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SPECIAL ISSUE ARTICLE

Rhenium and yttrium ions as antimicrobial agents against multidrug resistant *Klebsiella pneumoniae* and *Acinetobacter baumannii* biofilms

A.J. Slate[1], L. Shalamanova[2], I.D. Akhidime[1] and K.A. Whitehead[1]

1 Microbiology at Interfaces Research Group, Manchester Metropolitan University, Manchester, UK
2 Cardiovascular Group, Manchester Metropolitan University, Manchester, UK

Significance and Impact of the Study: The metal ions, molybdenum, rhenium, thallium and yttrium were tested against both *Klebsiella pneumoniae* and *Acinetobacter baumannii* in planktonic and biofilm forms. This research demonstrated that all the metal ions may be effective antimicrobial agents. However, molybdenum induced high levels of cytotoxicity, whilst, there was no significant difference in the toxicity of the other metal ions tested. When considering the results for the antimicrobial efficacy and biotoxicity of the metal ions, in conjunction with the known toxicity of thallium in certain chemical compositions, it was concluded that overall rhenium or yttrium ions may be effective antimicrobial agents, one potential application may be utilizing these metal ions in hospital surface cleaning formulations.

Keywords
antimicrobial resistance, bacteria, biofilms, hospital acquired infection, metal ions.

Correspondence
Kathryn A. Whitehead, Microbiology at Interfaces Research Group, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK.
E-mail: k.a.whitehead@mmu.ac.uk

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Abstract

Antimicrobial resistance presents major global concerns to patient health. In this study, metal ions of molybdenum, rhenium, yttrium and thallium were tested against bacteria in planktonic and biofilm form using one strain of *Klebsiella pneumoniae* and *Acinetobacter baumannii*. The antimicrobial efficacy of the metal ions was evaluated against the planktonic bacterial strains using minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations, whilst the efficacy of the metal ions against biofilms was tested using a crystal violet biofilm assay. Live Dead staining was used to visualize the antimicrobial activity elicited by the metal ions on the bacterial cell. The results showed that higher concentrations of the metals were required to inhibit the growth of biofilms (72·9 mg l^{-1} to 416·7 mg l^{-1}), in comparison to their planktonic counterparts. MICs of the metal ions (<46·9 mg l^{-1}) (planktonic cells) did not affect biofilm formation. Overall, rhenium and yttrium were effective antimicrobial agents. Molybdenum demonstrated the greatest level of biotoxicity. When taking into account these results and the known toxicity of thallium, it is possible that rhenium or yttrium ions could be developed as effective biocidal formulations in order to prevent transmission in healthcare environments.

Introduction

Hospital-acquired infections place a huge burden upon healthcare services, dramatically increasing mortality rates whilst also placing an economic strain on the health services. In the UK, hospital acquired infections affect around 1 in 10 patients during their stay in hospital (Breathnach 2005) and cost the NHS approximately £3000 per patient (Magill *et al.* 2014).

Antimicrobial resistance (AMR) often occurs due to over persistent use of antimicrobials, such as antibiotics. Excessive use of antibiotics in agriculture and the over-prescribing of antibiotics to patients have added a selective pressure on micro-organisms (Laxminarayan *et al.* 2013). A study from the Centre for Disease Control and Prevention (CDC), suggests that antimicrobial resistance accounts for around 23 000 deaths annually in the USA alone (CDC, 2013). *Acinetobacter baumannii* has been

estimated to account for up to 10% of all hospital-acquired infections (Joly-Guillou 2005). *Klebsiella pneumoniae* has been shown to be the second highest causative agent of hospital acquired infections, after *Escherichia coli,* possibly due to its prevalence in gastrointestinal infections (Vading *et al.* 2018). Furthermore, the analysis of 50 bacterial isolates from urinary catheters has demonstrated that 16% were *Klebsiella* spp., whilst 2% were *Acinetobacter* spp. (Niveditha *et al.* 2012).

