Removal of pathogens by functionalised self-assembled monolayers

M. de los Reyes, A. Keegan, P. Monis and P. Majewski

ABSTRACT

In this study, the removal of *Escherichia coli* (*E. coli*) and MS-2 bacteriophage with silica powder and crystalline sand particles with NH_2-functionalised self-assembled monolayers (SAMs) was investigated. This study demonstrated that the removal of *E. coli* and MS-2 bacteriophage in the company of SAM coated substrates increased substantially over the maximum period, where the highest concentration of SAM covered silica powder achieved the fastest removal of both microorganisms, reaching the detection limit of the assay at 20 minutes. Removal was minimal with SAM coated quartz and no removal observed for unmodified quartz sand. SEM (scanning electron micrograph) pictures clearly show *E. coli* on SAM surfaces. With a greater removal observed for larger SAM concentrations, it may be suggested that the higher the concentration of the SAM, the faster the removal of the microorganisms. The removal is believed to be due to electrostatic attraction of the microorganisms to the positively charged surface of the functionalised particles.

Key words | pathogens, self-assembled monolayers, silica, water treatment

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INTRODUCTION

Microbiological contamination in potable water has long been a major public concern; microorganisms such as coliform bacteria, viruses and pathogenic protozoa cause various diseases ranging from mild gastroenteritis to severe diarrhoea. Waterborne diseases infect approximately 250 million people each year causing 10–20 million deaths (Kim & Yu 2005, Leclerc *et al.* 2002). The majority of these cases occur in developing nations and rural areas where sanitation is limited and public health awareness less significant than in more developed countries. The health implications of drinking water quality and the continuing demand for aesthetically pleasing water has brought about a rapid proliferation in the types and numbers of point-of-use water treatment device available (Ashbolt 2004). Various water treatment technologies available for sanitising source water include UV radiation, chlorination, ozonation and solar heating (Snelling *et al.* 2006), and though these methods prove beneficial for disinfection, treatment can be costly, time consuming and require complex equipment. The use of self-assembled monolayers (SAMs) in this respect is to override these current issues to provide a cheaper option for water treatment that does not require energy input to operate. While microbiological adhesions to SAMs have been investigated in areas such as infection of biomaterials (Gray 2004), medicinal purposes (Ista *et al.* 1996; Weibel *et al.* 2005) and food packaging (Susmel *et al.* 2003), the application of functionalised SAMs for water treatment has not been studied widely (Zerda *et al.* 1985).

A SAM is a closely packed, highly ordered array of chained hydrocarbon molecules containing various numbers of CH_2 − units (Collins & Sukenik 1995). The SAM is simply described as a hydrocarbon with the general formula A-$(CH_2)_n$ − B. **B** represents the bonding group, such as trichlorosilyl (−$SiCl_3$) and trimethoxysilane ($Si(OCH_3)_3$) forming tightly covalent Si—O—Si bonds to the surface atoms of silicon and silica. However, bonding with titanium

doi: 10.2166/aqua.2008.034

and titania via Si-O-Ti bonds were also observed (Liu *et al.* 2006). **A** denotes the head group, chosen from among a number of possible species, such as sulfonate ($-SO_3H$) and amine ($-NH_2$), respectively. The length of the hydrocarbon molecules and the related thickness of the SAM are calculated based on the numbers of CH_2-units and were calculated to vary between about 0.6 nm (three CH_2-units) and about 2.5 nm (17 CH_2-units). Immersed into aqueous solutions, some of the head groups, such as sulfonate head groups, tend to deprotonate and to form negatively charged surfaces. Head groups such as NH_2 are known to deprotonate at high pH-values and to capture protons at low pH-values forming positively charged surfaces (Hansen 2001). Therefore, by carefully choosing the SAM and pH-value of the solution in which the SAM is immersed, negatively as well as positively charged surfaces can be obtained.

In this study, the methods focus on the use of NH_2 – functionalised SAMs on amorphous substrates to examine its ability to remove *Escherichia coli* (*E. coli*) and MS-2 bacteriophage from solution (Dulbecco's Phosphate Buffered Saline (DPBS)) over time.

