A new, simple approach to confer permanent antimicrobial properties to hydroxylated surfaces by surface functionalization†

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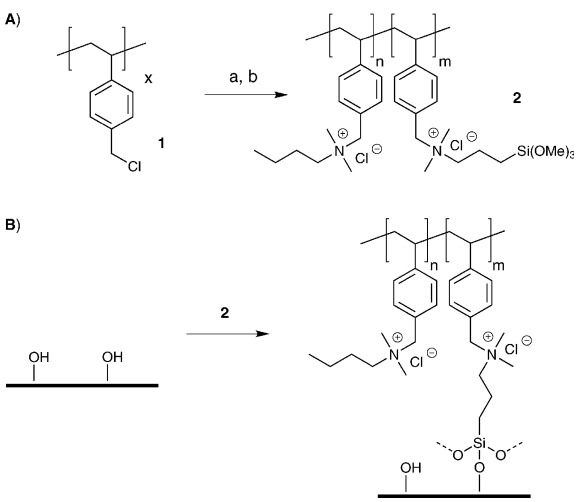
A new, simple method to obtain ultrathin polycationic monolayers on hydroxylated surfaces is described which uses a bifunctional copolymer comprising a reactive part (trimethoxysilane) and positive charges (quaternary ammonium salts) to confer antimicrobial properties.

This paper describes a versatile and experimentally simple technique for fabricating highly positively charged nanometric thin films on materials bearing hydroxylated groups (*e.g.* alcohols or silanols) on their outmost surfaces, such as glass, silicon wafer and cellulose, conferring on them the ability to kill bacteria on contact. A key feature of this approach is to use a bifunctional copolymer bearing simultaneously trimethoxysilane and quaternary ammonium groups; the former substituents anchor the polymer to chemically reactive surface groups[1]and the latter provide the cationic charges necessary for the biocidal behavior.

It is now well-established that the deposition of high molecular weight polymers bearing cationic groups confers antimicrobial properties to solid surfaces.[2,3] Such treatments are promising because they could provide a new approach to the control of bacterial proliferation. Contrary to the impregnation of materials with classical biocides (such as silver ions, low molecular weight quaternary ammonium salts...), the treatment is permanent, the surfaces are reusable and no molecules are released into the environment. The design of antimicrobial surfaces is in itself a matter of considerable interest for macromolecular chemists. They usually proceed either by physical adsorption[4,5] or by covalent grafting of small molecules[6] or polymer layers.[2,3] This latter approach with polymers provides, of course, a sturdier modification and eliminates the potential release of materials from the surface as is observed with physisorbed materials or surfaces grafted with some small molecules.[7] Two strategies have been developed for the grafting of polymers on surfaces: (1) preformed polymers are chemically grafted on the surface; (2) attached polymer chains are grown directly from the surface using atom transfer radical polymerization.[8] So far, however, the published examples of these strategies require (i) tedious multistep surface modifications; and (ii) the use of organic solvents. Therefore, there is a need for a less complicated approach that could be widely used in academic laboratories (by chemists and biologists) and, of course, in industrial applications.

In this context, we have synthesized a water-soluble cationic statistical copolymer containing specific silane functional groups capable of reacting with hydroxylated surfaces such as glass, textile, ceramics, oxidized plastics, *etc*. We have selected poly(vinylbenzyl) as the polymeric backbone since (i) it is commercially available; (ii) derivatives (chloride, bromide) of this polymer are easily modified *via* nucleophilic substitution; and (iii) poly[trialkyl(vinylbenzyl)ammonium chloride] is already known to present antimicrobial properties in solution.[9] Scheme 1 outlines the strategy used to synthesize the statistical copolymer **2**. The commercial poly(vinylbenzyl chloride) (M_n = 55 000 g mol^{-1}, PDI = 1.82) was first reacted with *N*,*N*′-dimethylaminopropyltrimethoxysilane (0.1 equiv. based on vinylbenzyl chloride moieties) in THF during 6 h at 50 °C. Then, *N*,*N*′-dimethylbutylamine in large excess in ethanol[10] was added to the reaction mixture, leading to the desired copolymer **2** in high yield (95%). The remarkable solubility of copolymer **2** in water (exceeding 10 mg mL^{-1}) shows that the reaction has been successful.

Antimicrobial polymeric monolayers (APMs) of the copolymer **2** on SiO_2 substrates were prepared by immersing



Scheme 1 Synthesis of poly[trialkyl(vinylbenzyl)ammonium chloride] **2** (A) and surface modification (B). Reagents and conditions: (a) *N*,*N*′-dimethylaminopropyltrimethoxysilane (0.1 equiv.), THF, 50 °C, 6 h; (b) *N*,*N*′-dimethylbutylamine, THF–EtOH, 50 °C, 12 h.

