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Study of the adhesion of *Staphylococcus aureus* to coated glass substrates

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Abstract The adhesion of *Staphylococcus aureus* was studied using a selection of laboratory-prepared and commercially available coated glass substrates using a simple methodology. Substrates were examined by scanning electron microscopy, atomic force microscopy and water droplet contact angles. It was found that microbial adhesion was independent of surface roughness, when this was of a lower magnitude than microbial size. It was also found that microbial adhesion was greater for hydrophilic surfaces than for hydrophobic ones, but that on a photoinduced superhydrophilic surface, microbes were more spread out—a potential benefit for more effective photocatalytic disinfection. It is suggested that hydrophobic and photoinduced superhydrophilic surface coatings both have potential as a means of reducing microbial fouling of surfaces.

Introduction

Microbes can be commonly found colonising surfaces in diverse environments, indeed it may be considered to be nature's default that microbes accumulate on surfaces, especially where there are sufficient nutrients and favourable conditions for growth [1]. Surface-attached microbes can form a biofilm; these comprise microbial cells in a growth phase which is significantly distinct from that of planktonic cells [2]. Biofilm microbes are very different from planktonic cells on a surface, they display an altered phenotype in terms of growth rates and gene transcription, when compared to planktonic cells of the same organism [3]. The structure of biofilms makes them a tenacious problem in a variety of settings, particularly healthcare, where biofilm contamination of biomedical devices is a major event in the aetiology of hospital-acquired infections [3, 4]. Biofilms are also a problem in the water industry, where supply pipe work can become constricted, corroded or contaminated by both benign and pathogenic microbes [1] and in oil extraction, where biofilm growth can initiate the degradation of the oil rig structure, as well as corroding oil pipelines [1]. It is accepted that there are three reasons why forming a biofilm might be advantageous to an organism: protection, nutrients and growth and genetic exchange [5–8].

The adsorption of microbes on to a surface is a complicated process, which is still under intense debate and study. On a basic level, it is accepted that there is an interplay of various short range attractive/repulsive forces such as electrical charge, electrostatics and Van der Waal's forces, this has collectively been summarised in the Derjaguin, Landau, Verwey and Overbeck (DLVO) theory [6, 7]. A recent article [8] has attempted to explain the adhesion of microbes to surfaces in physico-chemical terms and concludes that microbial attachment to a surface is governed by 'macromolecular physics and chemistry in the interfacial environment'. This can be linked to genetics because organisms with the ability to adapt their macromolecular structure can consequently adapt this physicochemical interaction, resulting in enhanced or reduced ability to adhere to a surface. These physicochemical interactions arise from the microbial cell surface structures, and the way that these interact with the substrate.

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One area of intense interest in biomaterials research is the creation of surfaces that resist the adsorption of microorganisms. Numerous candidate technologies have been studied, including, but not exclusively limited to, poly(ethylene glycol), diamond-like carbon films, zwitterionic polymer biomimetic surfaces and hydrophobic materials [9].

We have been involved in producing both hydrophilic and hydrophobic coatings on glass and evaluating the ability of certain films to kill bacteria under the action of light [10–16]. We have also developed a range of polymer materials that kill bacteria under the action of white light or laser light through mediation of gold nanoparticles and an organic dye [17–19]. In addition to the ability to kill bacteria on a surface, the ability to prevent bacterial adherence and biofilm formation is an alternative and perhaps preferable way to reduce the microbial load in the environment. This article examines the adhesion of microbes to a selection of coated glass substrates. The test substrates include both laboratory prepared sol–gel photocatalyst coatings and commercially available materials. Of particular interest are the superhydrophilic self-cleaning glasses Pilkington Activ™ and Saint-Gobain BIOCLEAN®. These were examined as it is thought that superhydrophilicity of these photocatalyst films might afford some reduction in microbial adhesion or a change in the way in which microbes aggregate. Superhydrophobic surfaces on the other hand are also thought to be suitable for reducing microbial adhesion to surfaces because they encourage water droplets to ball up, roll from the surface, taking dirt and microbes with them—a phenomenon known as the Lotus-Effect [20]. In light of this, a commercially produced hydrophobic material, Pilkington Hydrotech, was assessed alongside superhydrophilic materials. Studies of the adhesion of microbes to coated glass substrates have received minimal research attention, in particular sol–gel TiO_2 materials do not appear to have been studied at all [21].

