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Incorporation of Crystal Violet, Methylene Blue and Safranin O into a Copolymer Emulsion; the Development of a Novel Antimicrobial Paint

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Crystal violet, methylene blue, safranin O and 2 nm gold nanoparticles were incorporated into a copolymer emulsion paint and three separate paint systems were prepared; a three-dye system (crystal violet, methylene blue, safranin O and 2 nm gold nanoparticles), a two-dye system (crystal violet, methylene blue and 2 nm gold nanoparticles) and a one dye system (safranin O and 2 nm gold nanoparticles). The modified polymers were characterised by UV-Vis absorbance spectroscopy, IR spectroscopy and X-ray photoelectron spectroscopy. The three paint systems were moderately stable under aqueous conditions, with a limited amount of leaching of the dyes from the paint polymer into surrounding aqueous solution. Exposure of the three paint systems to a 28 W white light source induced the lethal photosensitisation of both *Staphylococcus aureus* and *Escherichia coli*. Moreover, both the three-dye and two-dye systems resulted in a 4 log kill against *S. aureus* under dark conditions, and a 1.5 log dark kill was obtained by the safranin O and 2 nm gold nanoparticle system.

1 Introduction

Nosocomial infections place a significant financial and social burden on healthcare associations throughout the world. The Centers for Disease Control and Prevention in the United States estimate that 1.7 million nosocomial infections occur in U.S. hospitals every year,[1] and that these infections contribute to almost 99,000 deaths every year. The treatment of these infections cost approximately $35-$45 billion annually.[2]

It is estimated that up to 80 % of all nosocomial infections are transmitted by touch.[3] Hand and surface disinfection serve as the first line of defence against nosocomial infections, however a significant proportion of staff, patients and visitors to healthcare facilities seldom practise the high standards of personal hygiene that are expected in a clinical setting. The hand-hygiene compliance rate of patients in hospitals in the U.S. was below 50 % in 2009.[4]

Frequently touched surfaces serve as a reservoir for the spread of pathogenic microorganisms in a clinical setting.[5] Typical surfaces that these organisms colonise include door handles, push plates, railings, tray tables, taps and IV fluid poles.[6] It is estimated that methicillin-resistant *Staphylococcus aureus* (MRSA) is prevalent on up to 27 % of all surfaces in a regular hospital ward, and up to 64 % of surfaces in specialist burn units.[6] In 2005, there were over 6500 recorded hospital deaths attributed to MRSA in the U.S.,[7] with over 250,000 hospitalisations associated with the organism in the same year.[7] Patients harbouring MRSA require a prolonged period of hospitalisation, usually in isolation, with the hospital stay extending an average of 26 days.[8] The associated cost of a prolonged hospital stay per patient is over $12,000 on average.[8]

Studies of MRSA have demonstrated that the bacterium can survive on a surface for up to 180-360 days depending on the environment and the surface that it is in contact with.[9] *Clostridium difficile* was attributed to almost 7500 deaths in the U.S. in 2008,[10] and is another prevalent microorganism found contaminating healthcare environments. *C. difficile* endospores may exist upon a surface for up to five months in an unused hospital room.[11, 12] According to Public Health England, up to 40 % of all nosocomial infections could be prevented if correct personal hygiene and disinfection protocol were followed.[13] It is apparent that more must be done to reduce the level of contamination, and to decrease the risk of nosocomial infections that are transmitted by touch.

There has recently been significant research interest in the development of self-sterilising surfaces, with the aim of offering an alternate method to eliminate microorganisms on medical devices and high-touch surfaces.[14-17] These novel antimicrobial strategies will work alongside current standard hospital protocol, in order to reduce the spread of infection.

Antimicrobial copper-alloy touch surfaces have demonstrated antimicrobial efficacy in clinical trials carried out in several countries, effectively reducing the incidence of nosocomial infections in hospital wards. In a four-year clinical trial conducted in the intensive care units of three major hospitals in the U.S., the majority of frequently touched surfaces, such as door handles, push plates, taps and tray tables, were made of copper-alloys.[18] Nosocomial infections in patients on these units were reportedly reduced by 58 %, when compared to patients in ICUs with non-copper touch surfaces.[18] A clinical trial carried out in a hospital in the UK in 2012 also employed the use of copper-alloy surfaces, and there was a reduction of over 90 % in microbial contamination on these high-touch surfaces, when compared to non-copper based surfaces.[19] It is important to recognise that if these copper-alloy surfaces were used by a healthcare facility, then all current furnishings and equipment would have to be replaced. This is an unrealistic intervention in the current economic climate, especially for U.K. hospitals who are part of the Private Finance Initiative scheme, where replacement units incur a 30 year lifetime cost. One way of overcoming this problem is to develop a product that can coat existing surfaces, such that they exhibit strong antimicrobial activity.

An interesting strategy recently employed in the development of antimicrobial materials utilises a modified form of photodynamic therapy (PDT), by the incorporation of photosensitiser dyes into polymeric materials. Research on PDT as an antimicrobial tool was neglected after the discovery and mass-production of antibiotics such as penicillin in the 1940s, given the extremely effective nature of these drugs.[20] In recent years, there has been a great acceleration in bacterial drug resistance, resulting in the development of alternative antimicrobial strategies in order to overcome the issue of more drug-resistant bacteria.[21] PDT has traditionally been used *in vivo* in the treatment of malignant and other diseased cells, however in recent years there has been growing interest in using this phototherapy to destroy microorganisms on a surface. Recently, Parkin, Wilson et al. reported the lethal photosensitisation of *Staphylococcus aureus* on a surface using a modified form of PDT with a basic thiazine dye, toluidine blue O,[22] demonstrating the potential of PDT as an anti-infective method for sterilising surfaces.

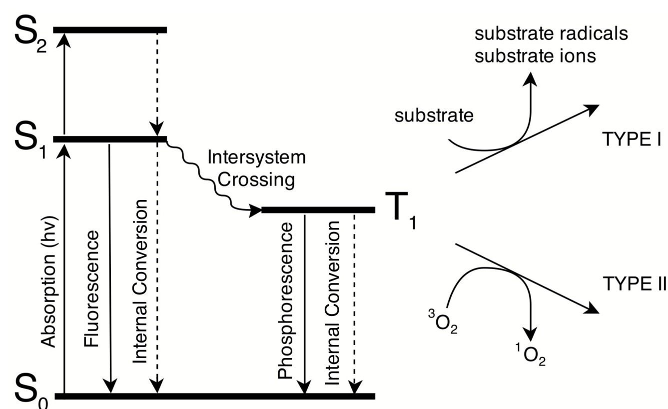

Fig. 1 A Jablonski diagram demonstrating the two different photochemical processes that can occur in photodynamic therapy.