Hospital equipment and surfaces can act as potential reservoirs for micro-organisms and the transmission of pathogenic bacteria to patients may lead to secondary hospital-acquired infections (Weinstein and Hota 2004). Several bacteria have been shown to survive for months on dry hospital contact surfaces, including *Acinetobacter* spp., which survived in a vegetative state for between 3 days to 11 months (final extinction was noted on day 343) and *Klebsiella* spp., remained viable between 2 h and 30 months (Wagenvoort and Joosten 2002; Otter *et al.* 2013). Therefore, there is an urgent need for effective biocidal regimes to be developed and deployed. This would involve the development of biocidal formulations with enhanced antimicrobial activity, but with little or no human toxicity at the same concentrations, in order to destroy possible bacterial reservoirs and reduce the exacerbated risk of transmission of infection in Hospital settings.

It has been suggested that conventional *in vitro* antimicrobial susceptibility testing methods which target sessile cells are not suitable for evaluating the efficiency of candidate antimicrobials (Weinstein and Hota 2004; Jacqueline and Caillon 2014). Several studies have shown that biofilms can demonstrate antimicrobial resistance greater than that exhibited by sessile bacterial cells (Bjarnsholt *et al.* 2005; Macia *et al.* 2014). Smith and Hunter (2008) reported that 80% of the methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* biofilms remained viable after the application of hospital biocides.

Metal ions may provide in part a biocidal solution as they have the ability to bind to and block functional groups (i.e. thiol groups) in bacterial cells (Stiefel *et al.* 2015). The interaction with such functional groups can displace and disrupt essential metals in bacterial enzymes, leading to dysregulation of protein synthesis, bacterial DNA synthesis and ultimately, the loss of cell membrane integrity resulting in cell death (Hobman and Crossman 2015). In previous work, we have demonstrated the antimicrobial activity of other metal ions in solution, however, this work did not look at either the biofilm or cytotoxicity effects of the antimicrobials (Vaidya *et al.* 2018).

Although such metal ions may be suggested for use as biocidal agents, that may be potentially used in hospital surface cleaning formulations, the properties of the metals is dependent on their form, ions can cause toxicity in animals and humans. Thallium is known to be more acutely toxic than other metals (Bannon 2015). The thallium ion (Ti^{+}) has a charge similar to that of potassium ions, allowing it to easily enter cells and compete with potassium-dependent biological systems, one example of this is Ti^{+} ions binding to Na/K-ATPase (Britten and Blank 1968; Cotton and Wilkinson 1980). Yet ^{201}thallium, is used in myocardial perfusion imaging with for detection and evaluation of coronary artery disease (Beller 1994). Thus, the extent of metal toxicity is ultimately dependent on its form. For example, the lethal dose, 50% (LD50) for thallium acetate in brown rats is 41·2 mg kg^{-1} (moderately toxic) and 16 mg kg^{-1} for thallium sulphate (highly toxic). In contrast, the transitional metals rhenium, yttrium and molybdenum are considered practically nontoxic (LD50 (single oral dose for rats) 5000–15 000 mg kg^{-1}) (Misra *et al.* 2003; Egorova and Ananikov 2017). Although molybdenum toxicity is rare in humans, there have been reports of molybdenum toxicity associated with dietary supplement overdose, which manifests with copper deficiency, gout-like symptoms and neurotoxicity (Vyskocil and Viau 1999).

In order to determine the possibility of the use of some lesser used metal ions as antimicrobial agents, this fundamental study tested the determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) crystal violet biofilm assay and Live Dead Staining of the biofilms against two known Gram-negative hospital pathogens, *Klebsiella pneumoniae* and *Acinetobacter baumanii.* The metal ions also needed to demonstrate low eukaryotic cellular toxicity, in order to determine if they could potentially be advantageous for use in novel biocidal formulations. Eukaryotic cytotoxicity was determined against the human renal proximal tubular HK-2 cell line. A renal cell line was chosen for this study, as it has been demonstrated that one of the main target organs for some metal toxicity is the kidneys, for example thallium has been shown to accumulate in the kidneys of poisoned people and to induce kidney lesions (Léonard and Gerber 1997).

Results and discussion

An antimicrobial resistant strain of both, *A. baumannii* and *K. pneumoniae* were used in these assays. *A. baumannii* demonstrated inhibition by Tetracycline 25 μg, Streptomycin 25 μg, Kanamycin 30 μg and Colistin Sulphate 100 μg whilst, *K. pneumoniae* demonstrated inhibition by Colistin Sulphate 100 μg, Nalidixic Acid 30 μg, Nitrofuratoin 50 μg and Streptomycin 25 μg (data not presented).