Materials and methods

Microbial adhesion procedure

Both commercially produced and laboratory produced coatings were prepared for study. The laboratory samples were sol–gel TiO_2 and Ag_2O/TiO_2 thin films deposited on standard microscope slides, the synthesis for which has been previously described elsewhere [22]. The commercially available materials were Float Glass, K-Glass™, Hydrotech, Activ™, SnO_2 and SiO_2 all from Pilkington Group Ltd. and BIOCLEAN® from Saint-Gobain Glass (SGG) UK Ltd. The commercially coated samples were all on float glass of typical thickness 3–4 mm. Samples under test were cut using glass cutters into coupons measuring 12 × 25 mm. This gives the coupons an available surface area of 3 cm^2. The active face of the samples was marked by scratching the surface at the top edge of a coupon with a diamond tipped pencil. Sample coupons were then cleaned using 70% isopropanol in distilled water solution. This solution washed off the dirt and debris left behind by the cutting process and is also a microbicide, disinfecting the surface before the experiment. All coupons were then irradiated by 254 nm light from an 8 W germicidal lamp (Vilber-Lourmat VL208-G, VWR International Ltd., Lutterworth, UK) for a period of 1 h. This step serves as a further decontamination process and affords the requisite UV illumination for the photocatalytic materials and removes any traces of the isopropanol.

A microbial suspension into which the coupons were to be placed was prepared from an overnight culture of the test organism prepared in nutrient broth (Oxoid Ltd., Basingstoke, UK) and grown aerobically with shaking at 37 °C. The organism tested was *Staphylococcus aureus* NCTC 6571. The overnight culture was prepared in 20 mL of nutrient broth, in a Falcon™ tube. This was then spun down to a pellet and re-suspended in an equal volume of sterile phosphate buffered saline (PBS) (Oxoid Ltd., Basingstoke, UK). This re-suspended overnight culture was then serially diluted 1,000-fold in sterile PBS. The diluted microbial suspension was then divided into 5 mL aliquots in sterile screw top universal bottles.

The first 2.0 cm of the coated glass coupons of test material were dipped aseptically into the 5 mL of microbial suspension and left there, vertically for a period of 5 min, before being carefully withdrawn from the suspension. The lower edge of withdrawn coupons was then tapped onto filter paper, and the coupon was placed onto filter paper, inactive side down, so that only the active surface was examined. At no time was the active surface blotted, or in any way touched. Coupons were then transferred aseptically to sterile petri dishes for an agar overlay. The overlay was carried out with 35 mL of molten Mannitol salt agar (Oxoid Ltd., Basingstoke, UK), made up to 75% of the recommended amount, poured at 42 °C. Coupons were allowed to dry before the overlay was carried out. Overlaid plates were incubated aerobically face up for 24 h at 37 °C. The process was repeated at least ten times for each coated surface.

Data acquisition and analysis

Incubated plates were photographed using a gel imaging system (Alphaimager) that results in monochrome images. The adherent viable colony forming units were counted manually on the digital images using OdoPlus freeware counting software. For the sake of image clarity and ease of

microbial colony counting, images were manipulated using Adobe Photoshop CS3. Results were tabulated and graphed in SPSS V15 statistical software [23].

Imaging

Samples with adherent microbes were also imaged by scanning electron microscopy (SEM). Microbes were fixed to the surface in glutaraldehyde and the sample sputter coated in gold. Images were obtained on a JEOL instrument with Oxford INCA software. Samples without adherent microbes were also analysed by atomic force microscopy (AFM). A Park Systems model XE-100 AFM was used in non-contact mode with a silicon tip and a scan rate of 1 Hz. Images were collected using the XEP software (Park Systems Inc., CA.) and analysed using XEI (Park Systems Inc., CA.).

Determination of sample hydrophilicity and hydrophobicity

Sample hydrophilicity/hydrophobicity was assessed by measurement of water droplet contact angles for all samples on a sessile 5 mL drop of distilled water, placed onto the samples from a 10 mL micropipettor. The droplet diameter was measured optically using a 20× Measuring Microscope (Peak Optics). The diameter and volume data were then used to calculate a contact angle for the droplet using a suitable computer program. Contact angle data was obtained on both UV irradiated (1 h, 254 nm) and non-irradiated samples to demonstrate the photoinduced superhydrophilicity (PSH) of the titania materials.

Results

The adhesion of microbes to a coated glass surface was determined by a simple procedure. Sterility of the samples before experiment was ensured by the utilisation of an isopropanol wash and UV irradiation. This UV irradiation process disinfects the surface of the coupons in addition to the 70% isopropanol wash. It also provides the UV pre-activation required for the photocatalytic materials (TiO_2, Ag_2O/TiO_2, Pilkington Activ™ and SGG BIOCLEAN®). Image capture by gel imaging camera produced monochrome images with microbial colonies clearly visible.

Adhesion of *S. aureus* NCTC6571

Some representative images of adherent *S. aureus* on all test substrates are shown in Fig. 1. The images a–i show colonies attached to the substrate coupon and in some cases embedded in the surrounding agar overlay.

