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Contact active antibacterial phosphonium coatings cured with UV light

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Described here is a simple and versatile approach for the preparation of antibacterial phosphonium-based coatings through the curing of a polymerizable tributylphosphonium monomer using UV light. The resulting surfaces resist bacterial growth and adhesion, even at high bacterial loadings up to 10^7 colony forming units per cm^2.

Infections caused by bacteria such as Escherichia coli (*E. coli*) and Staphylococcus aureus (*S. aureus*) are widespread and increasingly problematic on a global scale. These bacteria have the ability to survive, proliferate, and form biofilms on surfaces, and can be easily spread through direct contact.[1] Therefore, the development of robust and effective antibacterial coatings to reduce bacterial attachment, proliferation, and biofilm formation is of significant interest in order to decrease transfer through common surfaces such as doorknobs, elevator buttons, hospital beds and food packaging. Coatings for medical devices such catheters and implants also have great potential to reduce the bacterial infections that result from these objects.[2] A commonly employed approach to antibacterial surfaces involves the physical encapsulation of biocides such as antibiotics,[3–5] quaternary ammonium/phosphonium species,[6] or active metals on the surface or in the bulk material.[7–9] While this approach can be effective, some limitations include the release of toxic species into the environment, and the gradual exhaustion of the biocide, resulting in inactivity as well as subinhibitory concentrations of biocide near the surface and in the environment that will facilitate the development of bacterial resistance.[10] Therefore, the covalent immobilization of antibacterial agents is a particularly attractive approach for the development of long-lasting antibacterial surfaces.

In recent years there has been significant interest in the preparation of antibacterial surfaces based on immobilized poly(quaternary amine)s. These have been prepared by various processes including the covalent grafting of polymers onto the surface,[11,12] controlled polymerization,[13,14] photopolymerization,[15] or plasma polymerization from the substrate,[16] layer by layer assembly,[17,18] or simple painting on the surface.[19,20] While phosphonium-based small molecules and polymers have been established to exhibit antibacterial properties,[21–23] there are very few reports detailing phosphonium-based antibacterial surfaces.[24–26] Phosphonium salts are particularly attractive for surface applications because of their high thermal and chemical stability relative to ammonium salts.[27]

In this context, we describe the development of a new simple and versatile approach towards antibacterial surfaces using polymerizable phosphonium salts and UV curing. The physical and chemical properties of phosphonium cations allow them to be readily coated onto various surfaces in a solvent-free process and cured with UV light to create cross-linked polymer thin films (Fig. 1). It is demonstrated that the properties of the films can be tuned based on the phosphonium content and the curing conditions. The resultant coatings prevent both the growth and adhesion of bacteria on surfaces.

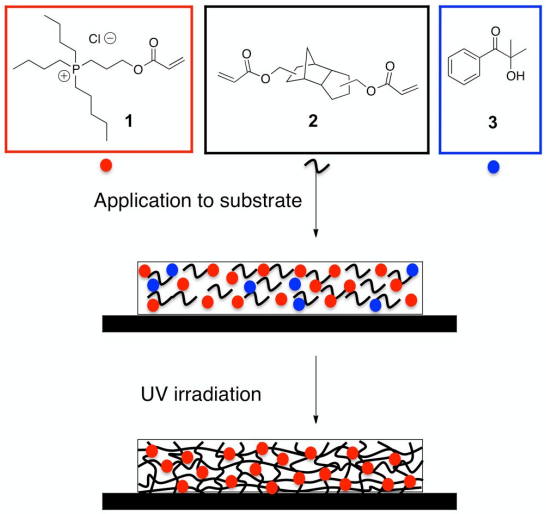

Fig. 1. Schematic showing the coating preparation process.

((3-Acryloyloxypropyl)tributyl)phosphonium chloride (**1**) was prepared as previously reported[28] and was selected as the phosphonium monomer for this study because the butyl chains were anticipated to provide an appropriate balance of hydrophobicity and hydrophilicity required for interactions with bacteria, and the acrylate functionality is known to exhibit high reactivity in UV curing processes.[29] The chloride counter ion results from the quaternization and acetylation steps used to prepare this molecule. Monomer **1** is a liquid at room temperature, allowing it to be cast on surfaces under solvent-free conditions. Tricyclodecanedimethanol diacrylate (**2**) and 2-hydroxy-2-methyl-1-phenylpropanone (**3**) were selected as the cross-linker and photoinitiator respectively, as they are both liquids, are widely used in UV curing and were found to effectively provide cross-linked thin films with **1**. Fixing the photoinitiator **3** concentration at 5 wt%, various weight ratios of **1** and **2** were formulated (Table 1), cast on substrates including

