Photocatalytic antimicrobial activity of thin surface films of TiO2, CuO and TiO2 /CuO dual layers on Escherichia coli and bacteriophage T4

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Photocatalytic antimicrobial activity of thin surface films of TiO_2, CuO and TiO_2 /CuO dual layers on *Escherichia coli* and bacteriophage T4

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Abstract

TiO_2 coated surfaces are increasingly studied for their ability to inactivate microorganisms. The activity of glass coated with thin films of TiO_2, CuO and hybrid CuO/TiO_2 prepared by atmospheric Chemical Vapour Deposition (Ap-CVD) and TiO_2 prepared by a sol-gel process was investigated using the inactivation of bacteriophage T4 as a model for inactivation of viruses. The chemical oxidising activity was also determined by measuring stearic acid oxidation. The results showed that the rate of inactivation of bacteriophage T4 increased with increasing chemical oxidising activity with the maximum rate obtained on highly active sol-gel preparations. However these were delicate and easily damaged unlike the Ap-CVD coatings. Inactivation rates were highest on CuO and CuO/TiO_2 which had the lowest chemical oxidising activities. The inactivation of T4 was higher than that of *Escherichia coli* on low activity surfaces. The combination of photocatalysis and toxicity of copper acted synergistically to inactivate bacteriophage T4 and retained some self-cleaning activity. The presence of phosphate ions slowed inactivation but NaCl had no effect. The results show that TiO_2/CuO coated surfaces are highly antiviral and may have applications in the food and healthcare industries.

INTRODUCTION

The photocatalytic properties of TiO_2 were first reported by Fujushima and Honda (1972) and have subsequently been the subject of much research, especially for the photochemical oxidation of pollutants. The photochemistry has been reviewed by Mills and Le Hunte (1997) and Hashimoto *et al.* (2005). The irradiation of TiO_2 with ultra-violet light promotes electrons from the valence band to the conduction band leaving a positively charged hole. The electrons and holes migrate and, at the surface, react to give reactive oxygen species such as ·OH and O_2^-. These in solution can react to give H_2O_2. The use of UVA activated TiO_2 for disinfection was first proposed by Matsunaga and co-workers (Matsunaga *et al.*, 1985). There have since been reports of the use of photocatalysis for the destruction of bacteria, fungi and algae. Most of the early work used suspensions of TiO_2 and planktonic organisms (Reviewed by Blake *et al.*, 1999). There have been relatively few reports on the antiviral activity of TiO_2 coated surfaces. The photocatalytic killing of *Escherichia coli* bacteriophage ms2 was demonstrated by Sjogren and Sierka, 1994. They were able to reduce the pfu count of a suspension of ms2 phage by a factor of 10 with TiO_2 and 10^3 with TiO_2 in the presence of $Fe_3(SO_4)_2$ with a 10 min irradiation at 2 mW m^2 in a continuously stirred batch reactor. The increased activity in the presence of $Fe_3(SO_4)_2$ was reported to be due to increased production of free radicals *via* the Fenton reaction. Phage ms2 was killed more slowly in suspension than *E. coli* (Cho, 2005). Poliovirus 1 was inactivated by a suspension of TiO_2 in treated wastewater and a 2 log inactivation occurred in 30 min starting with 3000 pfu ml^{-1} although the intensity of UV was not quoted (Watts *et al.*, 1995). The inactivation of *Lactobacillus* phage PL1 was reported by Kakita *et al.*, 1997 using a ceramic preparation coated with a mixture of oxides including TiO_2 and AgO. The photocatalytic inactivation of coliphage Qβ in a flow through reactor over immobilised TiO_2 (3 log reduction after 3 h) was slower than with suspended

TiO_2 (4 log reduction after 30 min) and broth introduced with the phage was shown to have an inhibitory effect (Lee *et al*., 1997, Otaki *et al*., 2000). Belhacova *et al*. (1999) used a similar system to study the inactivation of coliphage λ and a 4 log reduction was seen in 3 h but the phage was also sensitive to the UV used with a 4 log reduction after 4 h. A 6 log inactivation of phage ms2 and a 4 log reduction in an unspecified *Bacteroides fragilis* phage was seen in 60 min with an irradiation intensity of 5 mW m^{-2} in suspension (Armon *et al*., 1998). Intermittent irradiation gave a faster rate of inactivation than continuous irradiation (Laot *et al*., 1999). Although the latter authors used immobilised TiO_2, the inactivation experiments were studied in suspension.