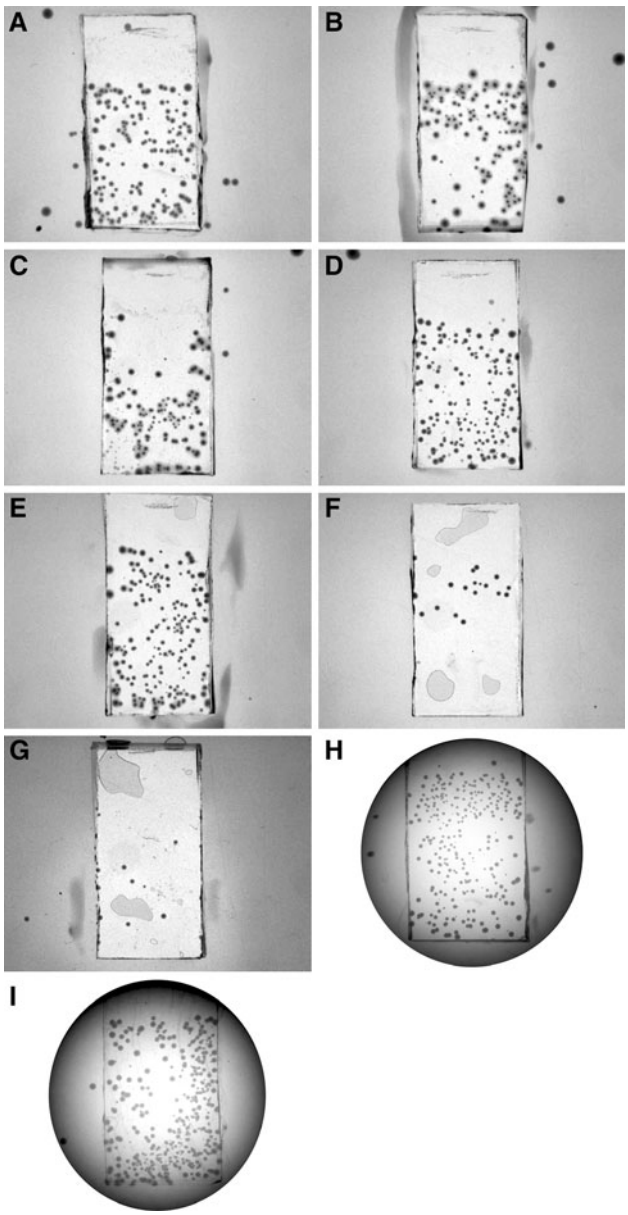

Fig. 1 Adhesion of *S. aureus* to 12 × 25 mm coupons of **a** Float Glass, **b** SiO_2, **c** SnO_2, **d** Activ™, **e** BIOCLEAN®, **f** K-Glass™, **g** Hydrotech, **h** sol–gel TiO_2 and **i** sol–gel Ag_2O/TiO_2 (Note figures **h** and **i** were captured through a microscope eyepiece, but the method was unchanged)

Table 1 and Fig. 2 show the adhesion data in tabular and graphical form, respectively. Because the data is not normally distributed, a box and whisker plot was used for the graphical representations. Any outlier data points are shown as open circles. It is clear that microbial adhesion is variable across the sample set, and there is significant variation within many of the sample's repeat measurements (box sizes vary). However, for the K-Glass™ and Hydrotech samples the lowest adhesion values were observed with minimal spread. The BIOCLEAN® sample has a wide

Table 1 Adhesion data for *S. aureus* on various substrates (standard deviations in brackets)

Sample	Mean adherent microbes (cfu/coupon)		Mean adherent microbes (cfu/cm^2)	
Float Glass	196	(68)	65	(23)
SiO_2	74	(43)	25	(14)
SnO_2	127	(45)	42	(15)
Activ™	228	(110)	76	(37)
BIOCLEAN®	161	(180)	54	(60)
K-Glass™	16	(7)	5	(2)
Hydrotech	9	(8)	3	(3)
Sol–gel TiO_2	125	(110)	42	(37)
Sol–gel Ag_2O/TiO_2	283	(99)	94	(33)

Data was derived from a minimum of ten separate experiments for each surface

spread of data such that the standard deviation calculation yields a result larger than the mean value.

SEM

The SEM was carried out on *S. aureus* that had adhered to a glass substrate and to sol–gel TiO_2 materials through the dip-coating procedure. Substrates were UV pre-irradiated and divided into two sets of samples before inoculation. The first set was kept in the dark for 6 h, the second set illuminated by 254 nm light for 6 h. Images of the bacteria on these surfaces are shown in Fig. 3.