MATERIALS AND METHODS

Self-assembled monolayers (SAMs)

To hydroxylate the surface of the substrates 20 g of either amorphous silica powder or crystalline sand particles, with diameters of 100 μm and 120 μm, respectively, were immersed in 200 ml of piranha solution (70% vol. H_2SO_4, 30% vol. of 30% wt H_2O_2, Ajax Chemicals) at room temperature under a fume hood for 5 minutes, followed by subsequent washes with Milli Q water five times at 100 ml and drying at 60°C. Formation of the SAMs was achieved by placing 20 g of either powder in 200 ml of toluene (Aldrich, USA) containing 12 ml vol of surfactant aminopropyltrimethoxysilane (APTMS, Sigma, Australia) and mixed for 5 minutes at room temperature. The powders were filtered through a Buchner funnel, washed three times with 70% ethanol, ultrasonicated for 15 minutes (SoniClean, Australia) and dried in an oven at 60°C.

Substrate materials

Amorphous silicon dioxide (SiO_2, analytical reagent, Malinckrodt Chemical Works, grain size range: 3–90 μm with $d_{10} = 7.6\,\mu m$, $d_{50} = 24.3\,\mu m$, $d_{90} = 49.1\,\mu m$, surface area $= 0.23\,m^2\,g^{-1}$) and quartz sand (grain size range: 100–450 μm with $d_{10} = 121.9\,\mu m$, $d_{50} = 239.3\,\mu m$, $d_{90} = 330.6\,\mu m$, surface area $= 0.02\,m^2\,g^{-1}$) inferred from the average particle size as determined by light scattering with a Malvern Mastersizer was used as precursor powder (Figure 1).

Escherichia coli (*E. coli*)

E. coli ATCC and ATCC 1559 were used in removal experiments. Tryptone Soya Agar (TSA) and broth were used for bacterial culture with incubation at 35°C, placed on a rotary shaker (Ratek, Australia) at 150 rpm as required. From an overnight culture, total culturable bacteria were estimated by performing serial dilutions in DPBS and 100 μl spread on dry TSA plates. Plates were incubated at 35°C for 18–24 hours. Plate counts were used to enumerate bacteria with counts between 20 and 300 colonies per plate utilised for the calculation of bacterial numbers. The number of colony forming units (CFU) per 100 μl was calculated by multiplying the mean of the triplicate counts for the selected dilution by the reciprocal of the dilution. A final count per ml was calculated by a 10-fold multiplication.

To monitor the removal of *E. coli* in the presence of silica powder and sand particles, a stock *E. coli* suspension was prepared. A total of 10^5 CFU/100 ml was placed into

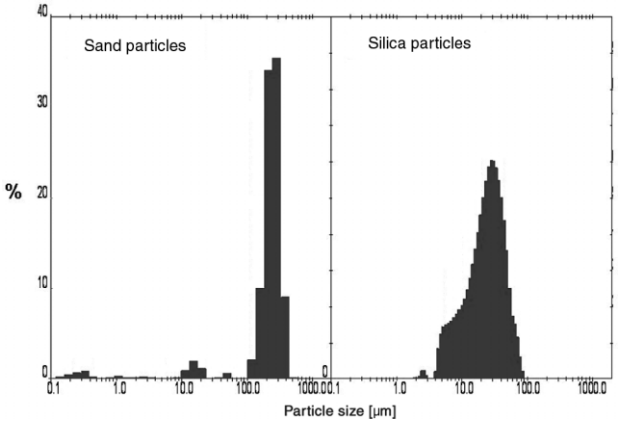

Figure 1 | Particle size distribution of the employed substrate materials.

100 ml of DPBS (pH ≈ 7.15). The suspension was mixed by vortexing and 20 ml aliquotted into four individual 50-ml beakers. Samples were taken at time zero before the addition of silica substrate to determine initial bacterial levels. Silica or sand was applied at the doses described in Table 1, each set on a magnetic stirrer at low speed. Due to the weight of sand particles, these experiments were performed in 50 ml TTP tubes (TTP labtech, United Kingdom) and mixed using a rotary wheel to maintain suspension of the particles.