More recently, research has examined the biocidal activity of thin films of titania anchored to solid surfaces (Kikuchi *et al*., 1997, Sunada *et al*., 1998, Kuhn *et al*., 2003, Yu *et al*., 2003) with a view to producing self-disinfecting surfaces. Viruses can be transmitted from hands to inanimate surfaces and *vice versa* (Ansari *et al*., 1988, 1991) and contaminated surfaces are implicated in the transmission of e.g. noroviruses (Widdowson *et al*., 2005). Thus the ability to eliminate viruses on photocatalytic self-cleaning /self sterilising surfaces may provide a useful additional mechanism of control of transmission of viral diseases along with conventional disinfection methods. A 2.2 log reduction in a suspension of *E. coli* phage Qβ was obtained after 1 h irradiation with near UV black light at an intensity of $3.6x10^{-3}$ W/cm^2 on TiO_2 immobilized on ceramic tiles (Lee et al., 1997). Kakita *et al*. (2000) showed killing of *Lactobacillus* phage PL1 by thin films of TiO_2 suspended in liquid and the mechanism of killing was reported to be via initial damage to protein of the capsid by ·OH, followed by damage to the phage DNA inside the particles (Kashiga *et al*., 2001).

Copper is well characterised for its antiviral activity (Sagripanti., 1992, Sagripanti et al 1993, Sagripanti and Lightfoote, 1996, Sagripanti et al 1997). In this report we demonstrate the antiviral activity in thin films on glass surfaces coated with TiO_2, CuO and TiO_2/CuO using bacteriophage T4 as a model and describe the relative killing efficiencies

of different TiO_2 coated surfaces compared to killing of *E. coli* on TiO_2. The photocatalytic activity of the various films to 'self-clean' were also assessed using stearic acid as a model pollutant.

MATERIALS AND METHODS

Microorganisms and culture conditions

Escherichia coli B NCIMB 9482, *E. coli* ATCC 10536 and bacteriophage T4 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen UK. *E. coli* strains were sub-cultured onto Nutrient Agar (Oxoid, Basingstoke, UK) and incubated at 37˚ C for 24h. Cultures were resuspended in Nutrient Broth (Oxoid) and kept on Microban® beads (TCS Ltd Merseyside, UK) at –70˚C. Prior to use, one bead was sub-cultured onto Nutrient Agar and incubated at 37º C for 24h. Broth cultures (100 ml Nutrient Broth in 250 ml Erlenmeyer flasks) were inoculated and incubated at 200 rpm and 37˚C for 16 h in a New Brunswick G24 orbital incubator (New Brunswick Scientific, St Albans, UK).
Stock bacteriophage suspension was produced by addition of bacteriophage T4 to a 24 h culture of *E. coli* B (NCIMB 9482) and incubation at 37˚C for a further 24 h. Remaining cells and cell debris were removed by centrifugation at 5000 x g for 10 min. Bacteriophage were centrifuged by centrifugation for 30 min at 20,000 x g in a Sorvall RC6 centrifuge. The pellet was washed 3x by centrifugation and resuspension, plaque count determined and diluted appropriately and stored at -20˚C.

Plaque assay

Phage was assayed by the double agar layer method (Gratia, 1936). Phage suspension (0.1 ml) was mixed with 0.2 ml overnight *E. coli* culture in nutrient broth (approx 2 x 10^8 cfu

ml^{-1}) and 5 ml molten soft agar (Nutrient broth + 6g l^{-1} agar; Oxoid, UK) and poured on to surface of a nutrient agar plate. When set the plates were incubated at 37˚C for 24 h and plaques counted.

TiO_2 coated glass

Samples of two commercially available CVD coated glasses were obtained from two different manufacturers. Both samples had a 15 nm thick TiO_2 layer and were cut into 20 mm squares. Preparation of CuO and CuO -TiO_2 dual layer coated glass was via atmospheric pressure chemical vapour deposition (Ap-CVD) as previously described (Yates *et al*., 2007). Sample cleaning was performed either by ultra-sonication for 40 min in 40 ml of 100% methanol in an ultrasonic bath (Beckton-Dickinson, NJ, USA) or by gently shaking in methanol. Samples were removed aseptically and placed in UVA transparent disposable plastic Petri dishes (Sterilin), film side uppermost. The coated samples were then pre-irradiated by placing them under a 40 W UVA bulb with a $2.24x10^{-3}$ W cm^2 output for 24 h. For some control experiments samples of the TiO_2 coated glass were inactivated by autoclaving at 121˚C for 30 min, otherwise float glass was used as a control.

