White Light-Activated Antimicrobial Paint using Crystal Violet

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Abstract

Crystal violet (CV) was incorporated into acrylic latex to produce white light activated antimicrobial paint (WLAAP). Measurement of the water contact angle of the WLAAP showed that the water contact angle increased with increasing CV concentration. In a leaching test over 120 h, the amount of CV which leached from the WLAAPs was close to the detection limit (<0.03 %). The WLAAPs were used to coat samples of polyurethane and these showed bactericidal activity against *E. coli* which is a key causative agent of healthcare-associated infections (HAIs). A reduction in the numbers of viable bacteria was observed on the painted coated polyurethane after 6 h in the dark and the bactericidal activity increased with increasing CV concentration (P <0.1). After 6 h of white light exposure, all of coated polyurethanes demonstrated a potent photobactericidal activity and it was statistically confirmed that the WLAAP showed better activity in white light than in the dark (P <0.05). At the highest CV concentration, the numbers of viable bacteria fell below the detection limit (<10^3 CFU/mL) after 6h of white light exposure. The difference in antimicrobial activity between the materials in the light and dark was 0.48 log at CV 250 ppm and it increased by 0.43 log at each increment of CV 250 ppm. The difference was the highest (>1.8 log) at the highest CV concentration (1000 ppm).These WLAAPs are promising candidates for use in healthcare facilities to reduce HAIs.

Keywords: crystal violet, acrylic latex, *E.coli* bacteria, white light-activated antimicrobial paint, water contact angle

1. Introduction

Healthcare-associated infections (HAIs) occur in surgery centers, dialysis centers, and long-term care facilities such as nursing homes, and community clinics [1]. Their effects vary from simply discomfort for patients to prolonged or permanent disability and in some cases death [2]. According to the US Center of Disease Control and Prevention (CDC) in the US, approximately 4 % of inpatients acquire HAIs, equivalent to 728,000 infections per year, with 75,000 of these patients dying during hospitalization [3, 4].

Since 2008, the CDC has implemented many strategies, including surveillance, inspection, and improved guidelines to reduce the rate of HAIs [5-10]. In particular, significant effort has been dedicated to prevent central line-associated bloodstream infections (CLABSIs), catheter-associated urinary tract infections (CAUTIs), and surgical site infections (SSIs), meticillin-resistant *Staphylococcus aureus* (MRSA) infections, and *Clostridium difficile* infections. Despite these actions, a reduction in infection rates was often not achieved, and in some cases, even increased [11].

Contaminated surfaces in healthcare facilities make an important contribution to HAIs. Pathogens are capable of surviving on surfaces of healthcare facilities for several weeks and they can be transmitted through touch by healthcare workers and patients [12, 13]. Vigorous cleaning and disinfection schemes can reduce transmission of healthcare associated pathogens [14, 15]. However, routine cleaning and disinfection is unable to completely remove the pathogens, which are adhesive and may reproduce rapidly in favourable conditions [16, 17]. Thus, it is necessary to develop alternative techniques to prevent or reduce pathogen transmission in healthcare facilities.

Use of antimicrobial surfaces is considered an alternative approach to inhibit the spread of HAIs in healthcare facilities as an additional measure to cleaning [18, 19]. Many techniques have

been suggested for application to soft and hard surfaces, including silver or copper coated surfaces [20-22], self-cleaning surfaces [23, 24] and enzyme or bacteriophage immobilized surfaces [25, 26]. In addition, the incorporation of light-activated antimicrobial agents (LAAAs) into surfaces is considered a promising technique [27-29]. Titanium dioxide (TiO_2), a well known LAAA, is capable of killing a range of microorganisms including bacteria, fungi, viruses, and algae, and it has been studied for application in medical devices such as film, glass, and polymers [30-33]. The antimicrobial activity of TiO_2 is frequently ascribed to $OH^{-\bullet}$ radicals and other reactive oxygen species (ROS) induced by the light source, causing damage to the bacterial cell membrane and cell wall [33]. However, TiO_2 demonstrates negligible photobactericidal activity under white light conditions as it is activated by ultraviolet (UV) light which comprises only a small proportion of the total radiation in sunlight and is virtually absent in artificial white lighting [34, 35]. In order to address this problem, many studies was recently performed to enhance photocatalytic efficiency and white light utilization of TiO_2 including using impurity dope and metallization [36-38]. Previous studies showed that incorporation of vanadium or silver and bromine extended photocatalytic range of TiO_2 from the ultraviolet to the visible region and they showed enhanced antimicrobial activities on bacteria under white light condition [39, 40].