Samples were taken every 20 minutes for a total of 100 minutes; 1 ml was removed from the reactor and placed in a 1.7 ml tube and centrifuged at 2,000 rpm (7.62 g units) for 60 seconds to remove the sand or silica particles, leaving bacteria in suspension. Samples were plated onto TSA plates (in triplicate) and spread evenly before overnight incubation at 35°C.

MS-2 bacteriophage

MS-2 bacteriophage stocks (ATCC 15597 B-1) were prepared in suspension (DPBS) using their host cells *E. coli* (ATCC 15597). Active cultures of the *E. coli* host were first cultivated for 24 hours at 35°C in #271 medium (American Type Culture Collection) (Strauss & Sinsheimer 1963). Total phage was estimated by preparing triplicate 10-fold serial dilutions of the phage stock in DPBS. A 100 μl aliquot of phage suspension was added to a molten agar overlay with 30 μl of an overnight *E. coli* culture and poured onto a dry TSA plate then incubated at 35°C for 18–24 hours. Plaque counts between 20 and 300 per plate were applied for calculation of phage number. The number of phage forming units (PFU) per 100 μl was calculated by multiplying the mean of the triplicate counts for the selected dilution by the reciprocal of the dilution. A final count per ml was determined.

To examine the removal of MS-2 bacteriophage, 100 μl of diluted phage culture (final concentration of 10^3 PFU/100 ml) was placed into 100 ml of DPBS (pH ≈ 7.5) with 20 ml of the solution placed into four separate 50-ml beakers. Samples were taken at time zero then silica or sand was applied as described in Table 1. Beakers were each set on magnetic stirrers (sand particles in 50 ml TTP tubes, spun on a rotary wheel); 1 ml of the sample was pipetted out of solution every 20 minutes (from 0 to 100 minutes), centrifuged for 60 seconds at 200 rpm (7.62 g units) and plated in triplicate, with 100 μl transferred to molten agar overlays with the addition of 30 μl of an overnight *E. coli* ATCC 15597 culture and poured onto dry TSA plates and set. Plates were incubated at 35°C for 18–24 hours.

Table 1 | Silica and sand substrate concentrations selected for experimentation

Volume of DPBS inoculated with *E. coli* (ml)	Substrate concentration (g)	Ratio (g l^{-1})	Surface ratio (m^2 l^{-1}) Powder	Sand
1. 20	0.0	0	0	0
2. 20	0.2	10	2.0	0.1
3. 20	0.4	20	4.0	0.2
4. 20	2.0	100	20	1.0

Data analysis

Statistical analysis was performed using Student's *t*-test on Microsoft Excel 2003. All p values <0.05 were considered to be significant.

Characterisation of SAM deposition

ToF-SIMS spectra were acquired using a PHI model TRFT™ II spectrometer (Physical Electronics, USA), with a high mass resolution of 9,000 and a high transmission of > 50%. The gallium (Ga^+) liquid metal primary ion source was operated with varying currents of 20, 60 and 600 pA, a pulse length of 15–60 ns and a separation rate of 11 kHz.

Silica powder and sand particles coated with SAMs were prepared for SEM (scanning electron micrograph) imaging by chemically fixing the bacteria with 2% OsO_4 for 30 minutes followed by dehydration with 70, 90, 95 and 100% ethanol for 10 minutes. Dry liquid CO_2 was used as the transitional fluid for critical point drying at 40 °C and 1,450 psi with an exchange temperature of 16 °C and 900 psi in an Energy Beam Scientific Polaron E300 critical point dryer. The dried samples were mounted on double-sided carbon tape and coated with platinum to generate a charge on the sample and imaged using a Philips XL 30 scanning electron microscope at 10 kV.

The zeta potential of the pure silica and SAMs coated silica powder were measured between pH values of 2.5 and 10. A Zetasizer Nano-ZS (Malvern Instruments, UK) was used to measure 0.5 g of the powder in 50 ml KCl solution. The different pH values were adjusted by adding HCl and NaOH, respectively.