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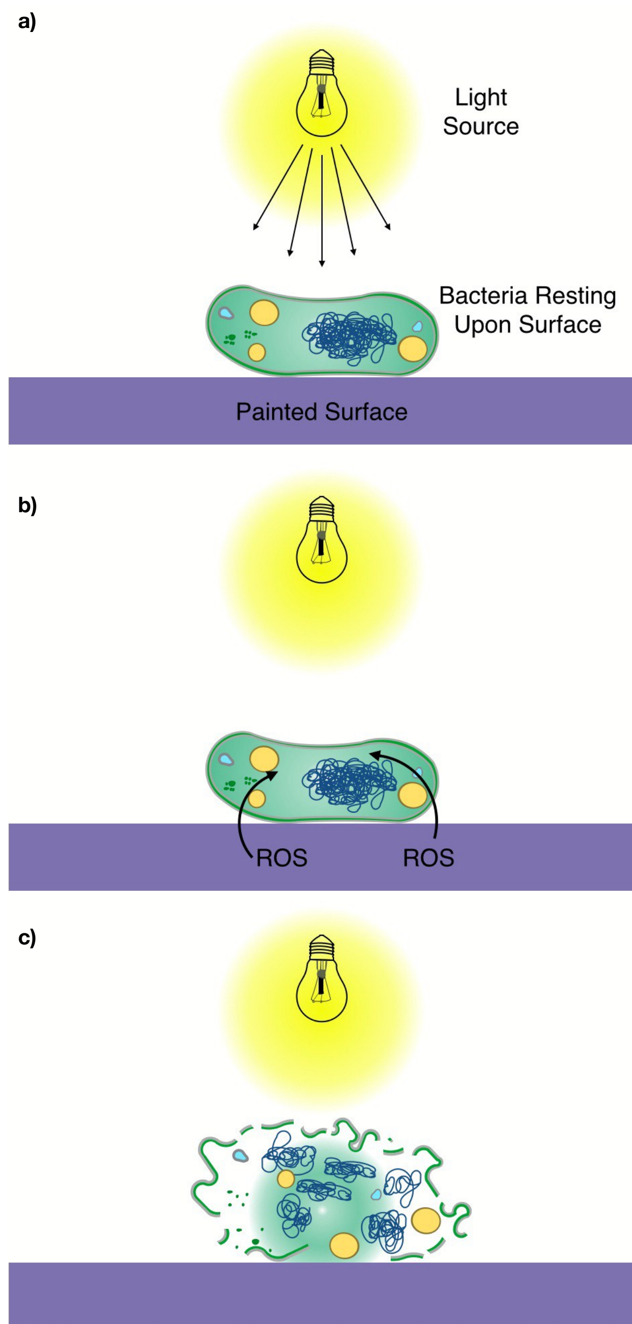

Fig. 2 The photodestruction of a bacterial cell upon a surface containing a photosensitiser species. (a) The surface is illuminated by a white light source (lightbulb) for an extended period of time. (b) The photosensitiser species within the painted surface is promoted to an excited triplet state, *via* the excited singlet state, where it then reacts either in an electron transfer process (type I) or an energy transfer process (type II) to produce singlet oxygen radicals and other reactive oxygen species (ROS). These ROS subsequently attack the bacterial cell *via* a mechanism of multi-site attack. (c) The ROS have destroyed the bacterial cell through oxidative attack, damaging the DNA, intracellular proteins and cellular membranes of the cell.

In PDT, the photosensitiser species exhibit bactericidal activity by means of the generation of reactive oxygen species, upon irradiation with light.[17] Upon photo-activation, the photosensitiser is promoted to the excited triplet state *via* the excited singlet state,[23] where it can then undergo two different photoprocesses; involving either an electron transfer with a substrate species such as water (type I), or an energy transfer with a triplet state oxygen species (type II).[24] This process of excitation, intersystem crossing and the subsequent reactions are shown in the Jablonski diagram in Fig. 1. The two photochemical processes produce a variety of reactive oxygen species including hydroxyl radicals (type I) and singlet oxygen radicals (type II).[25] These highly cytotoxic species initiate a mechanism of multi-site attack against the DNA, intracellular proteins and cellular membranes of the organism, killing it through oxidative damage. A mechanism of multi-site attack on the microorganism suggests resistance to these photoprocesses is highly improbable, since the bacterium would need to develop multiple simultaneous mechanisms of resistance in order to block the photo-destruction effected by PDT.[26] The destruction of a bacterial cell, through the generation of a reactive oxygen species, is shown in Fig. 2.

In clinical applications, it is imperative that the photosensitiser species that are used in an antimicrobial material do not demonstrate toxicity towards mammalian cells upon contact with the surface. Investigations into the effect of PDT against mammalian cells have indicated that the photosensitiser concentrations are not sufficient to damage them.[27] It is believed the nuclear membrane of these cells protects them from damage that is caused by the photosensitiser species and from the reactive oxygen species that are generated upon illumination.[27] Moreover, due to the large size of human skin cells relative to that of bacterial cells, it is likely that significantly higher photosensitiser densities would be required to effect significant photodamage, than that required to damage bacterial cells.[28] In addition, in applications where the photosensitiser is used in antimicrobial paints for touch surfaces, it is extremely unlikely that the generation of reactive oxygen species would exhibit toxicity towards human cells, as the surface would not generate reactive oxygen species during contact time.

Photosensitiser dyes such as methylene blue, toluidine blue and crystal violet have been shown to exhibit photo-activated antimicrobial activity upon immobilisation into medical grade polymers.[14, 15, 29-34] Furthermore, it has been shown that 2 nm gold nanoparticles enhance the antimicrobial activity of the photosensitiser dyes upon encapsulation within the polymer matrix, despite possessing no intrinsic cytotoxic properties of their own.[29] An EPR investigation suggested that the documented increase in photobactericidal activity that is caused by the nanogold is related to an increase in the production of the excited triplet state of the dye.[14] This rise in triplet state production will introduce more reactive oxygen species into the system, effecting a greater kill of bacterial cells due to an increase in oxidative damage.

Studies have demonstrated that these modified polymers induce the photo-destruction of both Gram-positive and Gram-negative bacteria upon photoactivation with short-term laser light irradiation.[30-32] The photosensitiser dyes, crystal violet and methylene blue, are of particular interest given that they have been shown to induce the lethal photosensitisation of Gram-positive bacteria on a polymer surface, upon irradiation with white light.[15, 34] Another dye that has displayed potent antimicrobial properties is safranin O, with reported bactericidal behaviour in complex media such as blood serum, blood and saliva.[35] Several patents have reported the dye's success as a photosensitiser *in vivo*, and shown there is selective destruction of bacteria in human and animal subjects.[36, 37] To our knowledge there are no known published studies of safranin O as a photosensitiser species outside the body.

In this paper, we report on the development of an antimicrobial paint, with potential use in a healthcare setting. Three individual paint samples were prepared using a combination of the three aforementioned light-activated photosensitiser dyes; crystal violet, methylene blue and safranin O, shown in Fig. 3. These samples were characterised using UV-Vis absorbance spectroscopy, infrared spectroscopy and X-ray photoelectron spectroscopy. Two of the three dyes selected for this study, crystal violet and methylene blue, cover a similar region of the electromagnetic spectrum, suggesting that there may be a potential saturation of ROS produced at wavelengths proportional to blue light. It is hoped that through the addition of safranin O, a red dye, to the system, that more reactive oxygen species will be produced, given safranin O occupies a different part of the electromagnetic spectrum.

Microbiological testing of the three dye samples was performed against both Gram-positive and Gram-negative bacteria, using a white light source to activate the antimicrobial properties of the dyes. Surprisingly, two of these paint systems demonstrated complete kill

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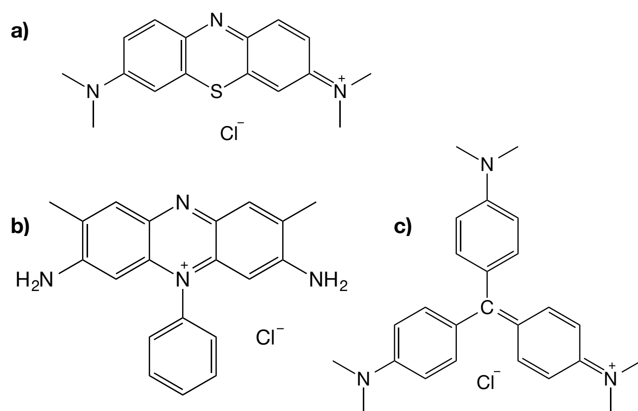

Fig. 3 Chemical structures of: (a) the phenothiazine photosensitiser dye methylene blue, (b) the diamino phenazine photosensitiser dye safranin O, and (c) the triarylmethane photosensitiser dye crystal violet.

of the Gram-positive bacterium *Staphylococcus aureus* in the dark, and, as such, are the best dark-active photobactericidal polymers reported to date. To our knowledge, this is the first report of an antimicrobial paint that uses photosensitiser dyes.