Crystal violet (CV), toluidine blue O (TBO), and methylene blue (MB) dyes, which are widely used in histological stains and for bacterial classification [41, 42], are also known LAAAs showing photobactericidal activity in white light. The dyes generate singlet oxygen ($^1O_2$) and ROS when they are exposed to the light source [43], and the $^1O_2$ and ROS cause adverse effects in bacteria such as loss of membrane integrity, inactivation of enzymes and DNA damage resulting in cell death [44-46]. The generation of $^1O_2$ and ROS is directly related to light intensity [47, 48]. In addition, the dyes have somewhat lower or no antimicrobial activity in the absence of

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light [49-51]. The antimicrobial activity of CV, TBO, and MB dyes is dependent on their concentration, the exposure time, and the intensity of the light source [52, 53]. Recent studies from our group have investigated the application of CV, TBO, and MB dyes to polymers to generate antimicrobial surfaces for use in medical devices including tracheal or urinary catheters and tubes for intravenous drips [54, 55]. We have shown that the dyes can be readily impregnated into polymers using a swell-encapsulation-shrink process, and the polymers showed potent photobactericidal activity in white light [44, 55]. In addition, incorporation of gold (Au) nanoparticles into a dye impregnated polymer significantly enhanced the photobactericidal effect [54, 56].

Although the techniques for synthesis of new materials mentioned above resulted in reasonable antimicrobial activity, most of them involve a multi-step process or long-term treatment for application to surfaces in healthcare facilities, and they are not applicable to all materials. For example, it is difficult for the chemical vapor deposition technique to be applied to heat susceptible materials, and the swell-encapsulation-shrink process can be applied only to soft polymers such as polyurethane and silicon. In the present study, a white light activated antimicrobial paint (WLAAP), which can be easily applied to surfaces in healthcare facilities, was developed. The WLAAP consisted of CV and a commercial acrylic latex which is commonly used as a component of household paint. The paint was characterized in terms of UV-vis absorbance spectra and water contact angle. The novel WLAAP was used to coat samples of polyether based thermoplastic polyurethane (PTPU) and antibacterial testing of these samples against *E. coli* showed that the WLAAP possessed significant activity in the dark and enhanced activity in the presence of white light.

2. Materials and Methods

2.1. Preparation of light activated antimicrobial paint

In this study, CV, which is a triarylmethane dye (Figure S1: Supporting information), were used to produce a WLAAP. As shown in Figure 1 (a), 100 mg of CV (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 10 mL of DI water and sonicated for 5 min to produce a CV solution of 10,000 ppm. With stirring for 5 min, the solution was mixed with acrylic latex (X935-33, AzkoNovel, Amsterdam, Netherlands) which is a copolymer of vinyl acetate and butyl acrylate. The CV paint solutions were prepared with various CV concentrations: CV 250, 500, 750, and 1000 ppm.

2.2. Characterization of crystal violet paint

For characterization of UV-vis spectra and equilibrium water contact angle of the CV paint, 1.5 mL of the paint was gently injected onto the surface of glass slides (2.5×7.5 cm^2), and air-dried for 24 h in the dark. The coating thickness was approximately 0.3 mm. The UV-vis absorption spectra for the paint coated slide glasses were measured using a UV-Vis Spectrometer (PerkinElmer Inc., Winter St., CT, USA). Absorption was measured from 400 to 900 nm.

The equilibrium water contact angle on the paint coated samples was measured. A droplet (~5 µL) of deionised water was dropped onto the surface of the samples by gravity from a gauge 27 needle, the samples were photographed side on and the images was analyzed using Surftens 4.5 software.

2.3. Leaching test

The stability of CV paint in solution was tested. Polyether based thermoplastic polyurethane (PTPU, American Polyfilm Inc, Branford, CT, USA) samples (1.5×1.5 cm^2) were coated by 0.6 mL of CV paint, and air dried for 24 h in the dark. The coated PTPU samples were placed in 5 mL of PBS solution for 120 h. UV-vis spectra of the solutions were periodically measured to determine if CV was leaching from the paint into the surrounding solution. The comparison

of the absorbance at 590 nm of PBS with a CV calibration curve enabled the determination of the released CV concentration.