Samples of glass coated with a 400 nm layer of Degussa P-25 TiO_2 by a sol-gel process (Mills *et al*., 2003) were obtained from Professor Andrew Mills, Strathclyde University, UK.

UVA inactivation

Inactivation of bacteriophage

Bacteriophage suspension was diluted to give approximately 2 x 10^8 pfu ml^{-1}. Fifty µl was added to the test samples and spread out using the edge of a flame sterilized microscope cover slip. Two ml sterile distilled water was added to the dishes to prevent desiccation. Four samples were exposed to UVA using UVA lamps at $2.24x10^{-3}$ W cm^{-2}. A sample was removed immediately and remaining samples removed at intervals. Four samples exposed

to UVA but covered with a polylaminar UVA protection film (Anglia Window Films, UK) to block UVA but not infra-red, acted as controls.

The samples were then immersed in 20 ml of sterile de-ionised water and vortexed for 60 sec to resuspend the bacteriophage. A viability count was performed by serial dilution and plaque assay.

Inactivation of bacteria

A frozen bead of *Escherichia coli* ATCC 10536 was thawed and subcultured onto nutrient agar (Oxoid) and incubated at 37˚C for 24 h and stored at 5°C. A 50 µl loopful was inoculated in to 20ml Iso-sensitest broth (Oxoid) and incubated for 24 h at 37° C. Cultures were centrifuged at 5000 x g for 10 min in a bench centrifuge and the cells were washed in de-ionised water three times by centrifugation and re-suspension. Cultures were re-suspended in water and adjusted to OD 0.5 at 600 nm in a spectrophotometer (Camspec, M330, Cambridge, UK) to give approx. $2x10^8$ colony forming units (cfu) ml^{-1}. Fifty µl were inoculated on to each test sample and spread out and irradiated and resuspended as before. A viability count was performed by serial dilution and plating onto nutrient agar in triplicate and incubation at 37°C for 48 h.

Photocatalytic oxidation of stearic acid

Stearic acid provides a good model for typical organic surface contamination and as such was used to monitor the extent of photocatalytic activity in the various films. The degradation of stearic acid was followed by FTIR (Bruker, Vector 22: Yates et al., 2006). Stearic acid (100 µl of 10 mmol l^{-1} in methanol) was spun coated onto the sample. After drying in an oven the sample was exposed to UVA (365 nm) with an intensity of 3×10^{-3}W cm^{-2}. The activity of the film was measured by the rate of reduction in selected stearic acid peaks in the IR region (3000 – 2800 cm^{-1}) in cm^{-1} min^{-1}.

Reproducibility

Each experiment was performed in triplicate and mean, standard deviations and T-tests performed using Micosoft Excel.

RESULTS

The sol-gel sample had the highest photocatalytic activity on stearic acid (0.3875 $cm^{-1}min^{-1}$), followed by commercial sample 2 (0.0185 $cm^{-1}min^{-1}$). Commercial sample 1 had the lowest activity of the plain TiO_2 samples (0.0101 $cm^{-1}min^{-1}$). The CuO-TiO_2 dual layer and CuO samples both had similar and relatively low activities (0.0015 and 0.0017 $cm^{-1}min^{-1}$ respectively).

In order to show to show the effects of UVA irradiation alone on the microbial coated samples, heat generated from the lamps, infra-red irradiation from the lamps, desiccation or of suspension of in deionised water during the timescale of the experiments on bacteriophage T4, inactivated TiO_2 coated glass was used as a control. The effects of UVA irradiation on phage viability are shown in Figure 1a. There was a one log reduction after 4 h which was a greater reduction than in the controls (with UVA blocked) which showed that there was some killing effect of the UVA alone. However, UVA did not completely kill the phage within the time of the experiments.