2 Experimental

2.1 Chemicals and substrates

The reagents used in the development of the materials were as follows: Vinyl acrylic copolymer emulsion paint, crystal violet (Sigma, UK), methylene blue hydrate (Riedel-de Haën), safranin O (Fisher, UK), 2 nm gold nanoparticles (2.49×10^{-7} mol L^{-1}, BBI International Ltd). In all synthetic work carried out, the water used was deionised (resistivity 15 MΩ cm) and the substrate was a glass microscope slide (VWR, UK).

2.2 Materials synthesis

2.2.1 System optimisation – paint concentration. Glass slides were dip-coated in various concentrations of emulsion paint for 30 s, which were made up of the following water-paint ratios: 1:0, 9:1, 8:2, 6:4, 4:6, 2:8, 1:9 and 0:1. The slides were withdrawn from the emulsion paint mixtures at a uniform rate of 120 cm/min, after which they were allowed to dry on a level surface (24 h).

2.2.2 System optimisation – dye concentration Glass slides (25 mm x 25 mm) that had been coated in the emulsion paint and air dried for 24 h were immersed in solutions of the three-dye system (CV/MB/SO/n-Au in water), for 2 h. These solutions were made up to the following concentrations,: 1×10^{-3} mol dm^{-3}, 5×10^{-4} mol dm^{-3}, 2.5×10^{-4} mol dm^{-3}, 1×10^{-4} mol dm^{-3}, 5×10^{-5} mol dm^{-3}, 2.5×10^{-5} mol dm^{-3}, 1×10^{-5} mol dm^{-3} and 5×10^{-6} mol dm^{-3}. The samples were subsequently removed from the solution and allowed to air dry on a level surface for 24 h, after which they were washed with deionised water and subsequently towel-dried.

2.2.3 System optimisation – duration of soaking period Glass slides (25 mm x 25 mm) that had been coated in the emulsion paint and dried for 24 h, were immersed in a crystal violet, methylene blue and safranin O solution (5×10^{-4} mol dm^{-3}) for various periods of time extending from 20 min to 24 h. After each set soaking period, the sample was removed from the solution and allowed to air dry for 24 h on a level surface, after which it was washed with deionised water and subsequently towel-dried.

2.2.4 Preparation of materials for antimicrobial testing. Four different samples, including a control, were prepared for antimicrobial testing.

a) Control sample: Glass microscope slides (25 mm x 25 mm) were immersed in deionised water for 2 h, before being air dried (24 h), washed and towel-dried.

b) Crystal violet, methylene blue, safranin O, 2 nm gold nanoparticle coated glass (CV/MB/SO/n-Au): Glass microscope slides (25 mm x 25 mm) that had been coated in emulsion paint and dried for 24 h were immersed in a crystal violet, methylene blue and safranin O solution (5×10^{-4} mol dm^{-3}, 10 % v/v 2 nm gold nanoparticles solution) for 2 h. The coated glass slides were then subsequently air-dried on a level surface for 24 h, washed (deionised water) and towel-dried.

c) Crystal violet, methylene blue, 2 nm gold nanoparticle coated glass (CV/MB/n-Au): Glass microscope slides (25 mm x 25 mm) that had been coated in emulsion paint and dried for 24 h were immersed in a crystal violet and methylene blue solution (5×10^{-4} mol dm^{-3}, 10 % v/v 2 nm gold nanoparticles solution) for 2 h. The coated glass slides were then subsequently air-dried on a level surface for 24 h, washed (deionised water) and towel-dried.

d) Safranin O, 2 nm gold nanoparticle coated glass (SO/n-Au): Glass microscope slides (25 mm x 25 mm) that had been coated in emulsion paint and dried for 24 h were immersed in a safranin O solution (5×10^{-4} mol dm^{-3}, 10 % v/v 2 nm gold nanoparticles solution) for 2 h. The coated glass slides were then subsequently air-dried on a level surface for 24 h, washed (deionised water) and towel-dried.

2.3 Material Characterisation

The UV-Vis absorption spectra of the coated glass slides used for microbiology were measured using a PerkinElmer Lambda 25 UV-Vis spectrometer, within the range of 400 – 800 nm. The absorption spectra of the three photosensitiser dyes were also recorded within the range 400 - 800 nm. The UV-Vis absorbance spectra of the seven samples that were immersed in a range of different concentrations of the CV/MB/SO/n-Au solution were measured. The concentration of the solutions ranged from 5×10^{-3} mol dm^{-3} to 5×10^{-6} mol dm^{-3}, and these measurements were carried out in order to identify an optimal concentration. The UV-Vis absorption spectra of samples immersed in 5×10^{-3} mol dm^{-3} solution for different periods of time were also measured in order to identify an optimal soaking period.

The infrared absorbance spectra of these coated slides were measured within the range 4000 – 400 cm^{-1}, with an accumulation of 15 scans per sample, using a Bruker Platinum ATR. X-ray photoelectron spectroscopy (XPS) analysis of an untreated painted glass slide, CV/MB/SO/n-Au, CV/MB/n-Au and SO/n-Au treated samples was carried out using a Thermo Scientific *K-Alpha* spectrometer to identify the different elements as a function of polymer depth, across the sample range.

2.4 Functional Testing

2.4.1 Water contact angle measurements. Equilibrium water contact angle measurements (~5.0 µL) on the coated glass slides used for microbiology, as well the control sample, were obtained. The contact angle measurement for each sample was taken to be the average value over 30 measurements, using a droplet of deionised water dispensed by gravity from a gauge 30 needle. The samples were photographed side on, and the data was analysed using FTA32 software.

2.4.2 Leaching test. The stability of a CV/MB/SO/n-Au coated glass slide in phosphate buffer saline (PBS, Dulbecco A), was investigated, in order to determine the extent of leaching of the three dyes. The coated glass slides (25 mm x 25 mm, as prepared for microbiology) were immersed in PBS (10 mL) for an extended period of time. The UV-Vis absorbance of the PBS was measured periodically to monitor the leaching of the dyes from the polymer into the surrounding solution. A comparison of the absorbance of the leaching solution with the calibration curves of the three dyes, enabled the determination of the concentration of these dyes that was released from the copolymer emulsion into the surrounding solution. The comparison used the maximum absorption peak values for each of the three dyes.

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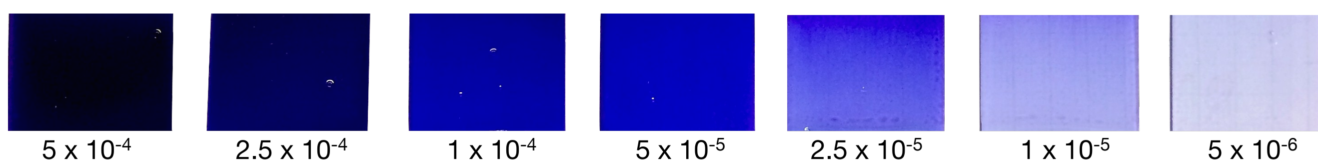

Fig. 4 Copolymer emulsion paint coated glass slides that have been immersed in (a) varying concentrations of CV/MB/SO/n-Au solution, ranging from 5 x 10^{-4} mol dm^{-3} to 5 x 10^{-6} mol dm^{-3}, for 2 h and (b) a 5 x 10^{-5} mol dm^{-3} solution of crystal violet for 1 h.