poly(ethylene terephthalate) (PET) and glass using a Meyer rod, and irradiated with a mercury light source. This process resulted in smooth films with surface roughnesses less than 2 nm, as measured by atomic force microscopy (AFM) (Fig. S1). The film thickness was ~25 μm. Adhesion testing using the Tape Test (ASTM D3359 – 09e2) indicated that the films generally exhibited strong adhesion to PET and lower adhesion to glass (Table S1). Beyond 47.5 wt% of **1**, the coatings did not adhere well to either substrate and delaminated upon washing, suggesting insufficient cross-linking.

Table 1. Effect of film formulation on accessible surface charge, as measured by fluorescein exchange.

wt% of phosphonium monomer **1**[a]	Accessible surface charge density (cations/nm^2)
< 20	None detected
20	5.6
25	34
30	91
37.5	120
47.5	250

[a]Formulation contains 5 wt% of photoinitiator **3** and the remainder is cross-linker **2** to make up to 100%.

The accessible surface charges on the coatings prepared from each formulation were quantified using the fluorescein exchange experiment.[14] It was found that when the content of phosphonium monomer **1** was less than 20%, the charge density was below the detection limit of the assay (approximately 0.01 cations per nm^2). However, as detailed in Table 1, from 20 – 47.5 wt% of **1**, the charge density steadily increased, reaching a value of 250 cations per nm^2 at 47.5 wt%. As several studies have suggested that cationic charge density is an important factor in achieving high antibacterial activity, further studies focused on the formulation containing 47.5 wt% of **1**.[14,30]

A major advantage of UV curing is the speed with which it can be performed, which is on the order of seconds, in comparison with hours for thermal polymerizations. However, a potential disadvantage of UV curing is the inability to achieve 100% polymerization of the alkene moieties, because of radical termination reactions and limited cure depth.[31,32] In particular, UV curing in air is subject to varying degrees of radical termination by oxygen and depends on the radical initiator and the growing polymer chain. To probe the efficiency of UV curing for the 47.5 wt% **1** formulation in air, the cure percentage was determined using attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy by comparing the intensity of the alkene peak at 810 cm^{-1} which disappears upon curing, to that of the carbonyl peak at 1720 cm^{-1} which does not change upon curing (Fig. S2). As shown in Table 2, 80 ± 5% curing was obtained in air. The corresponding gel content was measured to be 84 ± 2%. While these values are quite typical for UV curing,[31] and are acceptable for many applications, for antibacterial surfaces it is desirable to minimize the leaching of biocides from the surface as this can lead to ambiguity concerning the mechanism of action of bacterial killing and contribute to environmental contamination. Therefore, curing under an N_2 atmosphere was explored as a means of reducing radical termination by oxygen. This led to an increased cure percentage of 88 ± 4%, and an increased gel content of 88 ± 3%. The surfaces cured under N_2 were also noticeably harder and more durable. The application of a diamond tip surface profiler with a 0.5 mN force led to the etching of a 10 nm groove in the film cured in air, whereas the depth of the groove in the film cured under N_2 was indistinguishable from the surface roughness (Fig. 2; Fig. S1).

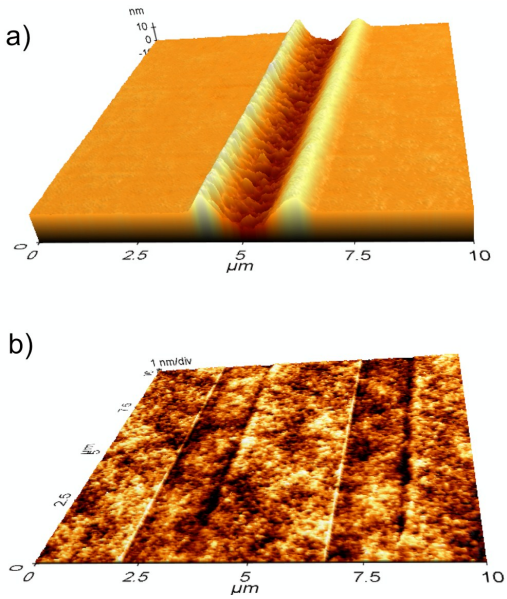

Fig. 2. AFM images following application of a diamond tip surface profiler with a 0.5 mN force to: a) a film cured in air and b) a film cured under N_2.