2.4. Antimicrobial testing

For antimicrobial testing, the CV paint coated PTPU samples were used. The antimicrobial activities of the samples were tested against *E. coli* ATCC 25922. The bacteria were stored at -70°C in Brain-Heart-Infusion broth (Oxoid Ltd., Hampshire, England, UK) containing 20% (v/v) glycerol and propagated on MacConkey agar (Oxoid Ltd., Hampshire, England, UK). 10 mL of BHI broth was inoculated with one bacterial colony and cultured at $37\,^{\circ}$C with shaking at 200 rpm. After incubation for 18 h, the bacteria were harvested by centrifugation ($21\,^{\circ}$C, 4000 rpm for 20 min), washed using 10 mL of phosphate buffered saline (PBS), and centrifuged again to recover the bacteria which were re-suspended in 10 mL of PBS. The washed suspension was diluted 1000-fold in order to obtain ~10^6 colony forming units per millilitre (CFU/mL). As shown in Figure 1 (b), 25 µL of bacterial suspension was inoculated on to the surface of each sample and a sterile glass cover slip (2.2×2.2 cm^2) was place on top to ensure good contact between the bacteria and the surface of the sample. The samples were loaded into petri dishes containing wet filter paper to maintain humidity, and exposed to white light, while a control set of samples was kept in the dark. After light exposure, the samples were place into 5 mL of PBS, and mixed using a vortex mixer for 1 min. The bacterial suspension was diluted, plated onto MacConkey agar, and incubated at $37\,^{\circ}$C for 24 hours. The colonies that grew on the plates were counted.

2.5 Statistical analyses

Correlation coefficients, linear regressions, and t-statistics of experimental data were calculated using the SPSS statistical software, version 12.0 (SPSS, Inc., Chicago, IL, USA).

3. Results and discussion

Various ratios of CV solution and acrylic latex (polymer) were employed to make WLAAPs, as shown Figure 2 (a). The paint changed from white to bright violet by adding CV into the acrylic latex, and the colour became more intense with increasing CV concentration. Figure 2 (b) shows the paint coated glass slides. Glass slides were coated by the paints containing different quantities of CV, and then air-dried for 24 h in the dark. After drying, the acrylic latex (control) changed from white to colourless, and the CV incorporated paints maintained their violet colour. However, in contrast with the solution state samples, the intensity of the paints did not increase with increasing CV concentration (250 to 750 ppm).

The UV-vis absorbance spectra of control and CV paints were measured over the wavelengths 400–900 nm. Since the absorbance values of paints containing >CV 250 ppm exceeded the detection limit of the UV-Vis Spectrometer, 10-fold diluted CV paints were used in this measurement. As shown in Figure 3, the colour intensity of the paints appeared lower after dilution. The absorbance spectra showed that all of the CV paints gave a main absorption at 590 nm, and the peak intensity and spectrum range of absorbance increased with CV concentration. The spectrum curves of the CV paints were different from the results of previous studies; the absorbance spectrum of the CV paint gave a normal distribution whereas the spectrum of CV incorporated into polymers presented bimodal distribution [54, 56]. This is probably due to difference in the solvents used. Depending on the type of solvent such as water, acetone, and methanol, the CV absorbance spectrum can change with different peak in the wavelength range of 500-650 nm [57]. Regardless of the CV concentration in the paint, all of the paints showed comparable spectra in agreement with results of a previous study [58].

In order to investigate the change of physical property by combination of crystal violet and acrylic latex, the water contact angle of control and CV paints was measured. As shown in Figure 4, the control gave water contact angles of 10.9 °. However, the contact angle increased

with CV concentration (y = 0.0042x + 10.449, R^2 = 0.8487), and it was the highest at CV 1000 ppm. Compared to the control, the highest increase was approximately 4.2 $^\circ$ at the highest CV concentration (P <0.01). This is explained by the fact that the increase of the CV portion in acrylic latex contributed to the change of water contact angle because CV is a relatively hydrophobic compared to the acrylic latex (control).

The CV release from the CV paint in PBS solution was determined using a UV-vis spectrometer. The CV paint coated PTPU samples were immersed in 5 mL of PBS solution for a total of 120 h and the CV release was measured at intervals of 24 h. Figure 5 shows the CV release from the paint coated samples to PBS solutions for 120 h. It was confirmed that all of paints released some CV into PBS solution after 24 h immersion and that the CV concentration was the highest (0.27 ppm) for the paint containing CV 1000 ppm (0.04, 0.07, and 0.14 ppm from paints containing CV 250, 500, and 750 ppm, respectively). Over a period of more than 100 h, additional CV leaching was not detected in all of the CV paints. The total amount of CV leached from the paints over a period of 120 h was minor (<0.03 %).