2.5 Microbiological Testing

2.5.1 Antimicrobial testing. The four glass slide samples prepared as detailed in section 2.2.4 were used in the bactericidal assay. The bacteria tested were *Staphylococcus aureus* NCTC 83254 and *Escherichia coli* ATCC 25922. These organisms were stored at -70 ˚C in Brain-Heart-Infusion broth (BHI, Oxoid) containing 20% (v/v) glycerol and propagated on either Mannitol Salt agar (MSA, Oxoid) in the case of *S. aureus* or MacConkey agar (MAC, Oxoid) in the case of *E. coli*, for a maximum of two subcultures at intervals of 2 weeks.

BHI broth was inoculated with 1 bacterial colony and cultured in air at 37 ˚C for 18 h with shaking at 200 rpm. The bacteria were recovered by centrifugation (20 ˚C, 4000 rpm, 5 min), washed in phosphate buffered saline (PBS, 10 mL) and again recovered by centrifugation (20 ˚C, 4000 rpm, 5 min). The bacteria were re-suspended in PBS (10 mL) and diluted 1000-fold to obtain an inoculum (~1 x 10^6 cfu/mL). The inoculum in each experiment was confirmed by plating 10-fold serial dilutions on agar for viable colony counting. Triplicates of each polymer sample type were inoculated with 50 µL of the inoculum, and were then irradiated for 4 h using a compact fluorescent non-integrated lamp (see section 2.5.2). A further set of samples (triplicate) was maintained in the dark for the duration of the irradiation time.

After the irradiation period, the inoculated samples were placed in PBS (950 µL) and vortexed (30 s). The washed suspension and ten-fold serial dilutions were plated on agar for viable counts. The plates were incubated aerobically at 37 ˚C for 48 h (*S. aureus*) or 16 h (*E. coli)*. Each experiment contained three technical replicates and the experiment was reproduced three times. The statistical significance of the data was analysed using the Student's t-test.

2.5.2 Specifications of compact fluorescent lamp for activation of photosensitiser dyes. A 28 W lamp (General Electric Lighting biax™ 2D) was used to irradiate the samples over a 4 h period, at a distance of 0.4 m from the lamp. The luminous flux of the light emitted was 3500 ± 250 lux.

3 Results

3.1 Materials Synthesis

Square glass slides were dip-coated in emulsion paint at various concentrations in order to determine which emulsion ratio resulted in the best coating of the emulsion paint. A low paint content (less than 10 %) did not yield a consistent coating of the glass surface, with inconsistencies in the depth of the emulsion paint varying from a thicker coating towards the edges of the glass slide, to a considerably thinner, or non-existent coating towards the middle of the glass slide. The higher paint/water ratio mixtures yielded a much more consistent and thicker coating of the glass surface, resulting in a more even coverage of the surface than the lower paint/water ratio mixtures. An optimum ratio of 9-parts-paint to 1-part-water was selected for the emulsion paint mixture.

The effect of concentration of the dipping solution was studied, in order to ascertain the optimal concentration for use in preparation of the samples for microbiology. The concentration of the CV/MB/SO/n-Au dipping solution was varied, using water as the solvent. The paint-coated glass slides were immersed in several solutions of this dye system, which varied in concentration (1 x 10^{-3} mol dm^{-3} to 5 x 10^{-6} mol dm^{-3}). These were then dried and washed; the resulting dye coated slides are shown in Fig. 4. The incorporation of all three dyes increased significantly with concentration, with a visible variation in the colouring of the paint film. The observed increased uptake of the dye is justified, given that there is a greater amount of dye molecules that can encapsulate into the polymer at higher concentration.

It is essential that the dyes embedded in the paint do not leach a significant amount upon contact, particularly within a healthcare environment. In clinical applications, disinfectant wipes will frequently come into contact with the paint-dye surface, as all hard surfaces in wards in most hospitals are frequently cleaned with sterilising wipes in order to reduce the potential for infection. The slides that were immersed in the 2.5 x 10^{-4} mol dm^{-3} and 5 x 10^{-4} mol dm^{-3} solutions were vigorously wiped with Clinell wipes, which are used to clean hard surfaces on the wards in UCLH NHS trust hospitals.[38] There was no notable leaching of the slides that had been coated in the CV/MB/SO/n-Au solution at both of these concentrations, upon contact with the Clinnell wipe. Therefore, a solution of 5 x 10^{-4} mol dm^{-3} of the three-dye solution (in water) was selected, given there was no apparent colour gradient or leaching when the surface was wiped with the Clinnell wipe.

The difference in dipping times of the glass slide in the three-dye solution was then studied, in order to further refine the procedure. The dipping time ranged from 20 min to 24 h. The most notable issue with the shortest dipping time was that there was a notable colour gradient of the three dyes within the polymer. This gradient seemed to disappear after a one-hour soaking period. Therefore a two-hour soaking period was selected for the preparation of the samples for microbiology, as this period of time would ensure an even distribution of the dyes within the polymer.

The four different dye-coated glass slides were prepared for microbiological testing. The crystal violet, methylene blue, safranin O and 2 nm nanoparticulate gold coated glass slide (CV/MB/SO/n-Au) was prepared by dip-coating a cut glass microscope slide (25 mm x 25 mm) in a 9:1 emulsion paint:water mixture for 30 s, after which the slide was withdrawn from the mixture at a uniform rate (120 cm/min). The slide was allowed to dry on a level surface (24 h) before it was immersed in the dye solution combining all three dyes and 10 % v/v 2 nm nanogold (2 h). The resulting product was then air dried (24 h), washed and towel-dried. The crystal violet, methylene blue and 2 nm nanoparticulate gold (CV/MB/n-Au) and the safranin O and 2 nm nanoparticulate gold (SO/n-Au) samples were also prepared in the same manner, using their respective dye solutions. It is believed that this procedure yields a stable product, which does not leach upon contact with a damp tissue and has a consistent coating of both paint and dye. It is thought that the 2 nm gold nanoparticles are distributed uniformly throughout the dye-coated surface.

3.2 Materials Characterisation

The UV-Vis absorbance spectra of the dye-coated samples prepared for microbiology were measured in the range 400 – 800 nm. Figure 5a shows the spectrum of a CV/MB/SO/n-Au sample that was prepared for microbiology, compared to the spectra of the three individual dyes; crystal violet, methylene blue and safranin O. It is apparent that the peaks within the CV, MB, SO, n-Au sample are characteristic of the three dyes, with both absorption maxima and shoulder peaks in the same wavelength region. The variance between the peaks within the sample, and the peaks from the dyes is less than 1 nm. The similarity between all of these peaks suggests that all three of the dyes have incorporated within the polymer system to some extent.

