A rapid screening assay for identifying mycobacteria targeted nanoparticle antibiotics

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Abstract
Antibiotic resistance is a serious problem. Nanotechnology offers enormous potential in medicine, yet there is limited knowledge regarding the toxicity of nanoparticles (NP) for mycobacterial species that cause serious human diseases (e.g. tuberculosis (TB) and leprosy). Mycobacterial diseases are a major global health problem; TB caused by Mycobacterium tuberculosis (Mtb) kills up to 2 million people annually and there are over 200,000 leprosy cases each year caused by Mycobacterium leprae (M. leprae). Few drugs are effective against these mycobacteria and increasing antibiotic resistance exacerbates the problem. As such, alternative therapies are urgently needed but most current assays used to assess the effectiveness of therapeutics against mycobacteria are slow and expensive. This study aimed to develop a rapid, low-cost assay which can be used for screening the antimicrobial properties of compounds against pathogenic mycobacteria and to assess the toxicity of three NP (silver [Ag], copper oxide [Cu(II)O], and zinc oxide [ZnO]) against a green fluorescent protein reporter strain of Mycobacterium avium subspecies paratuberculosis, a slow growing, pathogenic mycobacterial species causing paratuberculosis in ruminants. Fluorescence was used to monitor mycobacterial growth over time, with NP concentrations of 6.25–100 µg/mL tested for up to 7 days, and a method of data analysis was designed to permit comparison between results. Mycobacterial sensitivity to the NP was found to be NP composition specific and toxicity could be ranked in the following order: Ag > Cu(II)O > ZnO.

Keywords
Antibacterial activity, antibiotic resistance, drug screening, mycobacterium, nanomedicine

Introduction
A recent report from the World Health Organization (WHO) highlights the urgent need to tackle antibiotic resistance (WHO, 2014a). Novel compounds are required but there are great challenges in identifying new therapeutics. Nanotechnology holds great promise to improve human health and nanoparticles (NP [with one or more external dimensions between 1 nm and 100 nm]) make interesting antibiotic candidates. The ability to create nano-scaled materials allows inclusion of pre-designed functions such as improved medicines, targeted drug delivery, diagnostic tools and anti-microbial agents.

The anti-microbial properties of NP are well documented for gram-positive and gram-negative bacteria (Bondarenko et al., 2012; Das et al., 2013; Pasquet et al., 2014; Ruparelia et al., 2007). Such anti-bacterial properties have been exploited in a vast array of consumer products (e.g. food packaging) and clinical applications (e.g. wound dressings). The physico-chemical properties of NP (e.g. composition, solubility, morphology) are fundamental to their anti-bacterial activity (Puzyn et al., 2011). However, there is limited information available regarding the anti-bacterial activity of NP drugs against mycobacteria.

Some members of the genus Mycobacterium are responsible for serious infectious diseases of humans and animals such as leprosy, tuberculosis (TB), and paratuberculosis. There are 200,000 cases of leprosy reported annually (WHO, 2015a), TB continues to kill up to 2 million people each year (WHO, 2012, 2013, 2014b, 2015b), and paratuberculosis is endemic in many countries. The latter is very difficult to diagnose, treat, and control, costing the farming sector billions of pounds annually (Harris et al., 2002; Tewari et al., 2014). Mycobacteria are aerobic, non-motile bacteria with a waxy, complex, hydrophobic, thick cell wall rich in mycolic acids, making them highly impermeable to many solutes (Favrot & Ronning, 2012). The unique make-up of the physically resilient cell wall allows these mycobacteria to be resistant to many chemical disinfectants, antibiotic and chemotherapeutic drugs (Jarlier & Nikaido, 1994) and as a result they are notoriously difficult to kill (Cocito et al., 2013, 2014b, 2015b), and paratuberculosis is endemic in many countries. The latter is very difficult to diagnose, treat, and control, costing the farming sector billions of pounds annually (Harris et al., 2002; Tewari et al., 2014). Mycobacteria are aerobic, non-motile bacteria with a waxy, complex, hydrophobic, thick cell wall rich in mycolic acids, making them highly impermeable to many solutes (Favrot & Ronning, 2012).

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also resistant to a broad spectrum of antibiotic drugs similar to *Mycobacterium tuberculosis* (*Mtb*), responsible for TB in humans. Unlike *Mtb*, however, *Map* is classified as Hazard Group 2 by the UK Advisory Committee for Dangerous Pathogens and therefore has the advantage that it can be handled in a Containment Level 2 laboratory. In addition, a reporter strain capable of expressing green fluorescent protein (GFP) was available for the microplate assay developed in this study (Harris et al., 2002).