The antimicrobial testing of CV paint under white light and dark conditions was conducted against *Escherichia coli* which is a key causative agent of HAIs. 25 µL of this bacterial suspension, containing 7.8×10^4 CFU, was inoculated on to the surface of CV paint coated PTPU and then exposed to white light or placed in the dark at 20 $^\circ$C for 6 h. The intensity of white light was measured at a distance of 30 cm from the lamp using a lux meter (LX-101, Lutron Inc., Coopersburg, PA, USA). The light intensity was the highest at the center and it decreased with increasing distance from the center. The intensity of the light ranged from 3900 to 5300 lux (Figure S2).

CV dye used in this study as WLAAA has antimicrobial and antifungal activities. It inhibits bacterial growth through interaction between positive (GV^+) ions dissociated from CV and

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components of bacterial cells including lipopolysaccharide in the outer membrane of Gram negative bacteria, peptidoglycan and DNA [50, 59]. As shown in Figure 6 (a), the combination of CV and acrylic latex showed antimicrobial activity in the dark. After 6 h in the dark, a 0.12 log reduction in the numbers of viable bacteria was observed for CV 250 ppm, compared with the control, and the numbers of viable bacteria decreased with increasing CV concentration ($R^2 =$ 0.996, P <0.1) in the paint: 0.31 and 0.49 log reductions were obtained at CV 500 and 750 ppm, respectively, and at CV 1000 ppm, a 1.21 log reduction in the numbers of viable bacteria was observed. These results indicate that CV keeps its intrinsic antimicrobial property after being mixed with acrylic latex.

Figure 6 (b) shows the antimicrobial activities of CV paint in white light. As in the dark, the reduction in the numbers of viable bacteria remaining increased with increasing CV concentration after 6 h white light exposure ($R^2 = 0.811$, P <0.1). However, there was a statistically significant difference in the total bactericidal activity displayed by the materials in the light compared to the dark (comparison of all the concentrations in the light and dark: P <0.05). After 6 h of white light exposure, compared with the control, 0.36, 0.43, and 0.86 log reductions in the numbers of viable bacteria were observed at CV 250, 500, 750 ppm, and at CV 1000 ppm, the numbers of viable bacteria fell below the detection limit (<10^3 CFU/mL). Further, the difference in antimicrobial activity between the materials in the light and dark increased with increasing CV concentration in the paint. The difference was 0.48 log at CV 250 ppm and it increased by 0.43 log at each increment of CV 250 ppm. The difference between white light and dark conditions was the highest (>1.8 log) at the highest CV concentration (1000 ppm).

The increase in antibacterial activity of the CV paint in white light can be explained as follows: upon white light irradiation of CV paint, CV molecules inside the paint are excited to

a triplet state via an intersystem crossing from a slightly higher energy, shorter lived excited singlet state. The triplet state dye molecules can undergo one of two photochemical pathways [44]; quenching by molecular oxygen including the production of singlet oxygen ($^1O_2$), or interaction with biomolecules in the vicinity generating radical species. The generated $^1O_2$ and radical species can initiate multi-site attack on bacteria in the vicinity effecting cell death [55].

Although previous techniques have showed a good antibacterial activity when applied to surfaces, these techniques, including chemical vapor deposit, swell encapsulation shrink, and physiochemical modification, are not applicable to all substrates. WLAAP developed in this study can be easily applied for a wide range of substrates including paper, metal, glass, and hard and soft polymers (Figure S3). Moreover, WLAAP is readily fabricable and surface treatment using WLAAP is simple and easy. It is expected that WLAAP could be used for clinical care facilities including surgery centers, dialysis centers, and nursing homes and also applied in the home for decoration, and in toilet and kitchen surfaces. For real world application, adverse effects of the paint should be considered. CV has been widely used in medicine for over 100 years [50]. It poses a very low toxicity risk even in case of administration of high quantities [50, 60], however, it has been used in a diluted form as an antiseptic for external wounds for a century. Previous studies showed that CV is a useful agent for the elimination of MRSA on the skin [61, 62], and it did not produce any serious side effects or mild skin irritation [62]. The WLAAP showed it in solution leaching test that <0.28 ppm of CV was released from paints containing CV 250, 500, 750, 1000 ppm. This is much less than the concentration (lethal dose 50%: 420 ppm) representing a toxicity in rat oral test [63]. Moreover, the amount of CV used in WLAAP was relative minor compared to the concentration (20,000 ppm) causing dermal irritation or sensitization in long term clinical assessment [64]. Thus, it is unlikely that the WLAAP we report here will show toxicity towards humans.