The inactivation of bacteriophage T4 on commercial sample 1 is also shown in Figure 1a. There was a 2.5 log reduction after 4 h in the UVA irradiated samples whereas there was only a slight reduction in viability on the controls (less than 1 log). The experiments were relatively reproducible and this reduction was statistically significant when compared to the controls ($p<0.05$). The rate of killing on commercial glass 2 was higher and a >6 log reduction was obtained after 3 h (Figure 1a). Again this was significantly different from the controls ($P<0.05$).

However, when sol-gel coated P25 glass was used, the results were less reproducible and lower rate of killing was obtained in some experiments with a 6 log killing observed in 2-4 h on different samples (Figure 1b). Data for separate runs are given to show the variation between samples. Casual observation of the slides after the experiment showed some signs of damage to the coatings. The slides were examined by SEM there was clear evidence of damage to the surfaces with pits and grooves, some 10-100 μm wide, through the TiO_2 layer. The commercial samples had thinner layers of TiO_2 (15 nm) and showed no apparent damage during the assay process (not shown). The commercial samples were produced by CVD, which leads to hard, well adhered coatings. Sol-gel films by the nature of the method of manufacture generally tend to be more fragile and optically opaque (Mills 2003). Testing of samples cleaned by shaking rather than ultrasonication showed less damage and reproducible reductions in phage titre of $>10^5$ in 2 h (data not shown).

The killing of *E.coli* on TiO_2 followed a similar pattern to T4. There was a slight reduction on plain glass which was not significantly different from deactivated TiO_2 coated glass (Figure 1c). On commercial sample 1 there was only a slight and not significant difference between test and controls even after 4 h ($P>0.05$; Figure 1c) whereas a >5 log reduction was seen on sample 2 after 3 h ($P<0.05$; Figure 1c). The sol-gel samples were not tested with *E. coli* because of the damage to the coating.

The effects of resuspension medium on killing are shown in Figure 2. There was no decrease in the controls when water was used to resuspend the phage but there was a 1.5 log reduction in the controls with both saline and PBS ($p<0.05$). The test run with saline gave a >5 log reduction after 4h and with water and saline there were small numbers of survivors giving a >4 log reduction, significantly different from the controls ($p<0.05$). Inactivation on CVD CuO coated samples was more rapid with a >6 log reduction after 80 min with similar rates for both water and saline. The starting plaque count was 100-fold higher in these experiments. Killing with PBS was slower but there was no significant

difference after 80 min (P>0.05; Figure 3a). On TiO_2 over CuO the rate of killing was higher with both water and saline giving a >9 log kill after 80 min (Figure 3b). The rate of killing was significantly slower with PBS and there was only a 7 log reduction after 80 min (P<0.05). There was a 2 log reduction in the controls but there were no significant differences after 80 min (P>0.05).

Discussion

The sol-gel TiO_2 samples had the highest photocatalytic activity. This is probably related to the thickness of the coating which gives a greater UV absorption and hence increased activity (Jung, 2005) and to the increased surface area due to increased surface roughness, allowing more efficient exposure of the stearic acid on the TiO_2 surface. The sol-gel film had a much rougher surface (Ra=25 nm) and hence surface area than that of either of the commercial samples (Ra=2.6 nm and Ra=2.9 nm for commercial samples 1 and 2 respectively (measured using a Dektak 3ST surface profiler). As the commercial samples were of similar thickness and roughness, differences in activity are probably related to the inherent nature of the films. These differences could relate to low levels of dopants such as Na which are known to de-activate TiO_2 (Aubry *et al.*, 2007). CuO and TiO_2-CuO had 10-fold lower activity than those for TiO_2. Previous analysis established that, although the dual sample was deposited firstly with CuO and then TiO_2, the final result was of a surface of mainly Cu_2O and CuO on the surface with only a small amount of TiO_2 (Yates *et al*. unpublished data).

The TiO_2 coated surfaces were capable of inactivating bacteriophage T4 in thin surface films. Both commercially available CVD coated glasses showed killing of bacteriophage T4 and *E. coli* and sample 2 was able to achieve total killing after 3 h. These samples were more robust than the sol-gel coated samples tested which, although having a much higher chemical oxidising activity, had unreproducible killing times of 2-4 h. This was probably

due to damage to the coating during testing, the uneven surface resulting in uneven spread of liquid might affect reproducibility if surface defects are spread across the surface in an unreproducible and uncharacterised manner.