Fig. 5b shows the spectra of a safranin O dyed sample and the one-dye system (safranin O incorporated with nano-gold). The peak

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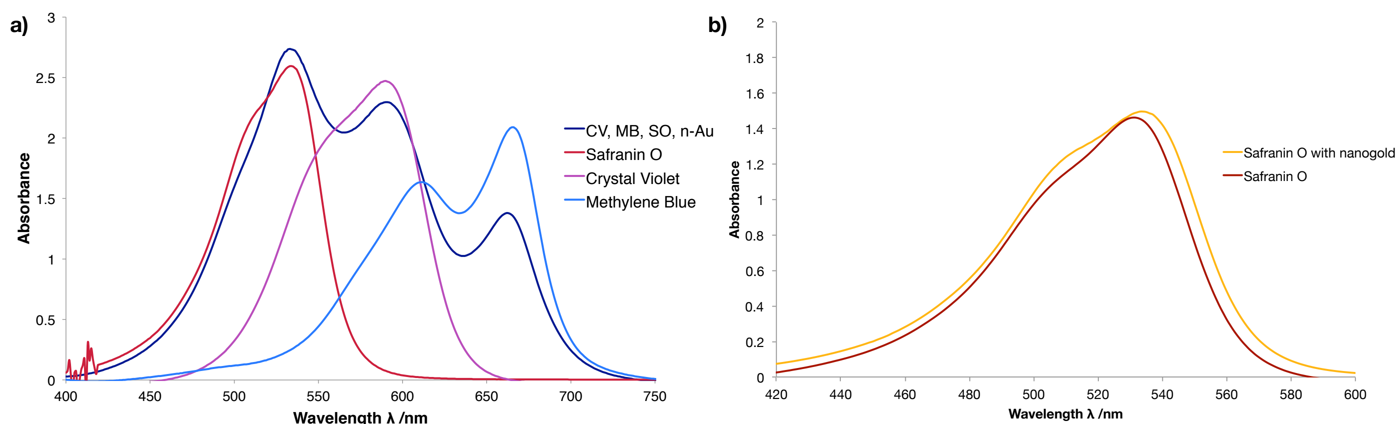

Fig. 5 (a) The UV-Vis spectra measured in the range 400-750 nm of the three separate dye species, crystal violet, methylene blue and safranin O, as well as the three-dye system that was prepared for microbiology: crystal violet, methylene blue and safranin O incorporated with 2 nm nanogold (CV/MB/SO/n-Au). (b) The UV-Vis absorbance measured in the range 420-600 nm of a sample coated with safranin O, and another sample coated with safranin O that has 2 nm nanogold incorporated within the polymer.

maxima, intensity and form are very similar, which indicates that the incorporation of nano-gold has no effect on the macrostructure of the dye and that its incorporation does not hinder or increase the uptake of the dye. Furthermore, it shows that there is no detectable absorption of the 2 nm gold nanoparticles in the visible region of the spectrum, given that the only peak present in the spectrum is caused by the dye. A nanoparticle must usually have a diameter, d > 4 nm, for surface plasmon resonance oscillations to give rise to nanoparticle colouration, therefore given the small size of these nanoparticles it is expected that there is no detectable peak in the visible region of the electromagnetic spectrum.[30]

The infrared absorbance spectra of the samples used for microbiological testing were obtained by ATR. The spectra (data not shown) demonstrated no significant difference across the sample range (4000 - 400 cm^{-1}), or when compared to a paint-coated glass slide, given that the strong absorbance bands of the emulsion paint polymer dominated over any signals attributed to the three different dyes within the systems. The similarity between the spectra of the three different dye-systems can be contributed to the high concentration of polymer present in each system, and the relatively low concentration of the dyes within these systems. The similarity between an untreated paint-coated sample and the dyed samples that were prepared for microbiological testing indicated that there was no effect on the polymer after the incorporation of the three dyes.

X-ray photoelectron spectroscopy was used to identify the presence of different elements in the paint-coated, one-dye, two-dye and three-dye systems, at various depths within the polymer. Peaks attributed to the presence of carbon, oxygen, nitrogen, sulphur, silicon and chlorine were observed, with minimal variation in the elemental analysis for all the elements observed, except for carbon, sulphur and nitrogen. There was no peak correlating to the presence of gold within the spectra, due to the very low concentration of nanoparticles encapsulated within the dye-systems. The peaks for carbon were detected at 284.8 - 289.2 eV for all four samples, suggesting these peaks were caused by an organic C 1s orbital.[39] The peaks for sulphur were detected at 168.3 - 169.6 eV for the three samples that had sulphur present in their spectrum, suggesting these peaks were caused by an organic sulphur species.[40] The peak for nitrogen was detected at 399.5 - 399.9 eV for all four samples, suggesting this peak was caused by an organic nitrogen species.[41]

XPS depth profile data indicated a significant variation in elemental content between the three different dye systems and the polymer during the initial 15 s of analysis. It appears that the elemental compositions of all four samples are almost identical thereafter for all the elements that were observed. The relatively small difference between the elemental compositions of the polymer and the three dye systems implies that the polymer's structure dominates at greater depths, and that the dyes are in low concentration within the bulk of the polymer matrix. The variation between the three dye systems and the polymer at a shallow depth (<15 s sputtering) indicates that the elemental compositions near the surface are mostly caused by the dyes within the three paint-dye samples. This suggests that there could be a greater dye concentration towards the surface of the polymer than in the bulk of the system. The variation is most notable in the carbon content of the samples; the polymer sample has a carbon content almost 20 % lower than the three different dye systems at the surface, as shown in Fig. 6. This difference between the undyed surface and the three dyed surfaces lessens with increasing depth, until the elemental content of the dyed samples converges to an elemental composition that is very similar to that of the polymer. This initial variation in elemental composition and subsequent convergence to an elemental composition similar to the polymer at a greater depth is also observed for nitrogen and sulphur, albeit to a lesser extent than for carbon. This trend for nitrogen and sulphur (data not shown) further indicates that the dyes do not penetrate deep within the polymer matrix, and that the majority of the dyes exist at the surface of the paint. This is ideal as the dye is expected to have greater antimicrobial activity at the surface.

3.3 Functional Testing

3.3.1 Water contact angle measurements. The water contact angles of the samples used in microbiology, as well as a paint-coated slide, were measured under standard laboratory temperature and lighting conditions (Table 1). It is immediately apparent that the three different dyed samples all possess a significantly larger water contact angle than both the control glass slide and the paint-coated glass slide. Although they differ greatly from these two samples, they are all relatively similar to each other, suggesting that any of these three dyes will enhance the hydrophobicity of the surface to a similar extent. The paint-coated sample is notably more hydrophilic than the

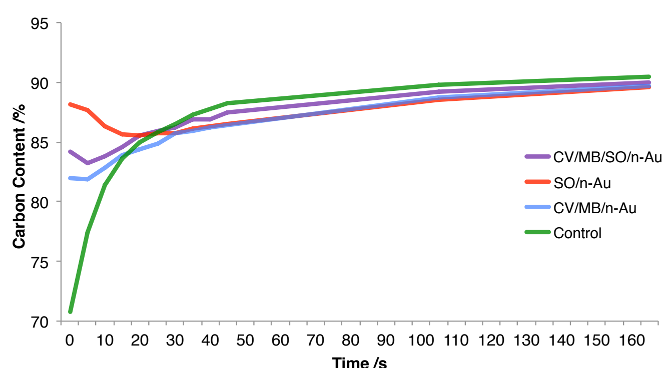

Fig. 6 The elemental content of carbon in the dye coated samples (CV/MB/SO/n-Au, CV/MB/n-Au, SO/n-Au) and a paint coated glass slide (control), obtained using XPS.

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Table 1 Average contact angle measurements (°) ± standard deviation of the >30 measurements made per sample, of water on the following glass coated surfaces: untreated glass slide, paint-coated slide, a painted slide coated with crystal violet, methylene blue, safranin O and incorporated with nano-gold (CV,MB,SO,n-Au), a painted slide coated with crystal violet, methylene blue, and incorporated with nano-gold (CV,MB, n-Au) and a painted slide coated with safranin O and incorporated with nano-gold (SO/n-Au).