Clear differences are observed between the samples that have been left in the dark for the course of the experiment and samples that were UV illuminated. Typically the UV illuminated surfaces show between 10 and 20% of the colonies of the non-irradiated surface. Notably, the titania materials have fewer adherent microbes—this is most likely due to the effect of UV light which activates the surface and renders it hydrophilic or superhydrophilic. Secondly, it can be seen in the samples not illuminated for the course of the experiment that there is a clear difference in the way in which the microbes are dispersed across the surface. It appears as though the microbes adherent upon the hydrophilic materials are in smaller groups than on the glass control and are more evenly dispersed. The microbes on the glass surface are collected together in larger groups and are unevenly dispersed with large areas of un-colonised space. Higher magnification images, with a larger inoculum concentration, were collected to further examine this and compare a TiO_2 sample with a glass control. This is shown in Figs. 4 and 5, in which both samples had been UV pre-illuminated before the experiment.

The nature of the surface greatly affected the morphology of the adherent colonies at high microbial concentration. On the glass (Fig. 4), the microbes tended to adhere in microcolonies with typically 20 units clustered together, with some degree of separation between them. There are few individual microbes that are adherent on the surface. In contrast, the adherent microbes on the sol–gel TiO_2 material (Fig. 5) were more dispersed. Although there was some

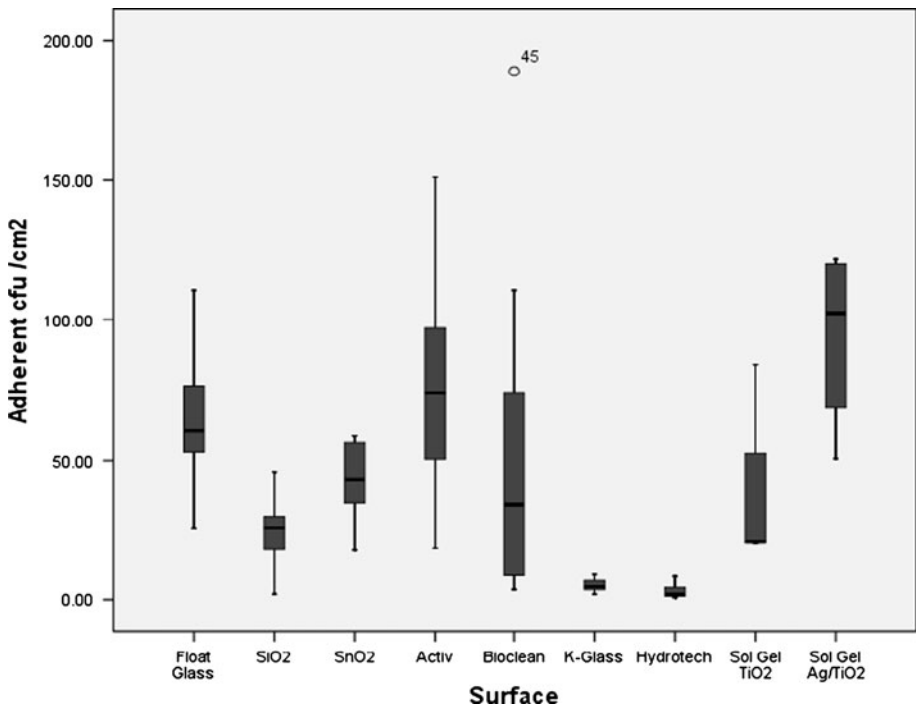

Fig. 2 Box and whisker plot showing adherent cfu/cm^2 of *S. aureus* NCTC6571 to coated glass substrates