Previous work has suggested that the killing mechanisms of UV activated TiO_2 may be different for bacteria and viruses, at least in suspension. Sogren and Sierka (1994) showed that *E. coli* was killed more rapidly than ms2 and Cho *et al.*, (2005) showed that free ·OH in suspension were more active at killing ms2 whereas surface bound radicals, superoxide and H_2O_2 were implicated in killing of *E. coli* as well as free ·OH . Inactivation of *Lactobacillus* phage PL-1 occurred via initial damage to coat proteins followed by attack on the DNA (Kashiga *et al.*, 2001). Inactivation of bacteria is probably due to membrane damage via lipid peroxidation (Kiwi and Nadtochenko, 2005). In the present study, the rates of killing of bacteriophage T4 and *E. coli* were similar on sample 2 but T4 was more sensitive than *E. coli* on sample 1. This may have been because the killing mechanisms were different. Studies on phage inactivation in suspension suggest that most phage particles are killed when bound to TiO_2 particles. (Koizumi and Tyer, 2002). For the inactivation of bacteria in suspension free radicals are probably only involved when particles of TiO_2 are attached to the surface of the bacteria (Horie et al., 1998a, b). H_2O_2 production may be required for killing of bacteria in suspension. On thin films, as in the present experiments, most organisms will be in contact with the surface, especially at low cell densities. The effects of use of different resuspension media showed that saline and water had similar kill rates but that PBS slowed the rate of killing, particularly on surfaces with TiO_2 and dual layers, possibly by inhibiting contact between the phage and the surface. The inhibition of photocatalytic killing of bacteriophage ms2 in suspension by phosphate has been attributed to inhibition of binding of phage to the TiO_2 particles by the phosphate ions (Koizumi and Taya, 2002). Phosphate has also been shown to reduce the

rate of photocatalytic killing of bacteria in suspension (Arana *et al*., 2002) by inhibiting contact between the bacteria and TiO_2 particles (Gogoniat *et al*., 2006).

Comparison of the photocatalytic decomposition rates for stearic acid with the microbial killing rates showed no overall trend between chemically different samples. The CuO and dual CuO-TiO_2, although of comparatively low photoactivity, showed faster microbial killing rates than for the TiO_2 samples. Enhanced killing was probably due to a combination of copper oxides giving toxic Cu^{+}/Cu^{2+} in solution and photocatalysis. However, considering just the three TiO_2 samples, it can be seen that the photocatalytic and biocidal activity increase in the same sample order. Possibly the greater self-clean ability of the sol-gel film (when not mechanically damaged) is clearing the dead cells off faster, which may otherwise mask the surface and hence letting the more UV light reach the surface. Also, this effect may be a small contributory part in reducing the cell killing time for the CuO-TiO_2 sample over that of the CuO.

The results suggest that the inactivation of coliphage T4 may be a more sensitive method to detect photocatalytic killing than using bacteria as we were able to distinguish between the two commercial samples more clearly. The assay was easy to use and gave reproducible results. The use of bacteriophage is much safer than using pathogenic viruses. As far as the authors are aware, this is the first description of the killing of viruses on thin films of TiO_2 and shows that such surfaces can be self-disinfecting. The results show that thin films of TiO_2 are antiviral and inclusion of copper increases the rate of inactivation. The surfaces may have applications in infection control.

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Legends to Figures

Figure 1 Photocatalytic inactivation of bacteriophage T4 and *Escherichia coli* on TiO_2 coated glass

Bacteriophage T4 suspension (50µl, 2 x 10^7pfu ml^{-1}) was spread over the surface of cleaned and pre-activated 2 x 2 cm TiO_2 coated glass squares in plastic Petri dishes and irradiated (365nm, 2.24 x 10^{-3} W cm^{-2}). Glass squares were removed after different times and phage resuspended in water and viability determined by plaque assay after suitable dilution. Controls were covered with a UVA absorbing plastic film. Inactivated coated glass was also used as a control to study any loss of viability during the experiment.

(a) Commercially available glass ■ inactivated glass test, □ inactivated glass control, ● commercial sample 1 test, ▽ commercial sample 1 control, ● commercial sample 2 test, ○ commercial sample 2 control.

(b) Photocatalytic inactivation of bacteriophage T4 on sol-gel TiO_2 coated glass ▽ control, ● run 1 ▲, run 2 ● , run 3 ○.