4. Conclusion

In this study, CV was incorporated into acrylic latex which is commonly used in household paint to produce WLAAP for healthcare or household applications. The combination of CV and acrylic latex resulted in a potent WLAAP that not only induced the lethal photosensitization (below detection limit at CV 1000 ppm) of *E. coli* within 6 h, but demonstrated strong bactericidal activity (1.21 log reduction in the numbers of viable bacteria at CV 1000 ppm) in the dark. Moreover, the CV in the WLAAP was shown to be stable over a 120 h period showing minimal leaching into the surrounding solution. It is anticipated that this potent WLAAP will be useful in healthcare applications to reduce surface bacterial contamination and thus the incidence of HAIs.

In this study, we did not investigate the amount of generated $^1O_2$ and ROS when WLAAP is exposed to what light source, and that which wavelength maximizes the generation. This is important to maximize the antimicrobial efficiency of WLAAP under light condition. Thus, we are considering an experiment of quantum efficiency measurement in future study.

Supporting information

Figure S1. Chemical structure of crystal violet.
Figure S2. Intesity distribution of white light.
Figure S3. Image of white light-activated antimicrobial paint coated metal, glass paper, and polymers.

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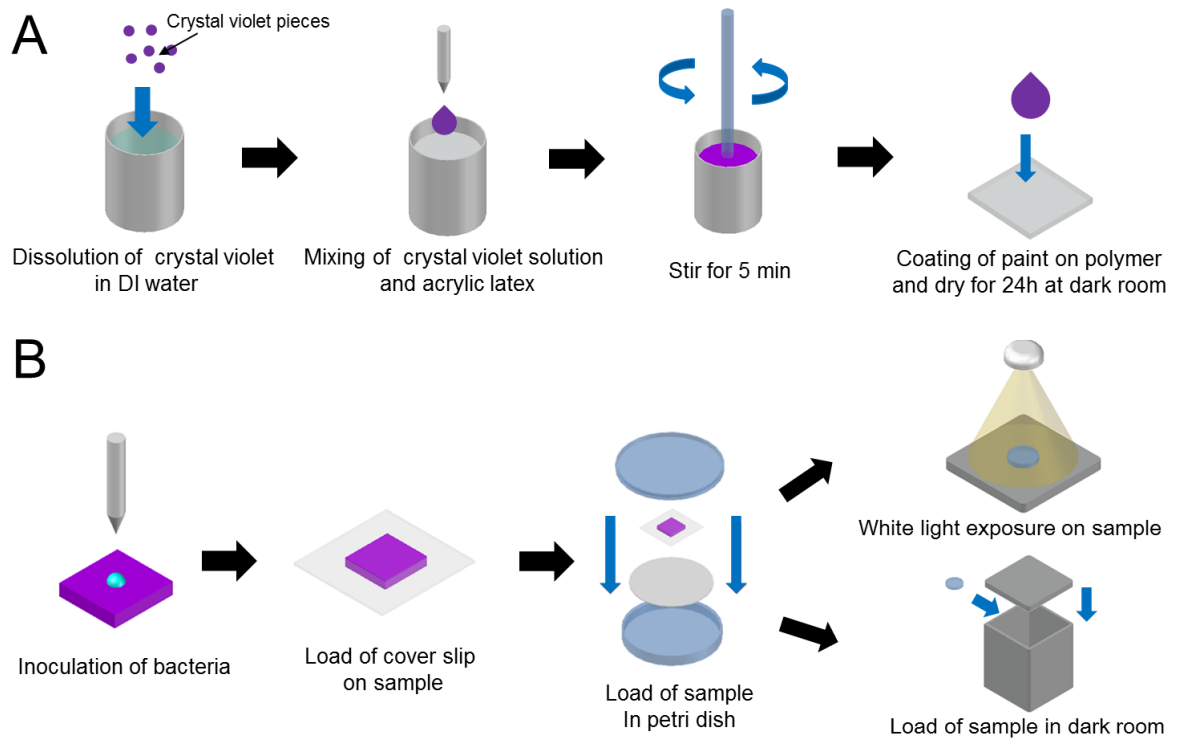
Figure legends

Figure 1. (a) Preparation of white light activated antimicrobial paint and (b) antimicrobial testing.