Sample	Contact Angle / °
Glass Slide	44.4 ± 1.4
Paint-coated slide	28.4 ± 0.9
CV/MB/SO/n-Au	75.1 ± 1.0
CV/MB/n-Au	73.6 ± 1.3
SO/n-Au	79.6 ± 0.9

glass slide, implying that the copolymer emulsion paint reduces the hydrophobicity of the surface.

3.3.2 Leaching Study The release of the crystal violet, methylene blue and safranin O from a CV/MB/SO/n-Au sample (prepared as detailed in section 2.2.4) into aqueous solution (PBS), was measured spectroscopically as a function of time. The concentration of the dyes that leached from the polymer was established, by comparing the optical density of the surrounding aqueous solution at 665 nm, 596 nm and 534 nm with a calibration curve for all three of the dyes (Fig. 7). The figure indicates that all samples released a limited concentration of dye into solution upon immersion. This leaching can be attributed to weakly bound surface adsorbed dye. The concentration of dye that released into solution rapidly plateaued with time and over the period of more than 120 h immersion in solution no further leaching was noted, demonstrating stability of the dye within the polymer.

3.4 Microbiological Testing

The antibacterial activity of a series of samples was tested against a Gram-positive bacterium and a Gram-negative bacterium, *Staphylococcus aureus* and *Escherichia coli*, respectively. The antibacterial activity was activated by irradiating the samples with a white light source, at 3500 ± 250 lux. Another set of samples was stored under dark conditions for the same duration as the light irradiation time period (4 h). The photobactericidal activity of the following samples was tested: a control glass slide, a three dye system (crystal violet, methylene blue and safranin O, incorporated with 2 nm nanoparticulate gold), a two dye system (crystal violet and methylene blue, incorporated with 2 nm nanogold), and a one dye system (safranin O incorporated with 2 nm nanogold). A painted glass slide was not selected as the control sample due to its hydrophilic nature. When the inoculum was placed upon a paint coated slide, the fluid would sheet across the whole of the slide. After a four hour irradiation period, the fluid had completely evaporated from the surface. This issue persisted despite changes to the irradiation period. It was ultimately decided that a non-coated glass slide should be used as the control.

Fig. 8 shows the photobactericidal activity of the three paint-dye samples when tested against Gram-positive and Gram-negative bacteria. Under dark conditions, none of the samples showed antibacterial activity against the Gram-negative bacterium *E. coli*. However, the three-dye and two-dye systems exhibited highly significant lethal photosensitisation of the Gram-positive bacterium, *S. aureus*, under dark conditions (>4 ± 0.3 log reduction in bacterial numbers; $P < 0.001$). Although the one-dye system did not exhibit complete kill, there was statistically significant bactericidal activity compared to the control sample, when both were tested in the dark ($P < 0.001$). Unlike the dyed samples, there was no significant reduction in the counts of either bactericidal activity for the glass slide control.

Irradiation of the dye samples (white light at 3500 ± 250 lux, 4 h) resulted in highly significant complete lethal photosensitisation of both *Staphylococcus aureus* and *Escherichia coli* ($P < 0.001$), with bacterial levels reduced below the detected limit after 4 hours of irradiation (>4 ± 0.3 log reduction) for both Gram-positive and Gram-negative bacteria. There was no significant reduction in the counts of either *S. aureus* or *E. coli* for the glass control.

4 Discussion

In this study, several paint coated surfaces that were incorporated with different light-activated antimicrobial dyes were examined, with the aim of preparing a self-sterilising painted surface. It has been previously shown that light-activated dyes such as methylene blue, crystal violet and toluidine blue O can be immobilised into medical grade silicone polymers and into medical devices such as urinary catheters, and that these polymers demonstrate excellent antimicrobial activity upon irradiation with light.[14, 15, 29, 30, 32, 34] Research has also demonstrated that the incorporation of 2 nm gold nanoparticles into a polymer enhances the antimicrobial properties of the light-activated photosensitiser dyes.[29-31, 34] In this work, previous research was extended upon, and in particular the use of methylene blue and crystal violet within a polymer system was further explored. Moreover, the incorporation of 2 nm gold nanoparticles into the paint polymer systems was investigated. The incorporation of a third photosensitiser dye, safranin O, into the polymer was investigated, as this dye has been reported to exhibit efficacious antimicrobial properties *in vivo*,[35, 36] and in complex media.[37]

The procedure adopted to incorporate 2 nm gold nanoparticles into the copolymer emulsion paint involved a process that is comparable to the “swell-encapsulation” method devised by Noimark *et al.* to incorporate a photosensitiser species into a silicone polymer.[14, 17] In the “swell-encapsulation” method, the silicone polymer is immersed in an organic solvent (typically acetone), which has been saturated with a dye species. The solvent induces expansion of the polymer, allowing the dye species and nanoparticles to incorporate within the polymer matrix.[14, 17] In the procedure adopted in the development of the antimicrobial paint, a paint-coated glass slide is immersed in a solution containing the relevant dyes and 10 % v/v 2 nm gold nanoparticles, using water as the solvent. It is believed that water promotes the expansion of the copolymer emulsion, in a similar manner to the “swell-encapsulation" technique. This polymeric expansion allows the dye species and gold nanoparticles to incorporate into the polymer system.

X-ray photoelectron spectroscopy of the three dyed samples indicated that the there was a high concentration of the dyes that were incorporated within the polymer at the surface, and a dilute bulk concentration, indicating a disproportionately small amount of the dyes had penetrated into the bulk of the polymer matrix. An antimicrobial surface should typically possess a high concentration of antimicrobial agents towards the surface, rather than an even distribution throughout the polymer. A higher surface concentration and lower bulk concentration will yield the greatest kill of bacteria, given that an increased surface concentration of a photosensitiser species

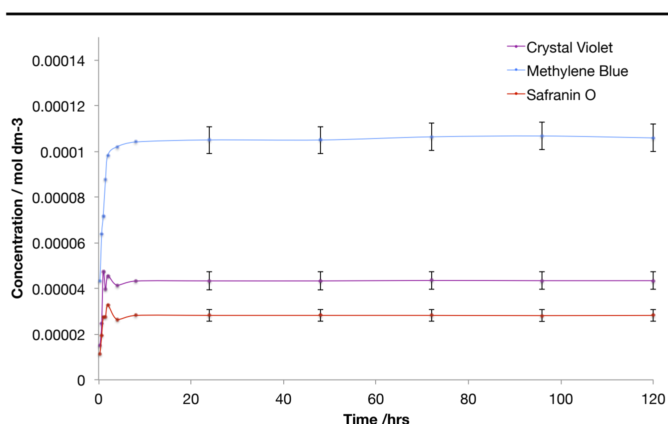

Fig. 7 Leaching of crystal violet, methylene blue and safranin O dyes (mol dm^{-3}) from a three dye sample that was prepared for microbiology, CV/MB/SO/n-Au. The sample was placed in PBS solution (15 mL), and the concentration of the solution was determined as function of time (hours).

