *E. coli* and the Gram-positive bacterium *S. aureus.* A white light source similar to those commonly found in UK hospitals was used to activate the antimicrobial activity of the dye-coated polymer.

Fig. 6 demonstrates that no reduction in the numbers of *E. coli* was observed for the untreated polymer in the dark after 6 h. Similarly, there was no detectable kill on the

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surface of the CV-encapsulated silicone samples stored in the dark for the same period of time. However, the samples containing CV showed a significant lethal photosensitization resulting in a statistically significant reduction in the number of *E. coli* (> 4 log reduction; P = 0.002) compared to the control elastomer sample after 6 h of exposure to a white light source emitting an average light intensity of 10500 ± 250 lux at a distance of 16 cm from the samples. These results indicate that the polymer with encapsulated dye is not toxic to bacterial cells without a light source but exhibits highly significant bactericidal activity in the presence of light.

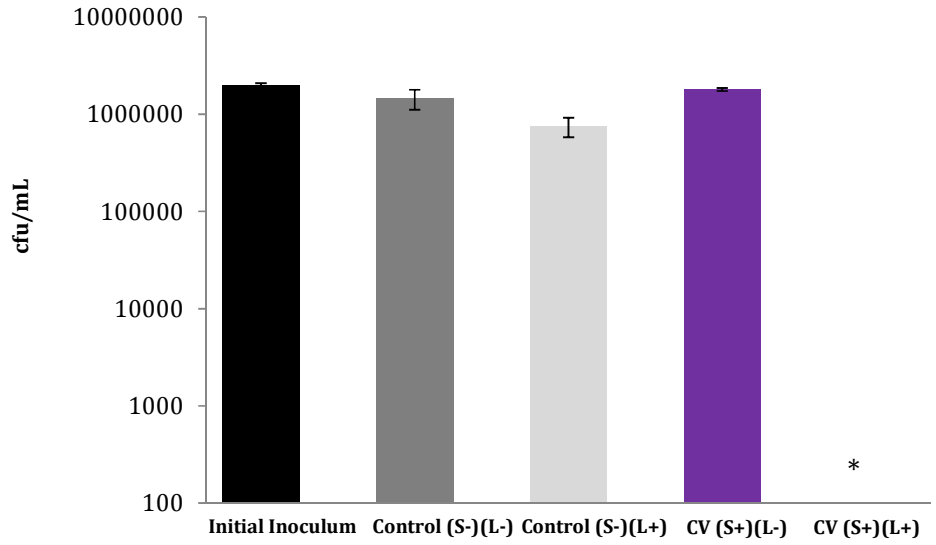

Figure 6: Numbers of *E. coli* on the surfaces of the polymers after 6 h exposure. Bars on the graph represent the: initial inoculum, control elastomer (control) and CV-encapsulated elastomer (CV). (L+ = white light exposure, L- = no white light exposure, S+ = CV dye present, S- = CV dye not present). The asterisk indicates where the bacterial numbers are below the detection limit of 100 cfu/mL.

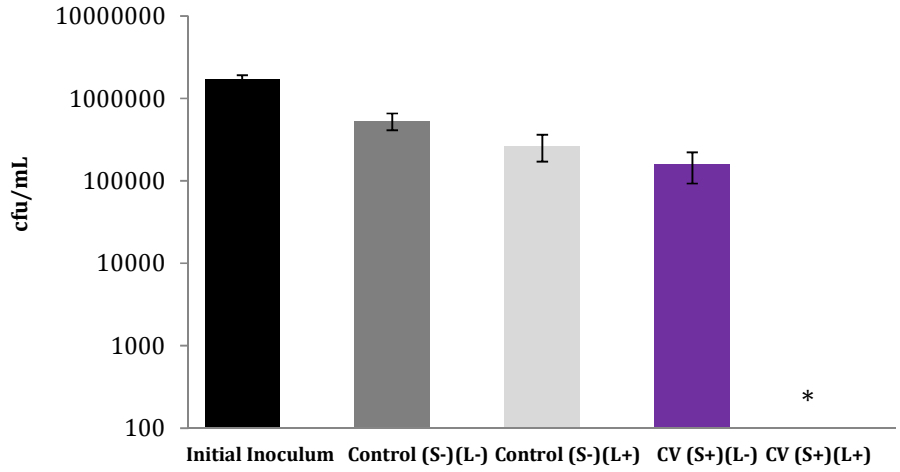

Figure 7: Numbers of *S. aureus* on the surfaces of the polymers after 2 h exposure. Bars on the graph represent the: initial inoculum, control elastomer (control) and CV-encapsulated elastomer (CV). (L+ = white light exposure, L- = no white light exposure, S+ = CV dye present, S- = CV dye not present). The asterisk indicates where the bacterial numbers are below the detection limit of 100 cfu/mL.