The MIC investigations on planktonic strains of *A. baumannii* and *K. pneumoniae* revealed that molybdenum

demonstrated the greatest bactericidal activity against both strains (6·5 mg l^{-1}; 6·2 mg l^{-1}) (Fig. 1a). This result was significantly different when compared to the other metal ions ($P < 0·05$). Rhenium ions were the next most effective inhibitory antimicrobial agent against *A. baumannii* and *K. pneumoniae* at 13·0 mg l^{-1} and 11·7 mg l^{-1} respectively.

The MBC investigations on the planktonic strains *A. baumannii* and *K. pneumoniae* revealed that against *A. baumannii,* there was no significant difference between the activity of the metal ion (180·0 mg l^{-1} to 208·3 mg l^{-1}). Yttrium demonstrated the greatest antimicrobial activity against *K. pneumoniae* (72·9 mg l^{-1}), although this was not significantly different the bactericidal activity of the other metal ions (Fig. 1b).

The crystal violet biofilm assay was used to quantify the antimicrobial effect of the metal ions at their predetermined MBCs. Against *A. baumannii* and *K. pneumoniae*, all the metal ions demonstrated a significant decrease in biofilm formation when compared to the control ($P < 0·05$) (Fig. 2) The exception was the use of the rhenium ions against *K. pneumoniae* which did reduce the amount of biofilm, but it was not significant when compared to the control (Fig. 2b).

Results from the Live Dead staining which enabled the antimicrobial activity of the metals to be visualized, revealed damage to the bacterial cell walls following treatment with the metal ions. Although thallium was found to provide the most damage to the cell walls of *K. pneumoniae* (85·12%) there was no significant difference in

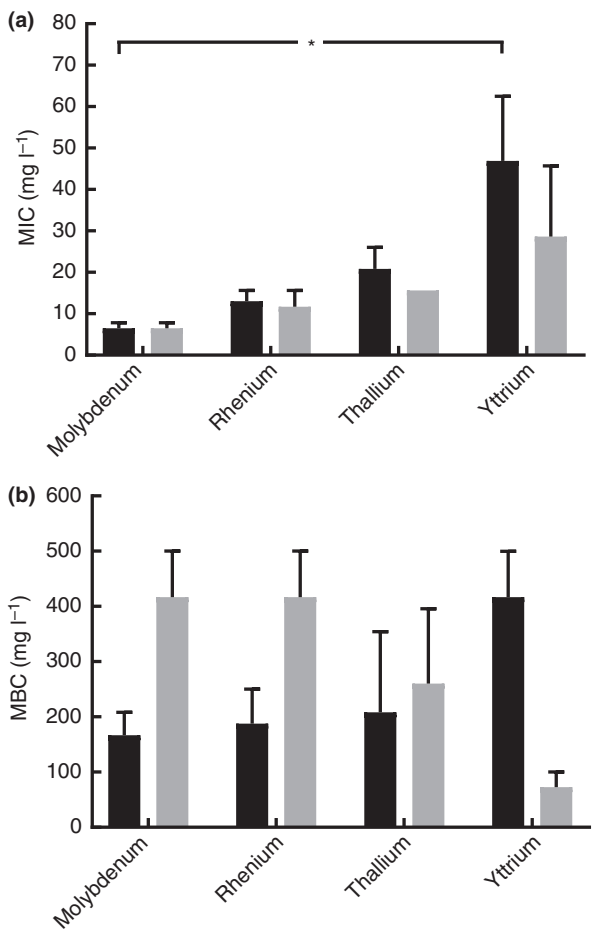

Figure 1 Minimum inhibitory concentrations (MICs) (a) and minimum bactericidal concentrations (MBCs) (b) of metal ion solutions against planktonic strains of *Klebsiella pneumoniae* and *Acinetobacter baumannii* ($n = 3$). *$P \leq 0·05$. Note the log difference in the *y*-axis (■) *A. baumannii* and (▒) *K. pneumoniae*.