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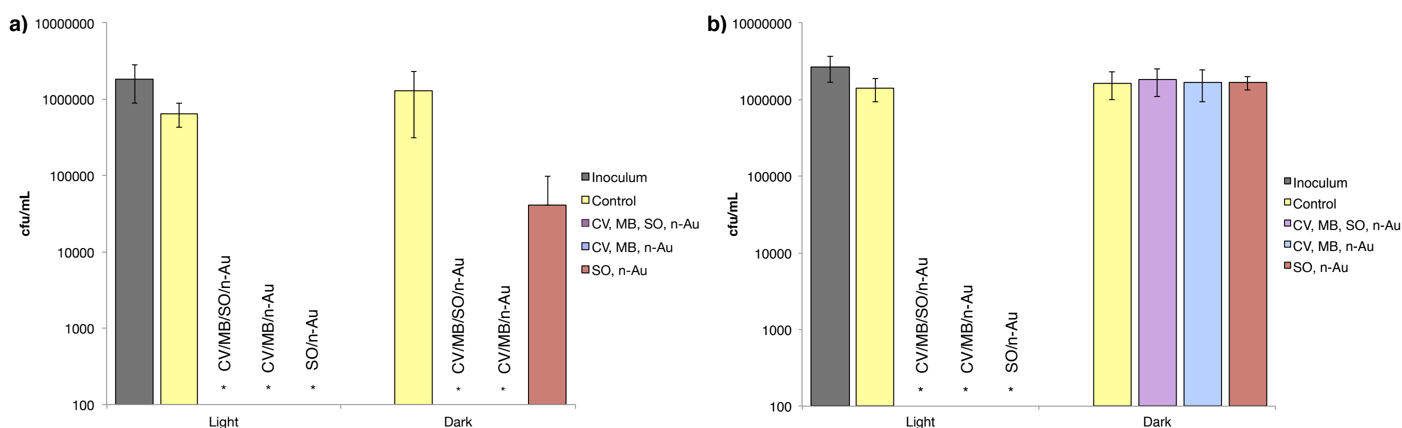

Fig. 8 Graph to show the lethal photosensitisation of (a) *Staphylococcus aureus* and (b) *Escherichia coli* upon irradiation with a white light source (3500 ± 250 lux for a 4 h period). The bars on the graph represent the initial inoculum, the control glass slide (control), the three dye system consisting of crystal violet, methylene blue and safranin O, incorporated with 2 nm nanogold (CV/MB/SO/n-Au), the two dye system consisting of crystal violet and methylene blue, incorporated with 2 nm nanogold (CV/MB/n-Au) and the one dye system consisting of safranin O, incorporated with 2 nm nanogold (SO/n-Au). The asterisk indicates where the bacterial counts are below the detection limit.

will yield a greater amount of reactive oxygen species at the surface, and therefore a greater bactericidal attack.

It is crucial that an antimicrobial paint does not leach or rub off upon contact with human skin tissue. The antimicrobial efficacy of the paint would be reduced upon instance of contact if the paint can be rubbed off, as there would be fewer antimicrobial agents on the surface. Moreover, there is an additional concern that the paint could potentially cause damage to human tissue if the dye were to rub off the surface and onto skin. Leaching studies demonstrated the polymer-embedded dyes are stable with respect to leaching. Limited release of dye into solution was noted after an extensive immersion time period and it can be speculated that the low concentration of dye released into solution was that which was weakly adsorbed to the polymer surface. The observation that these polymers are stable in solution, is significant for future applications in healthcare environments. However, if the situation arises that the dyes do leach out of the polymer upon human contact, for example, in push plate applications, it is vital that the photosensitiser and the photogenerated products do not induce the same phototoxic effects that are seen against the bacterial cells, on the host mammalian cells. *In vitro* studies analysing the instance of cell death for both mammalian and bacterial cells have demonstrated that an apparent therapeutic window exists, in which both irradiation time length and photosensitiser concentration only effectively destroy bacterial cells, and that mammalian cells do not experience similar damage.[14, 27, 42-46] These studies, coupled with the knowledge that the contact time periods between the paint surface and human skin are very short, suggest it is very unlikely that damage could occur to human skin cells if they were to come into prolonged contact with the dyes. Moreover, the reactive oxygen species generated by these dyes have a half-life of less than 1 μs,[29] with a reported diffusion distance of 10-100 nm. The size of an average human skin cell is 30 μm,[47] and so the likelihood that the singlet oxygen species will diffuse far enough to damage living human tissue is highly improbable. In the event damage does occur, it would be a short-term effect given that the outermost layer of the epidermis layer, the stratum corneum, consists of dead cells (corneocytes), which are constantly shedding. This continuous shedding means that the damaged cell would "shed" and be "replaced" by a non-damaged cell. Consequently, the toxicity of any antimicrobial paint towards mammalian cells should not be problematic in the potential use as a paint on contact surfaces in a clinical environment. Nonetheless, further investigations into the potential damage of the antimicrobial paint and its constituent dyes to mammalian cells are necessary in order to ascertain its suitability in a clinical setting.

Previous work carried out by Noimark anticipated that crystal violet and methylene blue would maintain their potency for ca. 10 years under luminous conditions equivalent to those in a hospital setting.[16] This particular study of the photostability of the species was conducted using a model that is similar to that conducted in our study of an antimicrobial paint; a dye or several dyes incorporated within a polymer. We therefore hope that the antimicrobial paint species developed in this study would exhibit a similar lifetime under constant illumination. It is important to note there are differences between the systems developed in this study, and those developed by Noimark *et al.*, particularly with regard to one of the dyes and the polymers that were used. There have not been any studies of the stability of safranin O under constant high-level luminous conditions, after it has incorporated within a polymer matrix, and it is vital for this to be further pursued before this dye can be considered as a possible component of an antimicrobial paint. Moreover, the stability of all three dyes within this specific polymer is vital to the paint's success, and should therefore be investigated.

In the UK, there are many stipulations that contractors must abide by when they design and fit new facilities in a clinical setting. Some of these stipulations include ensuring door handles, fingerplates and kick boards are made of stainless steel, or are significantly different in colour from the door itself.[48] There are further stipulations that any switches, controls, buttons of any kind are highly contrasting in colour to their surroundings, in order to assist those who are visually impaired.[48] These regulations also suggest walls and ceilings should vary in colour from the floors. The highly coloured nature of the paint developed in this study can be considered advantageous when it is evaluated as a commercial product in a clinical setting, given that it will compliment the regulations set forth by the body responsible for the maintenance and construction of the National Health Service in the UK, NHS Estates.

It is estimated that around 80 % of all nosocomial infections are transmitted by touch,[3] and that almost one third of these infections could be prevented, if correct infection control protocol were followed.[49] Frequently-touched surfaces act as a reservoir for the spread of infection and a multitude of different pathogenic species can exist on surfaces within a hospital ward. The Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus* are the most prolific bacteria found on these surfaces,[50] and are also common causes of nosocomial infection.[51] This study has demonstrated the effective destruction of both *S. aureus* and *E. coli* under light conditions. In the potential application of these paint-dye systems, to coat frequently-touched surfaces in a clinical setting, the antimicrobial properties of the photosensitiser dyes will be constantly activated by continued irradiation by the white light sources that illuminate the rooms within the building. Should a room not remain illuminated for all periods of the day, there would still be a continuous efficacious dark kill against *S. aureus*.