After washing to remove any unbound molecules, the measured cure percentages were 87 ± 1% and 97 ± 1% for the air and N_2 cured surfaces respectively, with remaining double bond content that corresponded to molecules of cross-linker with only one of the acrylate groups reacted. Both coatings had water contact angles of ~63°. This suggests that the surfaces were hydrophilic but not highly hydrophilic.[33]

Table 2. Properties of coatings prepared from 47.5 wt% of **1**, 47.5 wt% **2**, and 5 wt% **3** under air or N_2 atmospheres.

Curing atmosphere	Cure % (ATR-IR) before washing	Cure % (ATR-IR) after washing	Gel Content	Water contact angle
Air	80 ± 5%	87 ± 1%	84 ± 2%	63 ± 1°
N_2	88 ± 4%	97 ± 1%	88 ± 3%	65 ± 3°

Antibacterial testing focused on coatings cured under N_2 because of the increased curing efficiency and enhanced physical properties achieved under these conditions. N_2 cured surfaces containing 47.5% of phosphonium **1** were thoroughly washed by incubation in water for 12 h, with 3 changes of the water. This protocol was deemed sufficient to remove any unbound material as no further release of acrylate functionalities could be detected in the wash water by UV-visible spectroscopy (Fig. S3). This ensured that it was the actual activity of the surfaces that were being measured, rather than that of leachable phosphonium monomers or oligomers. This is particularly important, as recent

studies have suggested that some of the most active "immobilized" ammonium antibacterial surfaces may actually act through the release of unbound biocides.[34]

A suitable antibacterial test for surfaces of intermediate or low hydrophilicity involves placing the surface in contact with agar that has been innoculated with bacteria, as this minimizes issues associated with surface tension in bacterial suspensions and maximizes contact with the bacteria. To achieve this, a procedure based on the Kirby-Bauer protocol was used.[35] *E. coli* (ATCC 29425) and *S. aureus* (ATCC 6538) were selected as representative strains of gram-negative and gram-positive bacteria, respectively. As shown in Fig. 3 for *S. aureus* and Fig. S4 for *E. coli*, after 24 h of contact between the phosphonium surface and the bacteria-innoculated agar, no bacteria grew in the region of the phosphonium surface. Possible mechanisms of antibacterial activity that have been proposed for cationic surfaces include the disruption of the bacterial cell membrane[36] and the mobilization of metal cations that are important for membrane structure.[30] The former mechanism was proposed to be dominant for soluble polyphosphoniums[21] and surfaces with phosphoniums attached by long flexible linkers.[24] The mechanism of action of the current cross-linked phosphonium films and others with short linkers[25] are still unclear, though by analogy with surfaces having short quaternary ammonium chains,[37] may involve the ion exchange mechanism.

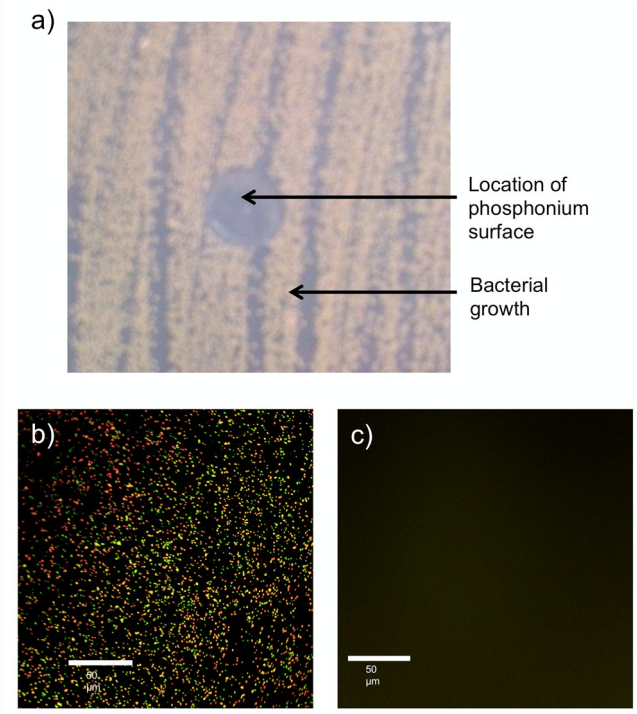

Fig. 3. Antibacterial testing results for a surface containing 47.5 wt% of **1** and cured under N_2: a) Image of an agar plate showing the absence of bacterial growth where the phosphonium surface was placed, following 24 h incubation with *S. aureus*; b) clean silicon wafer control and c) phosphonium surface following LIVE/DEAD® analysis after incubation of the surfaces with a suspension of *S. aureus* in PBS for 4 h. Live bacteria appear green in this assay, while dead bacteria appear red. No bacteria were detected on the phosphonium surface. Scale bar = 50 μm.