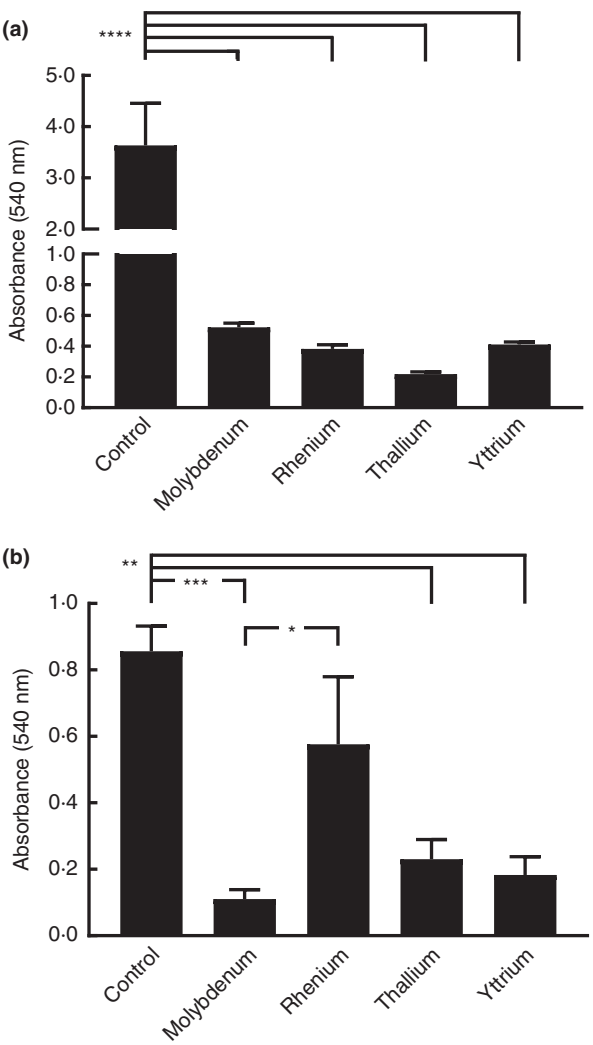

Figure 2 The optical density of (a) *Acinetobacter baumannii* and (b) *Klebsiella pneumoniae* crystal violet-stained biofilm material at 540 nm following incubation of the metal ion solutions at their minimum bactericidal concentrations concentrations ($n = 6$). *$P \leq 0·05$; **$P \leq 0·01$, ***$P \leq 0·001$.

the amount of cell wall damage produced between the thallium, rhenium and yttrium ions (Fig. 3).

Assays carried out to determine the toxicity of 50 mg l^{-1} of metal ions against the eukaryotic HK-2 cell line revealed that cell viability was significantly reduced by molybdenum (0·4%) followed by rhenium (42·3%), thallium (51·7%) and yttrium (54·1), as compared to the control cells (Fig. 4).

This study suggests that higher concentrations of metal ions are required to inhibit biofilms as opposed to their planktonic counterparts. This is in agreement with previous literature as it was demonstrated that a variety of metal ions tested against planktonic and biofilm-forming strains of *E. coli*, *S. aureus* and *P. aeruginosa* were 2–64 times less effective at destroying bacterial biofilms, in comparison to logarithmically growing planktonic bacterial strains (Harrisson *et al.* 2004).

In agreement with our work, Harrisson *et al.* (2004) demonstrated that molybdenum ions (MoO_4^+) showed antimicrobial activity against planktonic and biofilm-forming strains of *E. coli*, *S. aureus* and *P. aeruginosa*. Previous work has shown that mono/di-rhenium complexes have demonstrated antimicrobial activity against Gram-positive *S. aureus* and Gram-negative *E. coli* (Kumar *et al.* 2016). Rhenium ions were also tested in combination with ciprofloxacin (*in vivo*), where they destroyed bacterial cells by disrupting DNA gyrase synthesis (Lecina *et al.* 2014). molybdenum-rhenium coated struts have been used for applications within replacement heart-valve components (Beall mitral valves) (Ribeiro *et al.* 2016).

With regards to the antimicrobial activity of thallium, it has been reported that thallium ions and similar sized metal ions have the ability to replace sodium and potassium ions in biologically relevant systems. The metal ions can accumulate in the bacterial cells *via* the transporter excitatory amino acid carrier 1 (Tao *et al.* 2008). This transporter is essential and highly conserved, as its primary function is to mediate electrochemical transmembrane gradients. However, in contradiction to our eukaryotic assay results, it must be noted that thallium is an extremely toxic metal (Britten and Blank 1968; Cotton and Wilkinson 1980; Bannon 2015). This result may be due to the ionic form that the thallium is used in this study and an expanded investigation into reducing the biotoxicity of thallium in this form is currently underway.