It has been previously found that the phenothiazine dye methylene blue and the triaryl methane dye crystal violet demonstrate significant antimicrobial efficacy against both Gram-positive and

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Gram-negative bacteria upon laser irradiation, and effect limited destruction of the Gram-positive bacterium *Staphylococcus epidermidis* in dark conditions.[14, 15, 17] It has also been found that the light-activated antimicrobial action of these two dyes is enhanced upon incorporation of 2 nm gold nanoparticles into the polymer system.[14, 15, 29, 30, 34] EPR spectroscopy has suggested that this enhancement is due to an increase in the dye triplet state production in the presence of 2 nm gold nanoparticles.[14] In previous studies, the polymer used was a medical grade silicone polymer, and the dyes and nanoparticles were encapsulated via a novel “swell-encapsulation” method. In this study, the polymer used is an emulsion-based paint, and the dye was incorporated using a similar technique, whereby the polymer was immersed in a solution containing the dyes and nanoparticles, and these dyes were left to incorporate into the polymer matrix over a specified period of time. Consequently, in this study, the copolymer emulsion paint was incorporated with both crystal violet, methylene blue and 2 nm nanogold in order to determine if similar antimicrobial effects can be achieved with an alternative polymer system. Further to this system, the antimicrobial properties of an additional photosensitiser dye, safranin O, was studied, in order to ascertain whether a similar effect can be achieved using a single photosensitiser dye coupled with nanogold. This dye was also included in a third dye-paint system that was comprised of safranin O, along with the two other aforementioned dyes, crystal violet and methylene blue, in order to determine if a system containing three photosensitiser dyes could enact total destruction of both Gram-positive and Gram-negative bacteria in light and dark conditions. It should be noted that an emulsion paint copolymer sample encapsulated with 2 nm gold nanoparticles was not tested as a control, as it has been previously shown that samples consisting of 2 nm gold nanoparticles alone do not demonstrate any antimicrobial activity.[29]

The complete lethal photosensitisation of both Gram-positive and Gram-negative bacteria was observed in light conditions on the safranin O-nanogold samples. Furthermore, a significant reduction in the number of Gram-positive bacterium *Staphylococcus aureus* was observed under dark conditions with 1.5 ± 0.1 log kills achieved following a 4 h time period. Safranin O has historically been used as a biological stain in histology and cytology, given its propensity to colour all cell nuclei red.[52] In more recent years, this dye has been reported to exhibit potent antimicrobial properties with reported bactericidal behaviour in complex media such as blood serum, blood and saliva.[35]

Upon irradiation with light, all three dye systems, CV/MB/SO/n-Au, CV/MB/n-Au and SO/n-Au demonstrated lethal photosensitisation of both Gram-positive and Gram-negative bacteria, with a >4 ± 0.3 log reduction in bacterial numbers for all three dye systems. A limited reduction in bacterial numbers was noted for all three samples against the Gram-negative bacterium *E. coli*, in dark conditions. Although there was little effect observed on *E. coli* in the dark, the three-dye and two-dye systems were able to reduce the numbers of *S. aureus* to below the detection limit in the dark. The lesser susceptibility of *E. coli* to these three samples is unsurprising, given the structural differences in the Gram-positive and Gram-negative bacterial cell walls, and has been observed previously.[14, 24, 53]

It is apparent that all three of the paint-dye samples exhibit exceptional photobactericidal activity. The kills observed against *E. coli* are at least 1.9 (± 0.3) log greater than any noted in previous research involving a polymer that has been incorporated with a photosensitiser, and that has been irradiated with a white light source for a 3-4 hour period.[16] It is important to consider the fact that the irradiation time period of the samples in this report is 1 hour greater than the irradiation period used by Noimark et al., who achieved a 2.1 log kill against *E. coli.*[16] It is also worth noting that Noimark et al. reported a 4 log reduction in the numbers of *E. coli* after a 6 hour irradiation period.[16] This reduction implies that the antimicrobial paint product that has been developed exhibits a light-induced reduction in *E. coli* that is at least as great as the reduction reported by Noimark et al., if not greater.

The kills observed against *S. aureus* upon illumination of the systems, are significantly greater than those reported by Decraene et al. against *S. aureus* using a similar polymer system.[54] In their study two photosensitiser species, toluidine blue O and rose bengal, were incorporated into cellulose acetate, before the samples were inoculated with the organism and irradiated with a white light source for 6 h.[54] Decraene et al. reported a 2.7 reduction in the bacterial count after this time period.[54] The kill we report us at least 1.3 ± 0.3 log greater than this, despite the fact we have used a shorter irradiation period of 4 h. The dark kill against *S. aureus* is significantly greater than any other dark kill previously reported for a dye that has been incorporated within a polymer, with kills at least 2.1 (±0.3) log greater than those observed.[16] Despite not effecting complete kill against *S. aureus* under dark conditions, the photobactericidal activity of the safranin O-nanogold sample is still significant, with a 1.5 (± 0.1) log kill.

A light-activated mechanism of bacterial deactivation and destruction justifies the observed kill for the complete lethal photosensitisation of both the Gram-positive and Gram-negative bacteria under light conditions, however it does not explain the kill observed against *S. aureus* under dark conditions. The most probable cause for the dark kill is due to the inherent cytotoxic nature of the photosensitiser species against bacterial cells, and the enhanced effect the 2 nm gold nanoparticles have on these dyes.

There are growing concerns within the scientific community over the great acceleration in bacterial resistance to antibiotics in recent years.[55] Currently, more powerful antibiotic drugs are being prescribed in order to address this issue.[56] There is an increased demand for new, more potent antibiotics, and with a continued decline in their discovery, it is inevitable that bacterial resistance will exhaust all antibiotics on offer,[56] and that mankind may soon face a post-antibiotic era.[57] Due to the mechanism of multi-site attack associated with the strategy adopted in this study, it is anticipated that inducing further bacterial resistance will not be an issue for these antimicrobial paint systems.

5 Conclusions

In this study we have successfully incorporated crystal violet, methylene blue, safranin O and 2 nm gold nanoparticles into a copolymer emulsion paint using a simple immersion technique. The procedure was refined in order to ensure an even coating of the paint polymer on the surface and an even distribution of the dye and nanogold throughout the polymer. The three dyes were shown to display a moderate level of leaching under aqueous conditions over an extended period of time. XPS analysis suggested that there was a large amount of the dye molecules at the surface of the polymer, and that the bulk concentration was relatively low. This conclusion is in line with previous findings by Noimark et al., where fluorescence imaging detailed the high surface uptake of dyes into the polymers. This could suggest that a similar uptake may have occurred with the antimicrobial paint that has been developed.[33]

All three paint-dye systems demonstrated lethal photosensitisation of both Gram-positive and Gram-negative bacteria in light conditions, with the bacterial count reduced below the detection limit for both bacteria (>4 ± 0.3 log kill). To the author’s knowledge, the light kill observed against *E. coli* and *S. aureus* are as great as any previously reported kill. Moreover, both CV/MB/SO/n-Au and CV/MB/n-Au were shown to effect complete photodestruction of the Gram-positive bacteria in dark conditions as well (>4 ± 0.3 log kill). SO/n-Au was shown to effect significant antimicrobial activity under dark conditions, despite a lower concentration of leaching (1.5 ± 0.1 log kill). Indeed, to the author’s knowledge the dark kill demonstrated by CV/MB/SO/n-Au and CV/MB/n-Au are the greatest dark kills of a Gram-positive bacterium that we have achieved to date, demonstrating the promising potential of this antimicrobial paint product. It is hoped that these paint systems may potentially reduce the instance of nosocomial infections within a clinical setting, through the reduction of nosocomial infections that are transmitted by touch.

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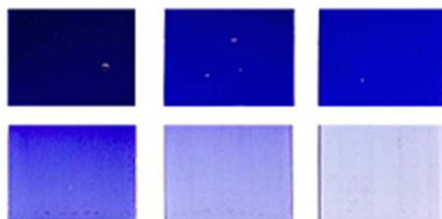

Crystal Violet, Methylene Blue and Safranin O were successfully incorporated into a co-polymer emulsion to make a potent antimicrobial paint.
80x39mm (72 x 72 DPI)

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