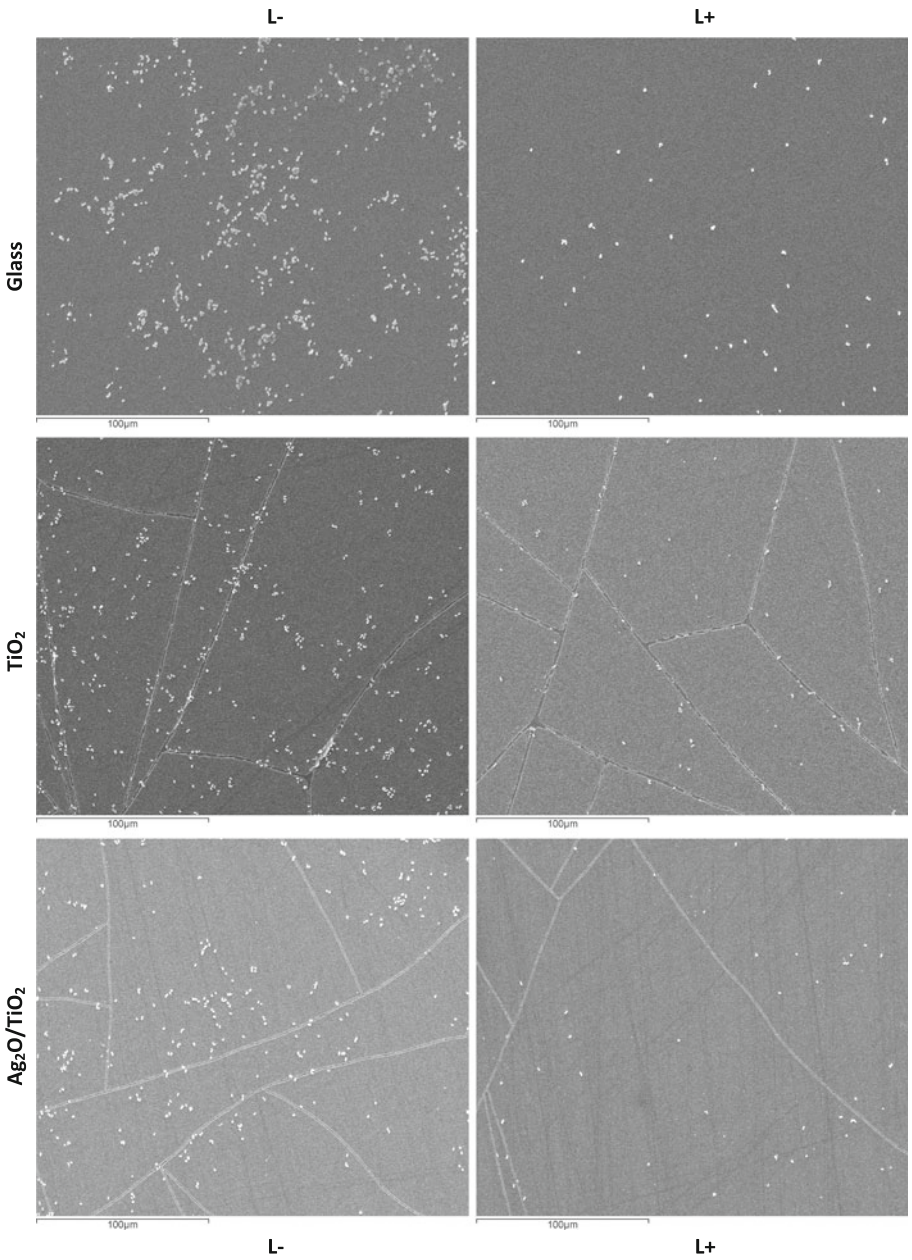

Fig. 3 SEM images of *S. aureus* on glass, TiO_2 and Ag_2O/TiO_2 under 254 nm illuminated (L+) and non-illuminated (L−) conditions

aggregation into microcolonies, the adherent microbes tended to be in smaller groups, or as single colonies. This may be a reflection of the comparative hydrophilicities of the materials. The TiO_2 is more hydrophilic, and demonstrates PSH after UV pre-illumination, when compared to the glass substrate which did not. This may prevent the aggregation of microbes in large microcolonies and encourage them to form only small adherent colonies—which would be more easily destroyed by the photocatalyst.

AFM

The AFM studies were performed on all of the samples to measure the surface roughness and assess if this was a major determinant in microbial adhesion. The intention was to observe the typical morphology of the surfaces and to calculate surface roughness (R_a) values as a means to explain the adhesion of microorganisms to the various substrates. R_a values were calculated in the software across multiple profiles of the surface, and the data points averaged.

Table 2 shows the calculated average surface roughness (R_a) of the samples given to two significant figures.

The different levels of roughness and surface morphologies can be clearly seen in the three dimensional AFM images in Fig. 6. Shrink cracks, resulting from the anneal process can be seen in the sol–gel TiO_2 examples (Fig. 6d), these are quite unusual, in that some are raised from the