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When tested against *S. aureus* in the dark for 2 h, there was significant kill with the polymer alone ($P < 0.01$). A significant increase in the antibacterial activity of the polymer in the dark was observed compared to the control sample when CV was present ($P < 0.01$). Importantly, exposing the CV-containing polymer to light resulted in the most potent lethal photosensitization with bacterial numbers reduced below the detection limit within 2 h ($P = 0.002$).

The difference in susceptibility between Gram-positive and Gram-negative bacteria has been observed in other studies with the latter generally more resistant presumably as a result of their more complex cell wall structure.[11, 17,19]

Previous studies have showed CV could be used as an effective LAAA. For example, Noimark *et al*. reported that CV-impregnated medical grade silicone polymer resulted in a 0.22 log reduction in the numbers of *E. coli* when exposed to 635 nm laser light for 13.5 min.[20] In addition, exposure of the same polymer to white light of 3750 lux for 6 h also exhibited some bactericidal effect.[21]

The data presented here shows that a relatively short white light illumination time of CV-PDMS is more effective at killing the Gram positive bacterium, *S. aureus*, than either methylene blue-polyurethane or toluidine blue-polyurethane (2 h vs. 24 h).[10]. The difference in efficacy may be correlated with the diffusion distance of ROS. The diffusion distance of singlet oxygen has been previously estimated as around 0.2 microns within the polymer.[20] Since MB and TBO are distributed evenly throughout the polymer, the dyes incorporated within the polymer will not contribute to antibacterial activity and only dye near the polymer surface will play a role. Therefore, we speculated that high concentrations of CV on the surface of the polymer would lead to increased production of ROS thereby increasing the kill of surface-loaded bacteria.[21] Additionally, CV accumulated on the polymer surface may be responsible for the dark kill of *S. aureus* observed because the bacteria are directly in contact with high surface concentrations of the antibacterial agent.

In this work the efficiency of a triarylmethane photosensitizer, CV, against two bacteria commonly encountered in UK hospitals was investigated when activated by a fluorescent lamp similar to those used in these environments. The results showed that polydimethylsiloxane coating including CV is potent at killing these pathogenic microbes. CV was chosen for embedding in the coating since it is able to absorb light strongly at many of the prominent wavelengths emitted by the white light source.

CV has antimicrobial, anti-fungal properties[13] and it has been used to treat infected wounds and superficial skin infections as well as for the treatment of infections involving methicillin-resistant *Staphylococcus aureus* (MRSA).[22, 23] The World Health Organization recommended the use of CV to treat skin problems such as superficial wounds and fungal infections.[24]

PDMS has been utilized widely in many applications including microfluidic channels, lubricants, defoaming agents, gas separation membranes and catheters due to its

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properties.[25] We showed that CV could make PDMS antimicrobial towards representative Gram-positive and Gram-negative bacteria that are significant causes of nosocomial infection under ambient light conditions. Thus, this self-disinfecting surface can be employed in a wide range of application areas from healthcare environments to electronic devices including tablet and mobile phone covers and computer keyboards. The intensity of the white light source utilized to activate the photo-antimicrobial properties of the polymer was 10500 ± 250. The lighting conditions in this study can be compared to the brightness of different locations in UK hospitals, as recommended by the Department of Health (Table 2).[17, 26, 27]

Table 2: Recommended light intensities for various areas in the UK healthcare environments.[17, 26, 27]

Environment	Light intensity /lx
Operating theatre	10 000-100 000
Pathology lab	8000
Ward corridors	≥ 200
A & E examination room	1000

Therefore, it is anticipated that it would be more efficacious to employ light-activated antimicrobial coating in examination rooms and operating theaters, where light intensities are highest.

However, it is also possible to achieve significant kills in areas of lower light intensity if the illumination time is prolonged. In addition, it should be noted that in these experiments, very high bacterial loads (~3.8 x 10^4 cfu cm^{-2} for *E.coli*, ~4.6 x 10^4 cfu cm^{-2} for *S.aureus*) were used to examine the antibacterial effect of the polymer, much higher than the levels reports on contaminated hospital surfaces (up to an equivalent of 3060 cfu cm^{-2} with average values of <100 cfu cm^{-2}).[21]

Conclusion

In this work light-activated antimicrobial PDMS polymer was prepared successfully using a facile swell-encapsulation-shrink method utilizing CV. When photo-activated by a standard hospital light source, the CV embedded polymer was potent at killing suspensions of *S. aureus* in just 2 hours and *E*. coli in just 6 hours, with a minimum of a 4 log reduction in the numbers of both bacteria. Consequently, it is clear that the presence of CV in PDMS extends its use in antimicrobial applications.

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