Figure 2. (a) Control and crystal violet paint solutions, and (b) the paint coated glass slides

Figure 3. UV-vis absorption spectra of control and crystal violet paints. The paints containing crystal violet 0, 25, 50, 75, and 100 ppm were coated to glass slides, and they were measured within the wavelengths 400–900 nm.

Figure 4. Water contact angles on the paints containing crystal violet 250, 500, 750, and 1000 ppm. The paints were coated to polyurethane samples (1.5×1.5 cm^2) and water contact angles, and then water contact angle on them were measured.

Figure 5. Leaching testing of crystal violet paints in PBS solution. The paints were coated to polyurethane samples (1.5×1.5 cm^2). The samples were immersed in 5 mL of PBS for 120 h.

Figure 6. Antimicrobial activities of control and crystal violet paints on *E. coli* bacteria: (a) 6 h incubation in the dark, and (b) 6 h incubation in white light. Polyurethane samples (1.5×1.5 cm^2) were coated using crystal violet paint. *E coli* was placed in contact with the samples and exposed to light intensity ranging from 3900 to 5300 lux. In all tests, the temperature was maintained at a constant 20 $^{\circ}$C in an incubator.

Figure S1. Chemical structure of crystal violet

Figure S2. Intensity distribution of white light which the paint coated samples are irradiated to. The intensity was measured at a distance of 30 cm from 28 W hospital fluorescent lamp using a lux meter. Colour scale bar corresponds from low (light blue) to high light intensity (light purple).

Figure S3. White light-activated antimicrobial paint coated (a) metal (aluminum), (b) glass, (c) paper (d) hard polymer (polystyrene), and soft polymer (polyurethane) plates.