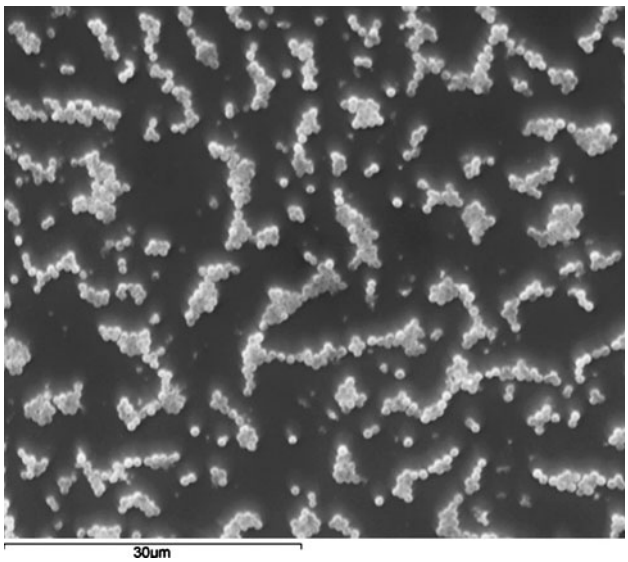

Fig. 4 SEM image of *S. aureus* on glass substrate. *Scale bar* 30 µm

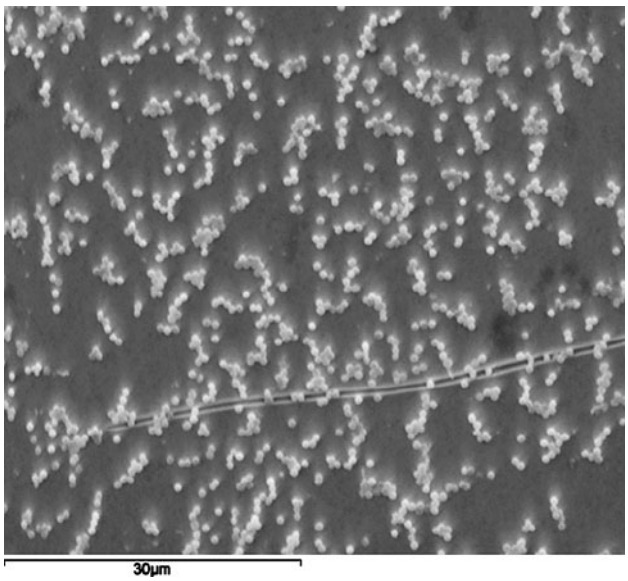

Fig. 5 SEM image of *S. aureus* on a sol–gel TiO_2 coating. *Scale bar* 30 µm

surface by approximately 100 nm—this leads to the high roughness measurements.

Water droplet contact angle

The comparative hydrophilic/hydrophobic properties of the materials were assessed by measurement of the contact angle of a water droplet on the surface. Low contact angles arise from the spreading of the water droplet, demonstrating the affinity of the surface for water. Conversely a high contact angle arises from the tendency of a droplet to not spread, and to remain stationary. Contact angles can be influenced by both the chemistry and the morphology of the surface. For example, a rough surface with cracks and channels can allow water to spread more easily than on a smooth surface of the same chemical composition. Also the chemistry of the surface, for example hydroxylation, resulting from photoactivity of a material can cause hydrophilicity, and the enhanced tendency for a droplet of water to spread. This is known as photoinduced hydrophilicity. To see if any of the samples demonstrated photoinduced hydrophilicity, contact angles were recorded before and after a period of 254 nm UV irradiation. The results are shown in Fig. 7.

Table 2 Average surface roughness (R_a) of samples

Sample	Average surface roughness R_a (nm)
Float Glass	3.1 ± 0.75
SiO_2	0.72 ± 0.30
SnO_2	17 ± 15
Activ™	4.9 ± 1.1
BIOCLEAN®	5.2 ± 0.29
K-Glass™	5.7 ± 0.82
Hydrotech™	1.4 ± 1.8
Sol–gel TiO_2	25 ± 8.8
Sol–gel Ag_2O/TiO_2	19 ± 12

Values quoted to two significant figures

The graph shows that most samples do not demonstrate photoinduced hydrophilicity, since the before and after measurements were not significantly different. This study showed that only the titanium dioxide coated surfaces-Pilkington Activ™, sol–gel TiO_2, sol–gel Ag_2O/TiO_2 and SGG BIOCLEAN® demonstrated photoinduced hydrophilicity. The sol–gel materials tended towards superhydrophilic behaviour with contact angles of ca. 15° and the commercial materials performed slightly better around 10°. The other sample of particular interest was the Pilkington Hydrotech, which was very hydrophobic, with a contact angle of ca. 130°.

Discussion

It has been observed for dental acrylics that surface roughness can directly affect the level of microbial adhesion on a surface [24]. Rougher surfaces harboured more microbes on them and hence adhesion to the surface was greater than for smoother surfaces. However, in the study presented in this article it can be seen that there is no direct correlation between the roughness of the surfaces and the level of microbial adhesion. Whilst this may appear in contradiction to published study, the level of roughness