RESULTS AND DISCUSSION

ToF-SIMS

Table 2 shows a summary of positively charged secondary ion intensities obtained from both silica powder and sand particles coated with and without SAMs. As the amine functional group is terminated on the self-assembled monolayer, its appearance and intensity on the spectrum (not shown here) would largely imply the successful chemisorption of the SAMs on the surface. However, NH_2 at $m/z = 16$ is not of use in ToF-SIMS spectra therefore NH_4^+ at $m/z = 18$ and NH_3^+ at $m/z = 17$ were used as indications for the presence of amine termination on the surface of the particles.

Overall, analysis clearly indicates the deposition of amine groups on both surfaces. It should be noted, though, that the strength of the silicon at $m/z = 28$ outweighs the coverage of the SAMs suggesting a possible incomplete coating on the original oxidised silicon surface. Contamination of NH_2-groups on the surface of unmodified silica powder is difficult to discern, but is believed to be a nitrogen-containing defect materialised from sample handling, preparation and possible chemical reactions with the atmosphere due to prolonged storage in this environment. In contrast, amine was not detected on sand particles without SAMs, but clearly observed on particles with SAMs, indicating successful attachment on the surface.

Zeta potential

The measurements clearly show that the NH_2-SAM provides a strongly positive zeta potential at pH values below about 9. At pH 7 the zeta potential was increased from −24.2 to 47.2 mV (Table 3). Although the non-coated silica particles exhibit a slight amine contamination as indicated by the ToF-SIMS analysis, the zeta potential of the material is still negative. The reason for this phenomenon may be that the identified amine groups are differently bonded in the amorphous silica compared with the amine groups at the tip of the SAM surface and their positive charge is compensated by silica resulting in the negative total charge.

Removal analysis

The removal of the microorganisms over a 100-minute period was determined for three substrate concentrations (with or without SAMs) by direct colony counts performed in triplicate. The $\log_{10}$ values of the average counts were used to calculate the removal of the microorganisms relative to the time = 0 count for each experimental condition (i.e. removal = $\log_{10}$ (count for time = X) – $\log_{10}$ (count for time = 0)).

Escherichia coli removal with silica powder

Figure 2 demonstrates the removal of *E. coli* in the presence of silica powder with and without SAMs to determine the removal attributed to the SAMs coating as a function of time. This data illustrates that *E. coli* removal was achieved with each concentration of silica powder in respect to control observations. Control reactions demonstrated no loss of bacteria due to processing over the 100-minute

Table 2 | ToF-SIMS peak intensities obtained from silica powder and sand particles coated with and without SAMs

	Silica particles	Silica particles + SAM	Sand particles	Sand particles + SAM
Si ($m/z = 28$)	619142	323802	24062	15622
NH_3 ($m/z = 17$)	1054	1703	0	254
NH_4 ($m/z = 18$)	756	3175	0	541
$NH_3 + NH_4$ Si (%)	0.3	1.5	0	5.1

Table 3 | Zeta potential of the silica and SAMs coated silica powder

pH value	SAMs coated silica powder (mV)	Silica powder (mV)
2.5	54.3	− 16.6
3	50.4	–
3.5	50.2	–
4	52.3	–
5	51.7	− 23.5
6.2	50.4	− 23.9
7	47.2	− 24.2
8	36.3	–
9	13.3	–
10	− 13.8	− 28

period ($p > 0.05$). Specifically, the ability to remove bacteria was found with unmodified silica powder (a maximum of 1.6 $\log_{10}$ removal attained for the 20 m^2 l^{-1}, Figure 2), but compared with this, SAMs coated silica powder exhibited a more efficient removal of the bacteria; the detection limit of the assay (2.3 $\log_{10}$ removal) was reached at 20 minutes with 20 m^2 l^{-1} silica with SAMs, followed by 4 m^2 l^{-1} at 60 minutes (2.3 $\log_{10}$ removal), however 2 m^2 l^{-1} only reached a 1.6 $\log_{10}$ removal at 100 minutes. As no further removal was seen for the 2 $m^2 l^{-1}$ silica with SAM concentration, it is postulated that the substrate was approaching saturation point, where removal ceases due to the unavailability of binding sites on the surface. This level of removal is reiterated in Figure 2. Theoretically attachment to unmodified silica powder should not occur as no prior alterations were made to induce a charge on its surface, therefore all samples with silica powder (with no SAMs) should be similar to those obtained with the control. As contamination of the unmodified silica powder with $-NH_3/NH_4$ was discovered within the ToF-SIMS spectra, this may have aided silica powder (with no SAMs) in the removal of the bacteria.