(c) *Escherichia coli* suspension (50µl, 2 x 10^7cfu ml^{-1}) was spread over the surface of cleaned and pre-activated 2 x 2 cm TiO_2 coated glass squares in plastic Petri dishes and irradiated (365nm, 2.24 x 10^{-3} W cm^{-2}). Glass squares were removed after different times and resuspended in water and viability determined by plate count after suitable dilution.

Figure 2 Effects of resuspension medium on photocatalytic inactivation of bacteriophage T4 on commercial TiO_2 coated glass.

Phage was resuspended in water, saline or PBS and inactivated on commercial sample 2.

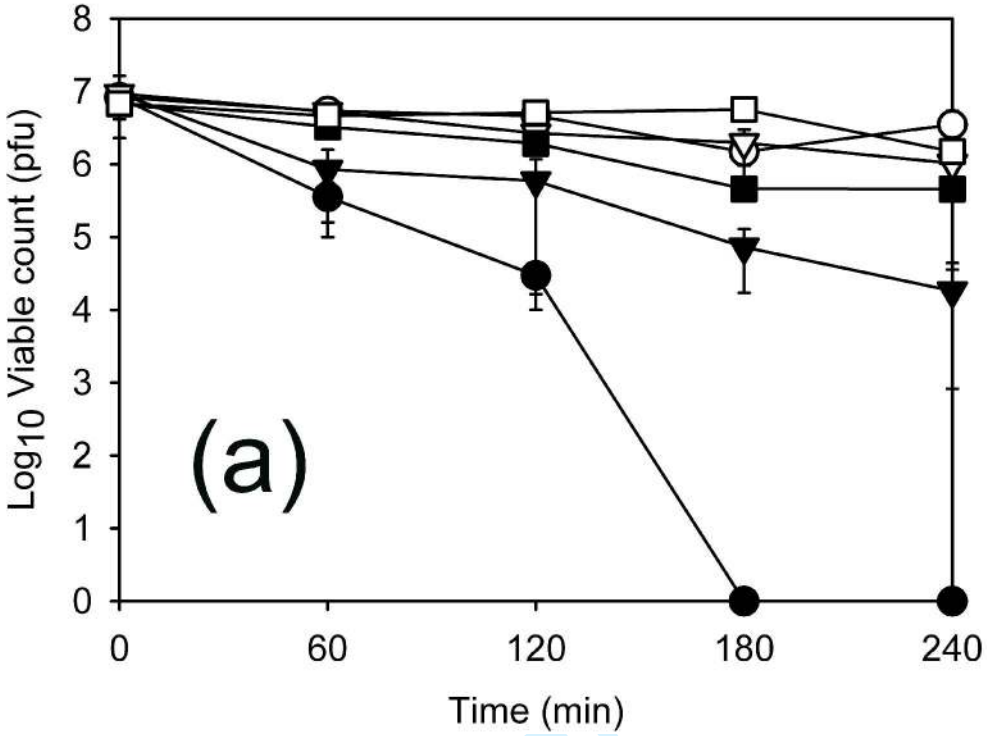
● water test, ○ water control, ▼ saline test, ▽ saline control,■ PBS test, □ PBS control.