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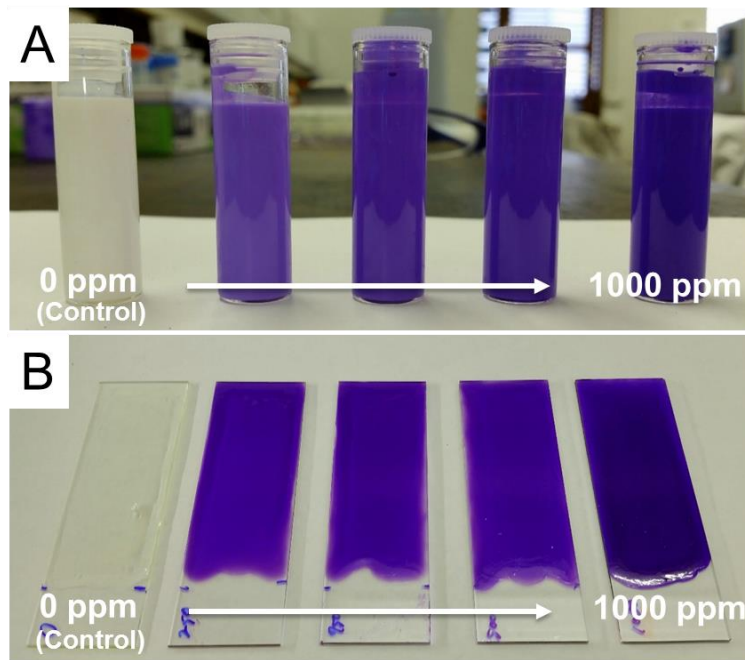
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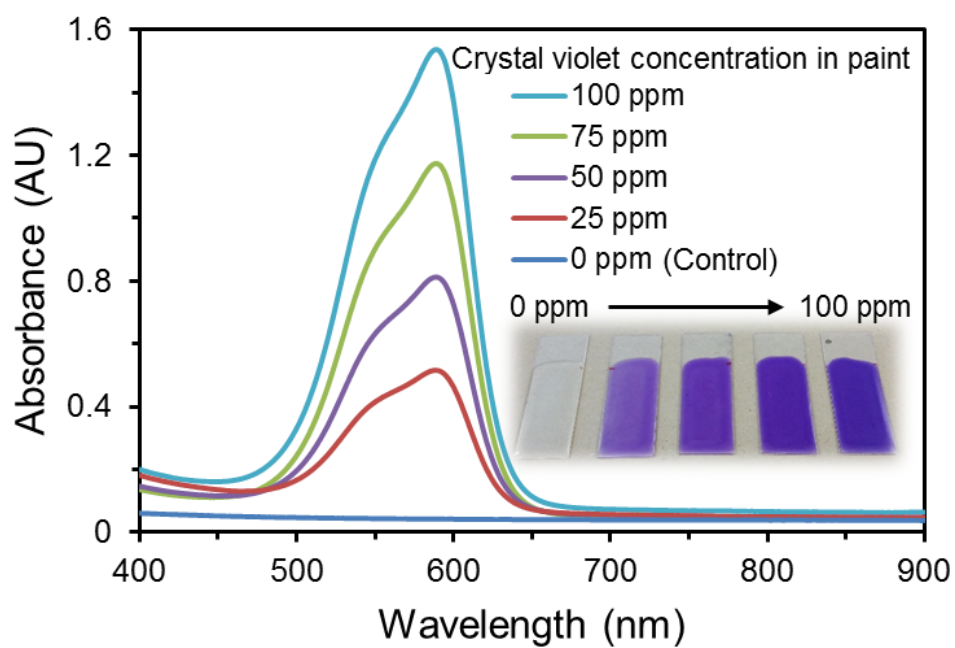
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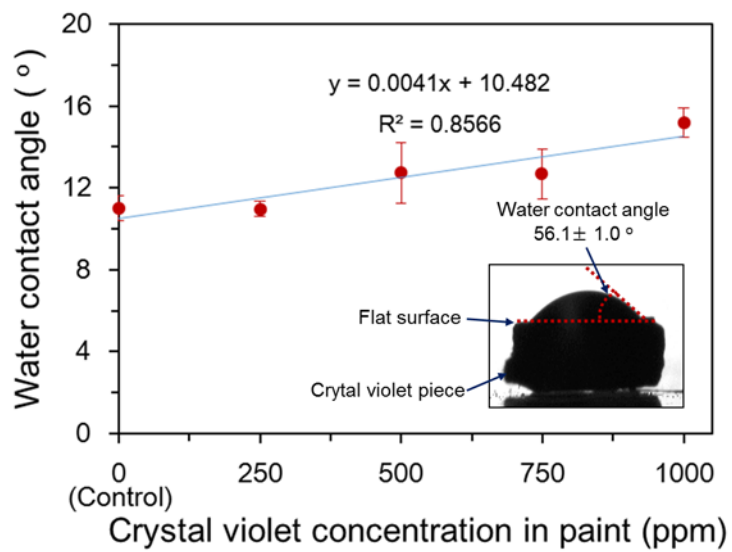