Escherichia coli removal with sand particles

Figure 3 displays the capability of sand particles to remove *E. coli* from suspension. Each unmodified sand particle concentration demonstrated no significant difference ($p > 0.05$) in data, indicating no bacterial removal due to the sand particles alone. Where SAM coated sand particles were introduced, a steady increase in bacterial removal was observed, which was directly attributed to the presence of the SAMs. Increased concentration of the SAMs coated particles possessed larger removal capacity, however, none reached the detection limit as observed with SAM coated silica powder. After the maximum test point, 0.1 m^2 l^{-1} only reached a 1.16 $\log_{10}$ removal, followed by 0.2 m^2 l^{-1} with a 1.37 $\log_{10}$ removal and 1 m^2 l^{-1} achieving a 1.67 $\log_{10}$ removal. The consistently lower removal of SAMs coated sand particles and slow attachment of the bacteria can be explained by the interaction of the *E. coli* with the surface structure of the particles. Taking into consideration the surface areas of both substrates, silica powder has a higher surface area compared with sand particles and thereby a higher binding capacity. As *E. coli* has a size of approximately 1 μm, it could be easily attached or filled in the microstructure of similar size. Silica powder also has up to 20 m^2 l^{-1} in total area while sand particles only have 1 m^2 l^{-1}, therefore there is 1/20 the amount of sand particles as there is silica powder suspended in solution.

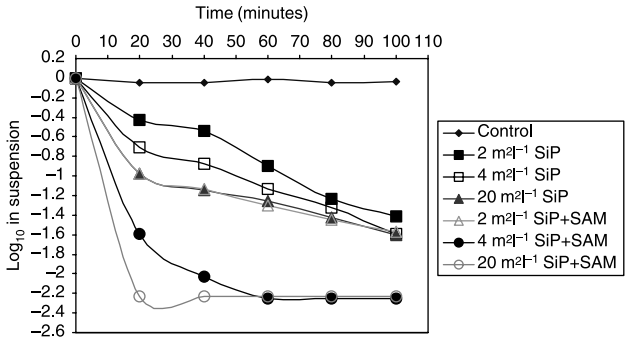

Figure 2 | $\log_{10}$ removal of *E. coli* with silica powder and silica powder coated with SAM.

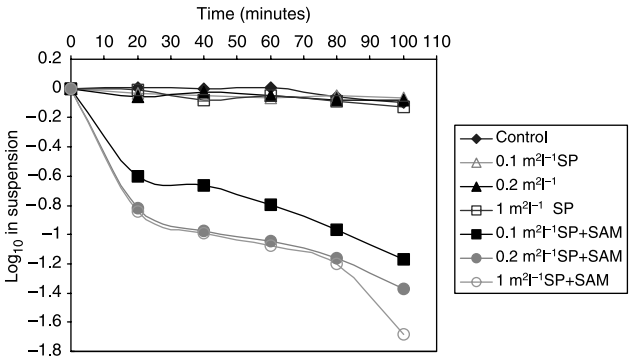

Figure 3 | $\log_{10}$ removal of *E. coli* with sand particles and sand particles coated with SAM.

Comparison of the results of the experiments clearly shows that the SAMs coated silica powder is much more efficient in removing the bacteria than the sand particles. This fact is presumably due to the 20 times larger surface area of silica powder compared with the sand particles.

MS-2 bacteriophage removal with silica powder

Owing to the results obtained for *E. coli* removal, MS-2 bacteriophage removal was set at 60 minutes duration.