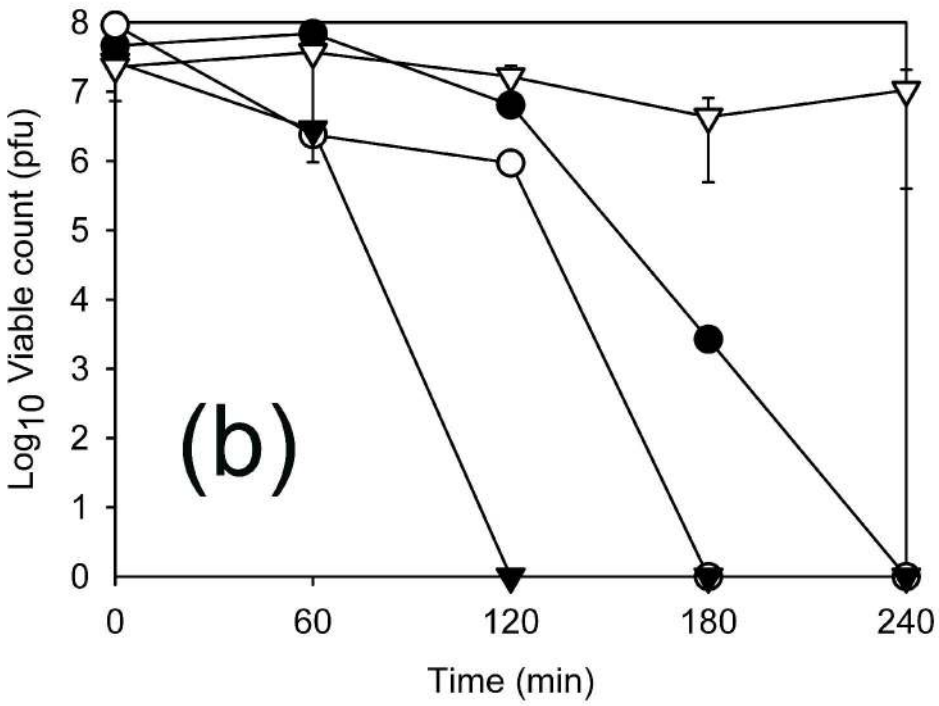
Other details as for figure 1.

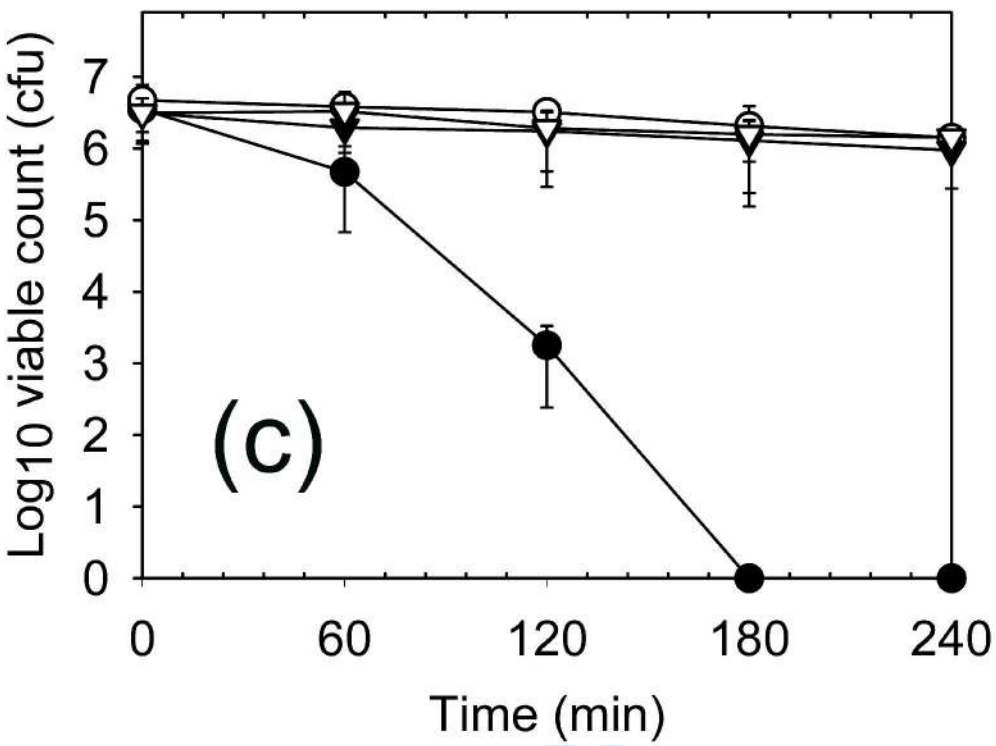
Figure 3 Effects of resuspension medium on inactivation of bacteriophage T4 on CVD CuO and hybrid CuO/TiO_2 coated glass.