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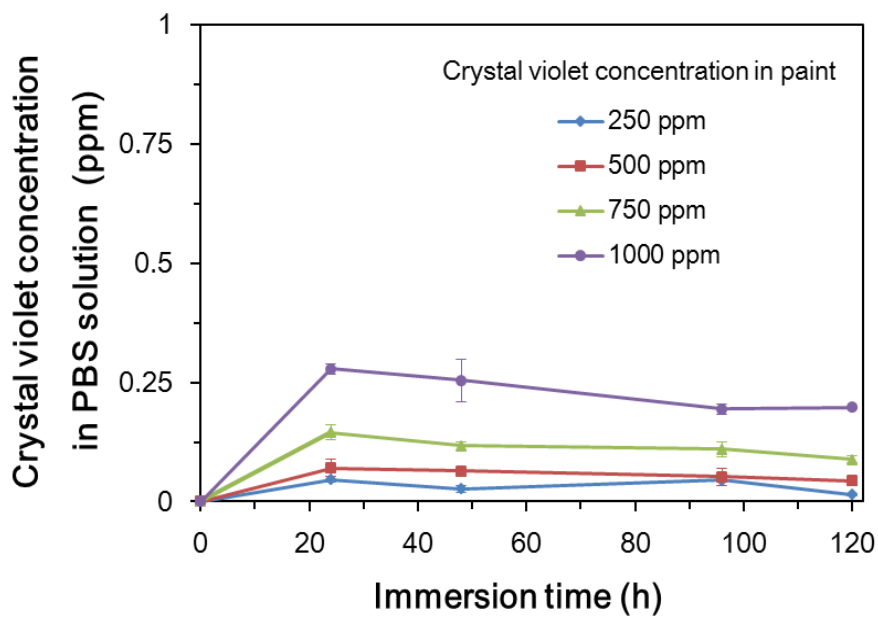
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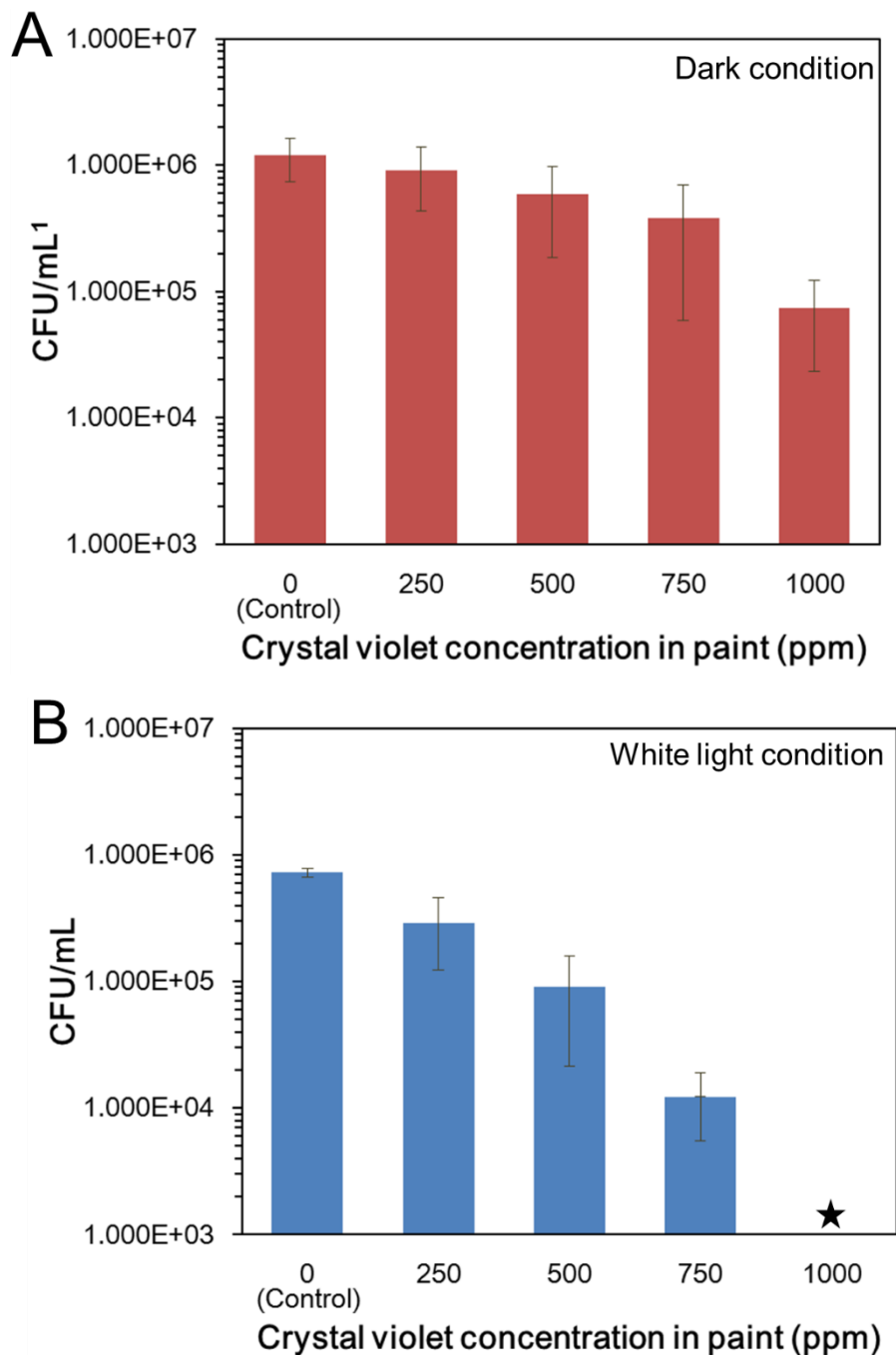
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[1] Colony forming unit/ mL

★ Below detection limit of 1000 CFU

TOC/Abstract graphic