A steady decline in phage numbers was evident for unmodified silica powder (Figure 4) with a maximum 0.54 $\log_{10}$ removal achieved by $4\,m^2\,l^{-1}$ and a 0.70 $\log_{10}$ removal by $20\,m^2\,l^{-1}$. In the presence of SAM coated silica powder, $20\,m^2\,l^{-1}$ reached the maximum detection limit removing 2 $\log_{10}$ at 20 minutes. This was followed by $4\,m^2\,l^{-1}$ reaching a 2 $\log_{10}$ removal at 60 minutes with only a 1 $\log_{10}$ removal by $2\,m^2\,l^{-1}$. A correlation between the binding natures of *E. coli* and MS-2 bacteriophage is evident with results obtained from both silica powders with and without SAMs in Figure 3. Both substrates succeed in removing bacteriophage, with silica powder coated SAMs reaching the detection limit at the first time interval of 20 minutes with $20\,m^2\,l^{-1}$.

MS-2 bacteriophage removal with sand particles

The original experimental procedures established for *E. coli* removal were employed for the removal of MS-2 bacteriophage with SAM deposited beach sand.

No significant differences were observed for unmodified sand particle concentrations, with values varying only between 0 and 0.4 log removals, which are regarded as very minimal changes (Figure 5). These values follow the same insignificance as the control values ($p > 0.05$). Pronounced differences were observed for all SAM covered beach sand concentrations, with each obtaining high significance against t = 0, control values and subsequent time points thereafter ($p < 0.05$). After the maximum test point, $0.1\,m^2\,l^{-1}$ achieved a 0.77 $\log_{10}$ removal, followed by $0.2\,m^2\,l^{-1}$ with a 1.02 $\log_{10}$ removal and $1\,m^2\,l^{-1}$ with a 1.22 $\log_{10}$ removal. Again, these values can be attributed to the amount of surface area available to act as binding sites.

The interaction of bacteria, viruses and proteins with functionalised surfaces has been studied widely and the results of this investigation do not contradict with existing knowledge (Zerda *et al.* 1985; Doyle *et al.* 1995; Fletcher 1996). However, the attachment of the microorganisms onto SAM coated particles in considerably agitated water is remarkable, because it indicates a strong interaction between the SAMs and the microorganisms. At the pH of the water samples, the microbial compounds studied here are negatively charged, whereas, the NH_2-terminated SAMs are positively charged (Schreiber 2000). It is therefore assumed that the removal of the organic matter is mainly due to a strong electrostatic attraction and immobilisation of the organic matter at the surface of the particles.

The results of the experiments show again the higher efficiency of the silica powder which is consistent with the results of the experiments on bacteria removal.

Removal mechanism

The main reason for the removal of the pathogens is believed to be the positive surface charge of the SAMs coated

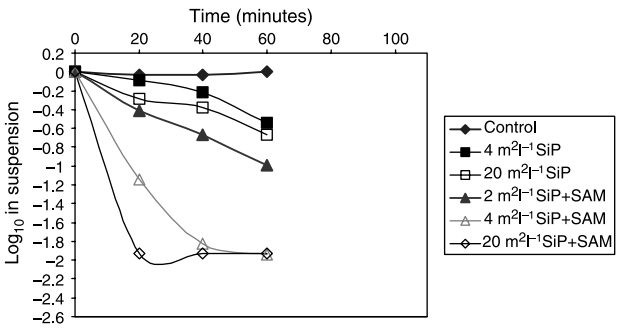

Figure 4 | $\log_{10}$ removal of MS-2 bacteriophage with silica powder and silica powder coated with SAM.

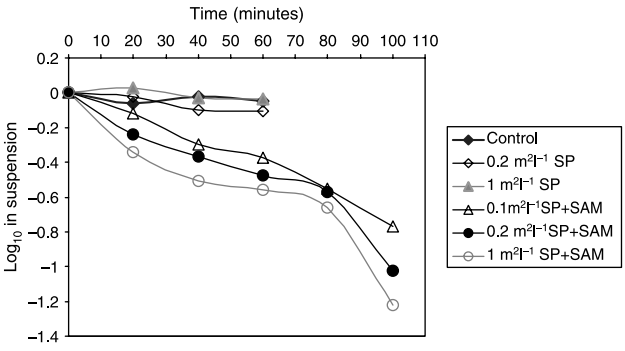

Figure 5 | $\log_{10}$ removal of MS-2 bacteriophage with sand particles and sand particles coated with SAM.