No zone of inhibition was detected for the surfaces in the above test, indicative that the observed activity was not the result of molecules leaching from the surface.[35] To probe this further, after the washing protocol described above was conducted, the surfaces were incubated in 0.3 mM phosphate buffer. The surfaces were then removed from this buffer and a suspension containing 10^5 colony forming units (CFUs) of *S. aureus* was added. Following plating of this bacterial suspension on agar, no inhibition of bacterial growth was detected in this solution in comparison to a control buffer that was not in contact with the surfaces. This confirmed that the activity was not due to the leaching of biocides. An additional problem that can be encountered in antibacterial surfaces is the adhesion of either live or dead bacteria to surfaces, which renders them inactive and can promote the growth of biofilms. To ensure that our surfaces resisted the adhesion of bacteria, a LIVE/DEAD® BacLight bacterial viability assay was performed using *S. aureus*. 100 uL of a PBS suspension containing 10^7 CFUs of bacteria per cm^2 was placed on the phosphonium surface. At time points of 4 and 24 h, the surface was gently rinsed with water and stained with SYTO 9 and propidium iodide. In this assay, live bacteria appear green due to the uptake of the dye SYTO 9, which permeates all bacterial membranes, while dead bacteria appear red due to the uptake of propidium iodide which permeates only damaged bacterial membranes and dominates over the fluorescence of SYTO 9. As shown in Fig. 3b,c and Fig. S5, no live or dead bacteria were detected on the phosphonium surfaces at either time point, suggesting that these surfaces can resist very high loadings of bacteria. In contrast both live and dead bacteria adhered to control silicon wafers under the same conditions. The ability of the phosphonium coatings to prevent bacterial attachment and colonization is very important as the colonization of bacteria on surfaces can lead to a faster development of resistance compared to that in solution, as resistance can be transferred by horizontal gene transfer.[38,39]

Testing was also performed to evaluate the potential toxicity of biocide **1** using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. C2C12 mouse myoblast cells, a model cell line, were found to exhibit > 80% viability at concentrations up to 0.2 mg/mL of **1** (Fig. S6). In comparison, benzyldimethylhexadecyl ammonium chloride, a common commercial antibacterial agent, was highly toxic to cells even at the lowest studied concentration of 8 μg/mL. Therefore, while the surfaces do not leach biocide following the initial washing step as all materials are subsequently covalently attached to the coating, these results suggest that phosphonium **1** may exhibit advantages over widely used ammonium biocides in terms of its toxicity to mammalian cells.

Conclusions

In conclusion, a new method was developed for the incorporation of antibacterial phosphonium polymer networks into coatings via UV curing. Optimal performance in terms of coating integrity and cationic charge were obtained for surfaces containing 47.5 wt% of the phosphonium monomer, and it was found that performing the UV curing under N_2 led to increased curing efficiency, higher gel content, and more durable coatings. The phosphonium-based coatings prevented the growth of *S. aureus* and *E. coli*, as demonstrated when they were placed in contact with bacteria-innoculated agar, and they also resisted the adhesion of both live

and dead bacteria at very high loadings of 10^7 CFUs of *S. aureus* per cm^2. Furthermore, the biocidal phosphonium monomer was found to be less toxic than a widely used ammonium biocide to mammalian cells. Overall, the results suggest that these new phosphonium coatings are highly promising for a wide range of applications ranging from medical devices to common objects such as keyboards and door handles. Further tuning of the coating formulation, casting process, and UV curing conditions to optimize the extent of curing and the coating properties, as well as further evaluation of the coating under a range of antibacterial testing conditions and should enable their development for these applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, additional AFM images, adhesion results, UV-vis spectra of surface washings, additional antibacterial testing results, MTT cell viability assay. See DOI: 10.1039/c000000x/

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Antibacterial phosphonium coatings were prepared by UV curing of phosphonium monomers. The simple approach and high stability of phosphoniums relative to ammoniums makes these coatings promising alternatives to ammonium surfaces.