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the substrates in an aqueous solution of the copolymer **2** at 1 mg mL^{-1} and the solution was allowed to evaporate to dryness. APMs were then cured at 120 °C for 1 h. The excess of unreacted polymer present on the surface was easily removed by several washings with deionized water. Fibrous cellulosic materials[11–13] such as cotton, wool, paper, *etc*. were similarly treated using this impregnation method, followed by a baking step at 120 °C in air. After washings with deionized water, the general aspects of the treated materials are identical to the untreated ones.

The presence of APMs on the treated Si/SiO_2 prisms and quartz slides was unambiguously revealed using IR-ATR and UV-Vis spectroscopy as well as by X-ray photoelectron spectroscopy (data summarized in the ESI†). The polymer-coated films were also characterized (data summarized in the ESI†). The density of ammonium groups on the treated surface is the key parameter that determines the efficiency of the antimicrobial properties. Indeed, it has been shown previously that there is a charge density threshold for biocidal cationic surfaces.[14,15] The density of the quaternary ammonium groups within the APMs was monitored by two methods: (i) measuring the absorbance of the benzyl group at 260 nm of a quartz slide functionalized with copolymer **2**;[16] (ii) by measuring the UV absorption at 501 nm of the fluorescein molecules used as counterions of the quaternary ammonium sites present on the APMs.[17] These two methods lead to similar values of the charge density, of the order of 10^{15} per cm^2, which corresponds to 0.5 μg cm^{-2} of immobilized copolymer. We have checked the stability of the APMs against washing with water or organic solvents (ethanol, dimethylformamide). Extensive washings, as well as prolonged immersion in water, do not lead to any detectable material loss, in agreement with the covalent bonding process.

The thicknesses of the APMs were measured by ellipsometry which shows that the grafting of copolymer **2** led to the formation of thin films on silicon wafer in the order of 6 nm. The surface topography of the APMs of the copolymer **2** on Si/SiO_2 wafers was examined by atomic force microscopy (AFM) in the tapping mode. The APM films were found to be very homogeneous along the surface of the material, with thickness variations not exceeding 0.4 nm from point to point (see ESI†).

The antimicrobial properties of the APMs were investigated by using the live/dead two-color fluorescence method that probes the permeability of the bacterial membrane as a marker of bacterial cell viability.[18] First, a suspension of Gram negative bacteria, *Escherichia coli* (MG 1655) or *Bacillus subtilis* (ATCC 6633), was deposited on glass slides treated with copolymer **2**, according to the protocol described above, and their viability was followed over time by epifluorescence microscopy. The time required to kill bacteria after contact is about 30 min for *E. coli* (Fig. 1).[19] APMs were also found to be active against Gram positive bacteria such as *Staphylococcus epidermidis* (ATCC 12228) and *Streptococcus mutans* (ATCC 25175D) (Table 1). The treated surfaces thus exhibit wide-spectrum antibacterial properties. Several materials, such as quartz, Si/SiO_2 wafer, cellulose (paper, cotton fabrics) and oxidized polydimethylsiloxane, were subjected with success to the same treatment, leading to antimicrobial surfaces (Table 1).

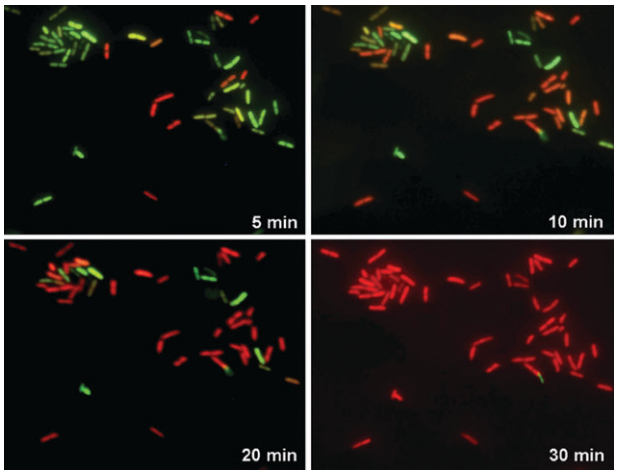

Fig. 1 Dynamic time-lapse imaging of bactericidal cell viability on treated glass surfaces. Fluorescence microscopy of *E. coli* (MG 1655) stained with fluorescent cell viability marker (live/dead assay, Invitrogen). Viable bacteria appear as green dots and non-viable as orange/red dots.