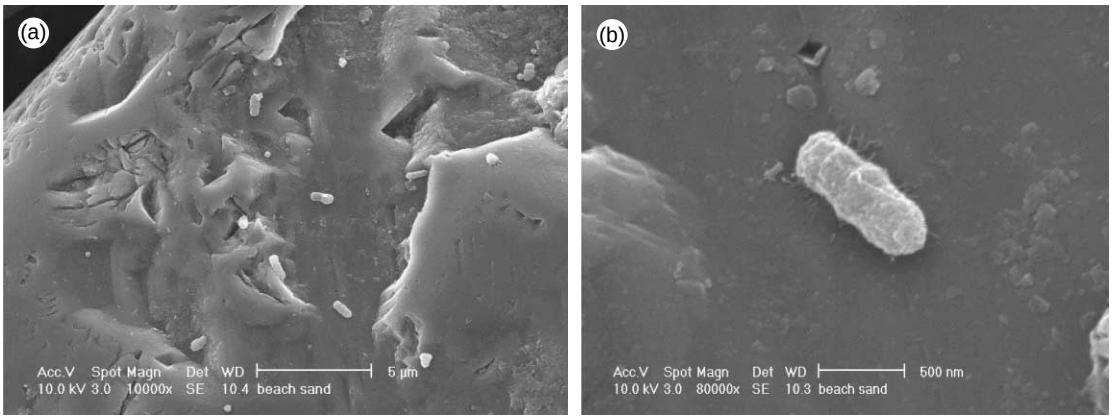

Figure 6 | (a) and (b) SEM images of *E. coli* on SAM coated sand particles.

particles compared with the non-coated particles. The chemistry of the amines is dominated by the ability of the lone pair on the nitrogen atom to capture protons forming NH_3^+-groups. This phenomenon is the reason for the positive charge of the particles coated with NH_2-SAM at pH values below about 9. As the pathogens are negatively charged at the pH of the water samples, the pathogens are attracted to the functionalised surface and immobilised.

SEM

A low-magnification (500 nm – 5 μm) scanning electron micrograph representative of two sites on the surface of one sand (with SAMs) particle sample is illustrated in Figure 6 (a) and (b). Investigations verified the presence of randomly distributed individual rod-shaped cells both the size (approximately 1 μm) and morphology of *E. coli*, confirming their presence on the SAMs modified particle surface. Focusing on Figure 6(b) demonstrates *E. coli* attachment with type 1 fimbriae (filamentous proteinaceous appendages) that act in adhesions involved in specific receptor recognition and colonisation, characteristic of *E. coli* binding properties (Van Loosdrecht *et al.* 1987). No MS-2 bacteriophage samples were analysed with the scanning electron microscope as documentation (Firtel & Beveridge 1995) suggest that viruses do not preserve well as a result of drying procedures and small sample size (≈ 30 nm). Distinction between phage and artefact at this level of magnitude would be difficult to discern.

CONCLUSION

In this summary, self-assembled monolayers on model surfaces (silica powder and crystalline sand particles) were used to assess its potential to remove *E. coli* and MS-2 bacteriophage. A large number of factors play an important role in attracting microbes to solid surfaces, with the most important aspects being surface charge, surface roughness, surface area and treatment time. The finer silica powder with about 20 times larger surface area compared with the sand particles is significantly more efficient in removing the species.

It is evident from the results that self-assembled monolayers are a novel, innovative method in the area of water treatment for the removal of pathogens. They are also easily scalable to industrial quantities due to the availability and smaller costs of the substrates. However, it has to be taken into account that microbes would grow with time in the presence of sufficient nutrients. After a period of time a biofilm may form on the SAMs which may cause a decrease of the efficiency of the material to remove pathogens from water.

Though results demonstrate that functionalised self-assembled monolayers are quite capable of removing *E. coli* and MS-2 bacteriophage, further detailed investigations are necessary to optimise the efficiency of the removal, especially by maximising the effectiveness of the material by tailoring the SAMs coating, and for implementation into existing water treatment technologies such as reactor beds and water filters.

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First received 25 February 2007; accepted in revised form 21 August 2007