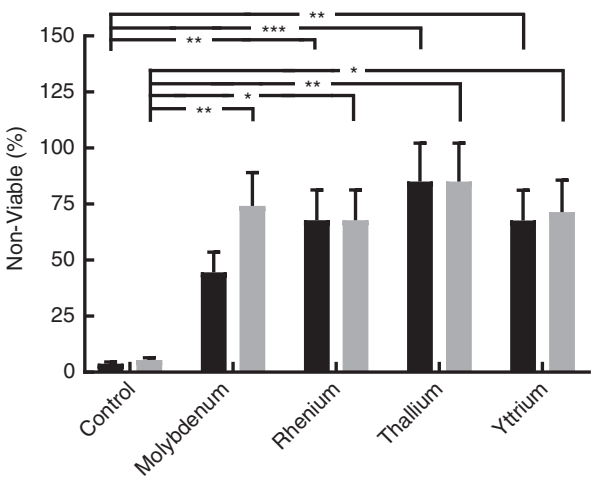

Figure 3 Percentage number of dead cells following Dead staining of the *Klebsiella pneumoniae* and *Klebsiella pneumoniae* biofilms after 7 days. *$P \leq 0{\cdot}05$, **$P \leq 0{\cdot}01$, ***$P \leq 0{\cdot}001$ (■) *A. baumannii* and (■) *K. pneumoniae*.

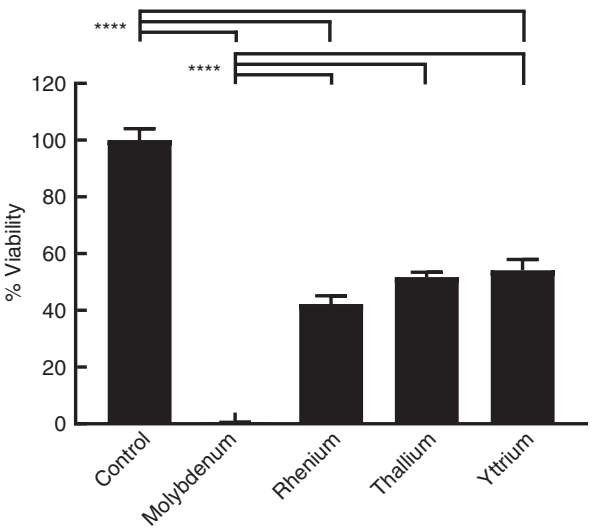

Figure 4 Viability of HK-2 cells exposed to 50 mg l^{-1} metal ion solutions for 26 h. The cells were bound to the surface before treatment. The viability is expressed as percentage of the viability of control cells (PBS in serum-free medium) ($n = 6$) ****$P \leq 0{\cdot}0001$.

Yttrium has also demonstrated antimicrobial activity. Yttrium fluoride nanoparticle complexes were evaluated as antimicrobial surface coatings, and the results showed that yttrium successfully inhibited both *E. coli* and *S. aureus* biofilm formation, due to surface adherence inhibition (Lellouche *et al.* 2012). The structural conformation of metals can vary (i.e. metal ions, nanoparticles and oxides); this results in a diverse range of antimicrobial activity for the same chemical species. However, this study provides evidence that using yttrium ions as a bactericidal component has potential.

In conclusion, considering the results for the antimicrobial efficacy, biotoxicity and the results reported by others in the literature, it is possible that metals such as rhenium and yttrium in ionic form could be developed into effective biocidal formulations for use in surface decontamination in hospitals to prevent the transmission of bacteria.

Materials and methods

Agar and broth preparation

The agar used in this study included nutrient agar (NA), nutrient broth (NB), for *K. pneumoniae* and tryptone soya agar (TSA) tryptone soya broth (TSB) for *A. baumannii.* (obtained from Oxoid, Hampshire, UK).