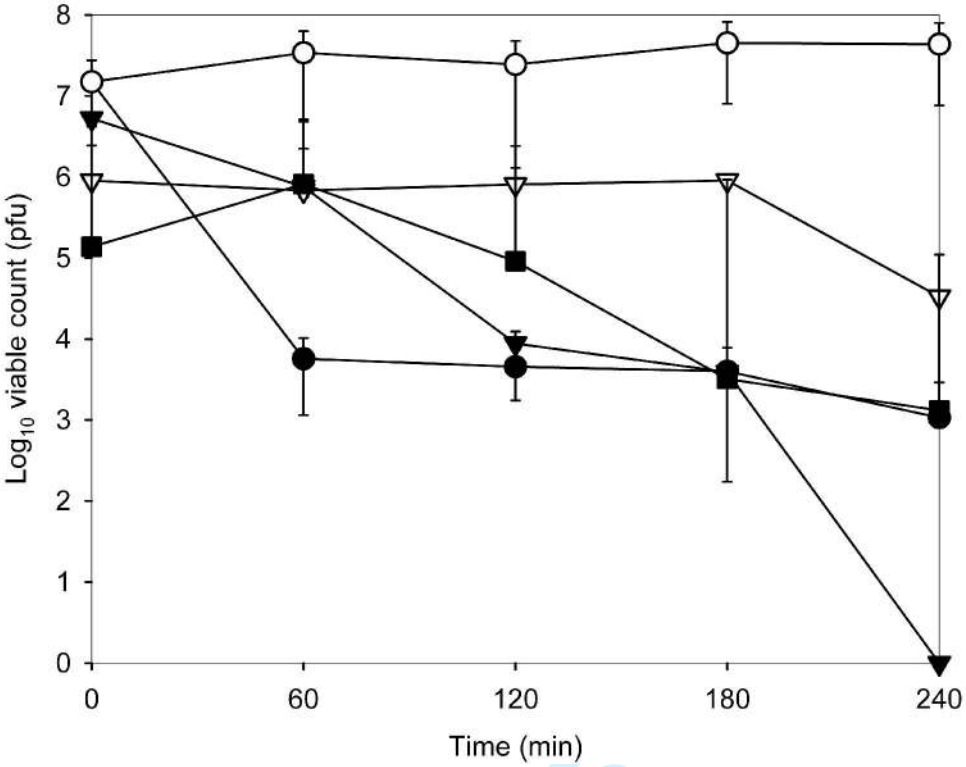
(a) CuO, (b) CuO - TiO_2 hybrid

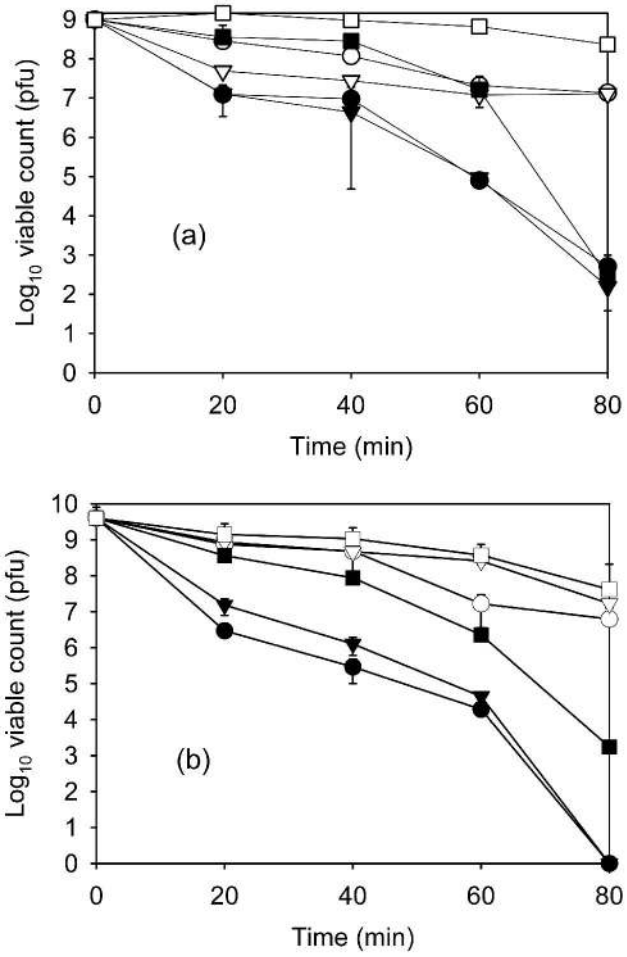
Other details as for figure 1.




(b)
Log10 Viable count (pfu)
Time (min)
0
1
2
3
4
5
6
7
8
0
60
120
180
240






Log10 viable count (pfu)
(a)
Time (min)
Log10 viable count (pfu)
(b)
Time (min)