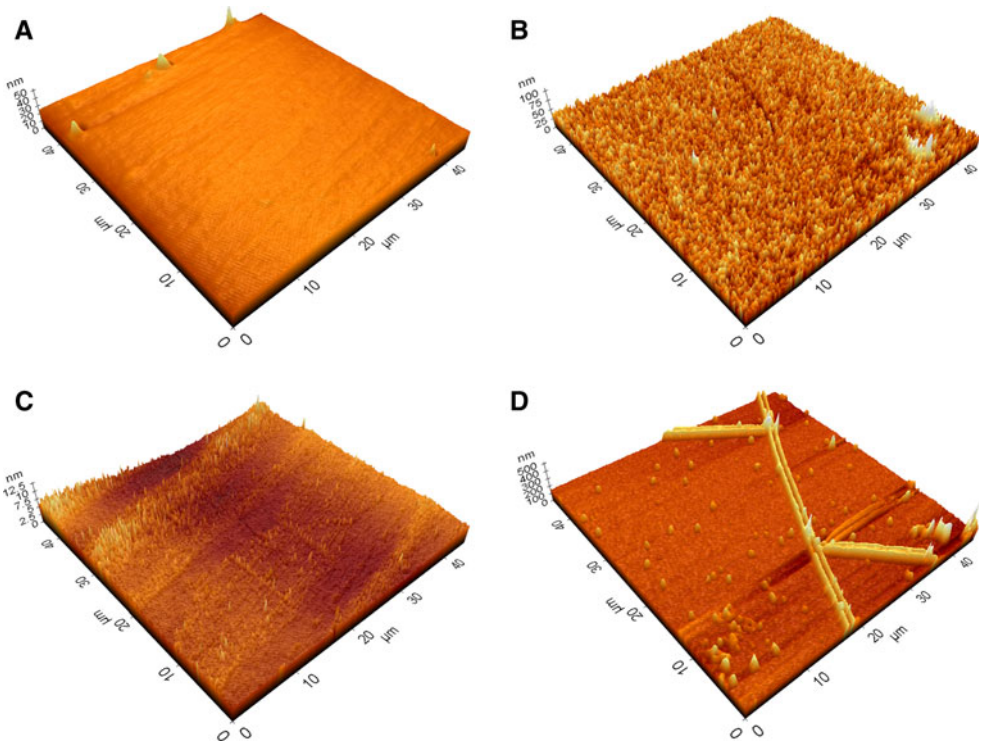

Fig. 6 Three dimensional AFM images of 45 μm square sections of **a** Float Glass, **b** Saint-Gobain BIOCLEAN®, **c** Pilkington Hydrotech and **d** sol–gel TiO_2 coated glass

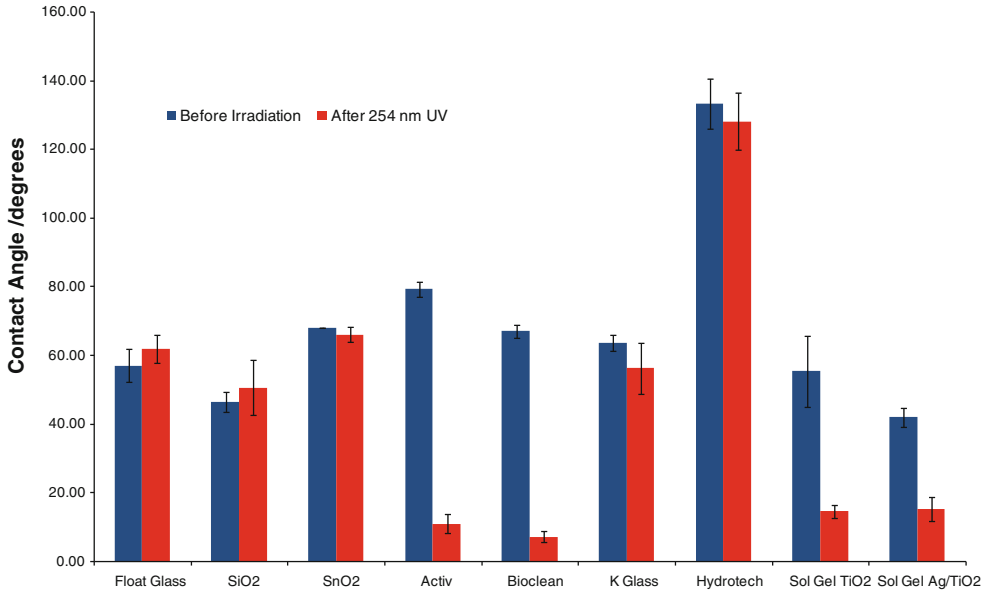

Fig. 7 Water droplet contact angles recorded on samples before and after 254 nm UV irradiation

recorded in this study and those published in the literature differ by three orders of magnitude. In the published study [24], the roughness of the materials was analysed by laser profilometry and on a micrometre scale. In this study, measurement of roughness was initially attempted by laser profilometry, but the surfaces were too smooth to be studied by this technique. It was only by utilising AFM methods that surface roughness could be calculated, and it was found to be on a nanometre scale. This has important implications, and can perhaps provide a rationale for the observed discrepancy between the findings in this study and the published literature. The typical size of a rod-shaped prokaryote is 1–5 μm long by 1 μm wide—*E. coli* cells for example measure 1 × 2 μm [1]. This means that the roughness 'experienced' by a microbe in the published literature case is of the same order of magnitude as the microbe's own size. However, in the case of the results from this study, the roughness is three orders of magnitude smaller than the typical dimensions of a prokaryotic cell. A microbe approaching the surfaces examined here is

considerably larger than the dimensions of the surface roughness, so it is of little direct consequence in determining if a microbe sticks to the surface or not.