Bacterial preparation

Acinetobacter baumannii (NCTC 12156) and *Klebsiella pneumoniae* (NCTC 9633) were recovered from −80°C stock cultures and transferred onto nutrient agar for 24 h at 37°C. Isolated bacterial colonies were transferred into 20 ml of the appropriate broth and mixed with a vortex shaker for 5 s. The broth suspension was transferred to a shaking incubator (200 rev min^{-1}) and incubated at 37°C for 24 h. In order to wash the bacterial cells, the cellular suspension was mixed with a vortex mixer for 5 s and then centrifuged for 10 min (1721 ***g***). The supernatant was removed and replaced with 20 ml of sterile distilled water and vortexed for 5 s, then centrifuged again at 1721 ***g*** for 10 min. The supernatant was discarded and 20 ml of sterile broth was added to the washed cells and vortexed for 5 s. The broth suspensions were standardized to an optical density (OD) of 1·0 at 540 nm (±0·1) to obtain bacterial concentrations of $5{\cdot}0 \times 10^8$ CFU per ml using a spectrophotometer (Jenway, Staffordshire, UK). The bacterial strains were tested using antimicrobial multidiscs (MAST, Liverpool, UK). One hundred microlitres of prepared bacterial suspension was spread across the agar and the antimicrobial multidisc was placed on top. The inoculated agar with the discs was incubated at 37°C for 24 h following which the zones on inhibition were measured to determine antibiotic sensitivity ($n = 3$).

Determination of minimum inhibitory concentration (MIC)

Bacterial broth preparations of *A. baumannii* and *K. pneumoniae* standardized to OD 1·0 were inoculated onto the appropriate broth. Triphenyl tetrazolium chloride solution (TTC) 1·5% (w/v) (Sigma, Dorset, UK) was prepared in sterile distilled water and mixed until the TCC had dissolved completely. The TCC solution was further diluted to give a final concentration of 0·15% w/v. One column acted as a positive control and another as a negative control. The 96 well microtest plate (MTP) was covered and sealed with Parafilm®, and the MTPs were incubated at 37°C for 24 h (aerobic conditions; without agitation). Following incubation, wells that contained living/viable cells had produced a purple colour, this was a result of the TTC becoming enzymatically reduced to 1,3,5-triphenylformazan. The first well in each column to display no colour change was taken as the minimum inhibitory concentration (MIC).

Minimum bactericidal concentration

To determine the minimum bactericidal concentrations (MBC), 20 μl of the bacterial suspension was taken from each MIC well showing no growth and also from the wells identified as the MIC and placed onto appropriate agar. The agar plates with the bacterial suspensions were dried for 1 h at room temperature and then incubated at 37°C for 24 h. Upon incubation, the lowest concentration that showed no growth was taken to be the MBC.

Preparation of stainless steel coupons

Stainless steel coupons 1 cm × 1 cm in size (304 bright annealed finish, Outokumpu, UK) were cleaned for use in the biofilm assays by submerging them for 10 min into 10 ml 100% acetone, dried at room temperature then transferred into 10 ml 100% methanol for 10 min and dried again. The coupons were placed into 70% ethanol for 10 min, dried and submerged into sterile distilled water for 10 min and dried at room temperature. Finally, the sterile coupons were placed in a sterile Petri dish where they were stored until needed.

Crystal violet biofilm assay

To produce biofilms on the metal surfaces, stainless steel coupons (1 cm × 1 cm) were placed into each well of a 12 well, flat-bottomed culture plate with the polished side facing upwards and 1 ml of broth suspension was pipetted into each well. The metal ions were added to the wells with a negative control well which included bacteria but not the antimicrobial agents. The 12-well plate lid was then closed and sealed using Parafilm® sheets to prevent the broth from evaporating during incubation. The inoculated well plates were incubated for 7 days at 37°C in aerobic conditions with no agitation. Following incubation, the broth was discarded and the coupons were dried for 1 h at room temperature in a type biosafety cabinet (Atlas Clean Air, Preston, UK).

The metals ions were diluted into sterile distilled water to obtain the predetermined MBC values. A negative control was also included, this included bacteria and not the antimicrobial agents. One millilitre of the metal ions was pipetted into a well containing the stainless steel coupon with the biofilm. Each metal ion solution and control was pipetted into individual wells, sealed using Parafilm® σ and incubated for 24 h at 37°C after which the metal ions

were discarded. Following incubation, the coupons were rinsed by pipetting 1 ml of sterile water gently against the coupon in the MTP well and dried at room temperature for 1 h in a type 2 biosafety cabinet.