We used micro-contact printing to control the spatial distribution of the APMs on a glass substrate and demonstrated that APMs kill bacteria on contact rather than by release of cationic polymer. Indeed, we have performed viability tests on polycationic surfaces micropatterned with an array of active and non-active stripes of 100 μm width.[20] The geometry of these micropatterns was first revealed by epifluorescence microscopy, using the electrostatic self-assembly[21] of carboxylic acid-terminated fluorescent latex beads (d = 0.2 μm) (Fig. 2A). The surface was then exposed to a bacteria inoculum and the cell viability was observed under an optical microscope after one hour of incubation. Fig. 2B shows alternate rows of live (green) and dead (red) bacteria, corresponding to the original chemical pattern. This suggests that the cationic polymers present on the surface do not diffuse laterally, once again in agreement with the fact they have been chemically grafted. It also shows that the interaction between the biocidal surface and the bacteria is purely local. Bacterial death occurs only when there is direct physical contact with the treated surface. Lastly, the bacteria once deposited on the surface do not diffuse laterally by Brownian diffusion, whether the surface has been chemically treated or not.

Table 1 Evaluation of the antimicrobial surface properties

Bacterium	Treated materials	Percentage of killed bacteria[a]
E. coli (MG 1655)[b]	Glass	99.7
B. subtilis (ATCC 6633)[b]	Glass	99.5
S. epidermidis (ATCC 12228)[c]	Glass	99.8
S. mutans (ATCC 251775D)[c]	Glass	98.0
E. coli (MG 1655)[b]	Cellulose (cotton fibers)	100
E. coli (MG 1655)[b]	Cellulose (paper)	100
E. coli (MG 1655)[b]	Oxidized PDMS[d]	99.9

[a] Quantitative data were obtained by counting with Image J the proportion of dead bacteria by fluorescence microscopy using a live/dead assay see (ESI†). [b] Gram negative strains. [c] Gram positive strains. [d] PDMS: polydimethylsiloxane.

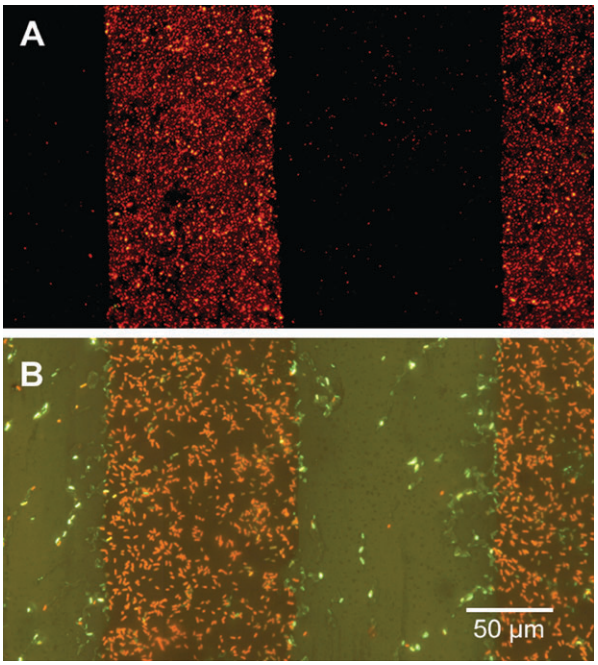

Fig. 2 Fluorescence microscopy images of an APM micropatterned glass slide. (A) Fluorescent carboxylic acid-terminated latex beads (id = 0.2 μm) adsorbed on glass slides micropatterned with APMs. (B) Bacterial viability assay* on glass slides micropatterned with APMs. *Note: viable bacteria appear as green dots and non-viable as red dots.

To conclude, this article describes a simple, inexpensive and widely accessible method for the preparation of bactericidal surfaces starting from hydroxylated materials (glass, cellulose...). The treatment is based on the use of a single component (a bifunctional statistical copolymer with silane reactive groups on one hand and positively charged groups on the other hand) that can be easily prepared separately and is deposited in the form of a dilute water solution on the surface to be treated. Materials of all sizes and shapes can be treated, provided they possess hydroxyls on their outer surface that chemically react with the silane moieties on the polymer. The antimicrobial treatment is efficient (death occurs in less than 30 min after physical contact is established) and works on both Gram positive and Gram negative bacteria. Because the biocidal molecules are chemically grafted to the substrate, the treatment is permanent and the surfaces can be reused after the bacteria present from the previous run have been removed by washing. Due to their extremely facile and versatile preparation as well as their stability, we expect that this bactericidal contact active surface will provide a powerful tool to understand the antimicrobial mechanism of cationic surfaces and should find applications in biotechnology or medical environments.

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