The principle physico-chemical interaction that has been examined in this study is the relative hydrophilic/hydrophobic nature of the surface. The photocatalytic materials all demonstrated PSH, with water contact angles in the region of 10–15° after UV illumination. The Pilkington Hydrotech sample with a near-superhydrophobic contact angle of ca. 130° was largely unaffected by UV illumination, neither were all other samples—which is as expected. Overall it was found that the most hydrophobic surface had the lowest level of adherent microbes, and further the superhydrophilic materials had the highest number of adherent microbes. In the region of contact angles around 60° there is great variation in the number of adherent microbes, this clearly shows that in a material which is neither hydrophilic, nor hydrophobic, there are other factors in play which determine the level of microbial adhesion. Incidentally, this reason is not surface roughness, as if only the materials with intermediate contact angles are considered, there is still no correlation between their roughness and the level of microbial adhesion. It is probable that the reason for the differences in microbial adhesion displayed by the samples studied is due to other physico-chemical interactions, such as Van der Waals and electrostatic forces between the surface and approaching microbes.

The SEM study of the hydrophilic materials has also shown one other aspect, which may assist in the function of the photocatalyst films as useful antimicrobial surfaces, despite their apparent tendency to permit adhesion of more microbes than other surfaces. Specifically, the tendency of the superhydrophilic surface to spread the microbes out across its surface, rather than allow their aggregation in larger clumps, is beneficial to the photocatalytic function of the coatings. By spreading across the surface, shielding of the photocatalyst from the light source is reduced, and the thickness of the microbial overlayer is reduced. This allows the photocatalyst to function more effectively, and ultimately allows the material to act as a more effective antimicrobial surface. In other words, the synergy between the photocatalysis and the PSH works in the favour of a better antimicrobial surface, despite the slightly higher tendency of these surfaces to adsorb microbes.

The results clearly show that there are two strategies for an antifouling surface, both having their own merits. If a surface which cannot be easily fouled is desired, then from the results of this study, a hydrophobic or superhydrophobic surface is required. These surfaces prevent the spread of water droplets and limit microbial attachment. With the superhydrophilic surfaces, we see that the level of microbial adhesion is significantly greater than for the hydrophobic surfaces. However, the adherent microbes are well dispersed on these surfaces, and because all of the superhydrophilic materials tested here are also photocatalysts, this is beneficial to their function. In certain situations, it could conceivably be possible to design products incorporating both kinds of surface into their design, such that for example microbes are physically removed from one area of a device by the hydrophobic surface, and collect in a region treated with photocatalyst, such that the microbes that are transferred are killed. Such a device would be a fusion of the two methodologies and incorporates the good points of both technologies.

Adhesion of microbes to TiO_2 photocatalyst surfaces has only been sparsely examined in the literature. Some studies [25–27] focus primarily on the antimicrobial properties. In these studies, it was, however, suggested that TiO_2 surfaces under UV illumination reduced microbial adhesion. Perhaps the best study of microbial adhesion to oxide thin films is that of Li and Logan [21]. In the Li and Logan study, it was shown that adhesion of *Pseudomonas aeruginosa* to thin films increased in the order float glass (Sn side) < float glass (air side) < TiO_2 (commercial) < SnO_2 < SnO_2:F. The study in this article correlates well with the findings from the Li and Logan study, in that TiO_2 materials appear to increase microbial adhesion compared to a glass control. In our study, we used a wider range of surfaces and surface types and a different microorganism. Furthermore we used prior UV irradiation to activate the surfaces.

Perhaps one of the most significant discoveries reported in this article is that preactivation of a titania surface has a marked effect on bacterial adhesion. For surfaces that were irradiated before exposure to bacteria showed a greater spread of the bacteria across the surface, more single colony forming units and a significant reduction, ca. fivefold in the actual number of bacteria adhering to the surface compared to the unactivated surface. The simplest explanation for this is that the surface before irradiation is not superhydrophilic.

Conclusion

It has been demonstrated that the adhesion of microbes to surfaces can be affected by numerous physico-chemical factors, and the complexity of microbial adhesion has been demonstrated. There is no one clear explanation for the behaviour of all of the materials with regard to adhesion of microbes to their surface. What has been demonstrated in this study is that at three orders of magnitude smaller than microbial size, surface roughness has no effect upon microbial adhesion. Similarly, water droplet contact angles do not provide an accurate indicator of the tendency of microbes to adhere to a surface either. However, one can

broadly say that a hydrophobic surface such as Hydrotech is fouled less easily, whereas a superhydrophilic surface such as the sol–gel materials is fouled more easily. When the contact angle is neither hydrophilic nor hydrophobic there is great variation in adhesion. This can only be explained by other physico-chemical factors not determined in this study. What is clear though is that both superhydrophilic and superhydrophobic surfaces can have their place in an antimicrobial coatings strategy, provided that the functional properties are considered in relation to the requirements of a coating in a particular setting, i.e., the fine line between self-cleaning and self-disinfecting must be considered.

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