One millilitre of 0·1% crystal violet was pipetted into each of the wells and was left to stand for 30 min to stain the biofilm. The stainless steel coupons were rinsed by gently pipetting 1 ml of sterile water (carefully so as not to disrupt the biofilm) over the coupons, before transferring them into a sterile 12-well culture plate.

To determine the optical density of the biofilms on the metal ions following incubation, the coupons were air-dried for 1 h, then 1 ml 33% glacial acetic acid was pipetted into the wells to solubilize the crystal violet-stained biofilm and left to stand for 30 min. One millilitre of the solubilized biofilm was taken from each well and pipetted into a cuvette and its OD was measured at 540 nm using a spectrophotometer (Jenway) ($n = 3$).

Live Dead staining

The bacterial suspensions were incubated on the stainless steel coupons for 7 days at 37°C. Following incubation, the inoculated broth was removed and discarded and the coupons were dried for 1 h at room temperature in a type 2 biosafety cabinet. The metals ions were diluted to their previously determined MBC values. One millilitre of the metal ions was added to each of the stainless steel coupons, this was left to stand for 10 min. The metal ions were then discarded and the coupons were dried at room temperature (1 h), in a type 2 biosafety cabinet.

A Live Dead® BacLight™ Bacterial Viability Kit (Fisher Scientific, Loughborough, UK) was used to carry out Live Dead staining of the biofilm which was then visualized using an epifluorescence microscope (Nikon, Surrey, UK). An initial staining solution was prepared using 30 μl of SYTO9®, 30 μl of Propidium iodide pipetted into 10 ml of filter-sterilized deionised water. The coupons had 1 ml of the prepared solution applied to the biofilms and were placed in the dark for 30 min. The solution was discarded and the coupons were dried at room temperature for 1 h in a type 2 biosafety cabinet, in the dark. The coupons were visualized using an epifluorescence microscope.

Epifluorescence microscopy

Using an epifluorescence microscope (Nikon Eclipse E600; Nikon) samples were visualized with ×100 objective and Type F immersion oil (Leica Microsystems, Milton Keynes, UK). CELL F (Olympus, Southend-on-Sea, UK) software was used to visualize and produce images of the samples and the percentage Live Dead coverage was calculated by quantifying the fluorescence emitted. To calculate Live Dead coverage, the images were visualized under different filters in the range of 510 nm–560 nm for the SYTO 9 stain and 590 nm–650 nm for the propridium iodide stain. The percentage number of viable and nonviable bacteria was quantified using the threshold function on the CELL F software.

Mammalian cell culture and viability test

Viability tests were carried out to determine the cytotoxic effect of the metals on immortalized human renal proximal tubular epithelial HK-2 cells (CRL-2190, ATCC, UK). Briefly, the HK-2 cells were maintained in humidified incubators at 5% CO_2 and 37°C in 50 : 50 DMEM : Ham's F-12 medium supplemented with 5 mmol l^{-1} glucose, 2·5 mmol l^{-1} glutamine, 10% foetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were seeded at a density of 9000 cells per well in 96-well plates and incubated for 24 h. The cells were then rinsed with phosphatebuffered saline and the growth arrested for 24 h in serum-free medium (SFM; 50 : 50 DMEM : Ham's F-12 medium supplemented with 5 mmol l^{-1} glucose, 2·5 mmol l^{-1} glutamine, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin).

The pH of the metal solutions was adjusted to pH 6·5–7 using 2 mol l^{-1} NaOH and the solutions were diluted 50 mg l^{-1} in SFM. Upon the 24 h growth arrest, the cells reached confluence of 100%. The SFM was aspirated and replaced with 200 μl of either 50 ng l^{-1} of metal ions in SFM or PBS in SFM for 23 h. Then 10 μl of WST-8 reagent (Cell Counting Kit-8; Dojindo Molecular Technologies, Peterborough, UK) was added to each well and the formation of soluble WST-8 formazan was detected after 3 h of incubation at 37°C by the use of a microplate reader at 450 nm wavelength.

Statistical analysis

A one-way ANOVA test was used to compare the treatment groups. Tukey's HSD post hoc test was carried out when significant P-values ($P < 0·05$) were detected. All n values indicate experimental replications.

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Anthony J. Slate and Liliana Shalamanova carried out the experimental work. Kathryn A. Whitehead devised the papers concept. All the authors contributed to the writing and development of this work.

Conflict of Interest

No conflict of interest declared.

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