There are great challenges associated with developing new antimycobacterial drugs, in particular anti-TB drugs. Pharmaceutical companies are reluctant to undertake new drug discovery or development because it involves exorbitant costs with insufficient profit margins or investment return. There are challenges in identifying new compounds that can kill both actively growing and persistent or latent bacteria, in evaluating new compounds and testing them in clinical trials. Generally, there has been an overall void in the discovery of novel anti-bacterial agents for the past 25 years (Boogaard et al., 2009; Silver, 2011; WHO, 2014a). The continual emergence of drug-resistant strains of mycobacterial species augments this problem. Pathogenic *Mycobacterium* species are slow growing and difficult to culture in *vitro*, in fact some like *M. leprae* cannot be propagated in the laboratory. The gold-standard in mycobacteria drug discovery continues to be based on bacteriological culture through colony counting on solid medium and determining minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs), thus being costly, labor intensive, highly variable and very slow (4–16 weeks) (Boogaard et al., 2009; Elguezabal et al., 2011; Hurdle et al., 2008; Kralik et al., 2012; Sharma et al., 2014). Consequently, diagnosis, assessment of antibiotic resistance, treatment and drug screening procedures are also costly and time consuming. Therefore, given the global situation, novel anti-bacterial agents are urgently required to fight infections and fill the void with new compounds. The discovery of safe, biocidal compounds to help fight human and animal infections is long overdue. NP may be suitable candidates to target mycobacterial infections, but their efficacy must first be screened and tested.

This study aimed to develop a rapid, inexpensive assay to screen the anti-mycobacterial effects of NP. The approach used was adapted, optimized and modified from previous work by Collins et al. (1998), employing the GFP reporter strain *Map* K10/GFP (Collins et al., 1998; Harris & Barletta, 2001). For the first time this micro-plate assay was used to effectively screen different concentrations of metal/metal oxide NP (*Ag*, copper II oxide [Cu(II)O], zinc oxide [ZnO]) for toxicity over a period of 7 days. It was hypothesized that the three NP tested would differ in their potency against the mycobacteria and that the relative toxicity of the different NP is related to their physico-chemical properties, such as their composition and dissolution rates. These NP were selected due to their reported antibacterial properties against a range of both gram-positive and gram-negative bacteria (Heinlaan et al., 2008; Mumper et al., 2013; Pelgrift & Friedman, 2013; Ramani et al., 2013). A wide range of concentrations was tested (6.25–100 μg/mL). Previously published MIC/MBC data for various microorganisms (Ansari et al., 2011; Ruparelia et al., 2007) and cytotoxicity data for cell lines (Arai et al., 2015; Kermanizadeh et al., 2013) aided in determining this range. Bacterial resistance to metal toxicity is low, thus making metal/metal oxide NP a logical area to explore when considering new compounds (Pelgrift & Friedman, 2013).

**Methods**

**Nanoparticle preparation and characterization**

The Ag (NM 300) and ZnO (NM 110) NP were obtained from the European Commission’s Joint Research Center (JRC) repository of NP, while the Cu(II)O NP were obtained from Sigma-Aldrich® (Poole, UK). Ag and ZnO NP have been extensively characterized previously (Kermanizadeh et al., 2013), whereas there is scarce literature on the characterization of Cu(II)O NP. All NP were sub sampled under sterile conditions and kept in the dark at ambient temperature until use. Supplier-related information and characterization data are presented in Table 1. NP were diluted in sterile 2% (vol/vol) fetal calf serum (FCS) prepared in sterile distilled water at a concentration of 1 mg/mL and sonicated for 16 minutes at 400W in a bath sonicator from Ultrawave® (Cardiff, UK) and then placed on ice (following the ENPRA (Risk Assessment of Engineered Nanoparticles) protocol (www.enpra.eu) (Jacobsen et al., 2010; Kermanizadeh et al., 2013). NP were then diluted in Middlebrook 7H9 medium supplemented with 10% (vol/vol) Middlebrook ADC enrichment (albumin/dextrose/catalase [Becton Dickinson, Oxford, UK]), 0.2% (vol/vol) glycerol, 2 μg/mL mycobactin J (7H9).

Agglomeration status, zeta potential, and hydrodynamic sizes were analyzed by dynamic light scattering (DLS) at a concentration of 100 μg/mL (Table 1) using a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., Worcestershire, UK) and transmission electron microscopy (TEM) on a Philips CM120 TEM (FEI, Cambridge, UK) was employed to visualize the NP in an aqueous environment.

**Metal salt controls**

For each metal/metal oxide particle utilized, a metal salt control was run alongside each experiment (in triplicate); silver nitrate (AgNO₃), copper sulfate (CuSO₄), and zinc chloride (ZnCl₂) (Sigma-Aldrich®, Poole, Worcestershire, UK). Salts were weighed and diluted in distilled water then serial diluted in 7H9, at a metal concentration matching the NP.

**Transmission electron microscopy**

TEM analysis was performed to determine the size, morphology and dispersion patterns of the NP in 7H9 at 25 and 100 μg/mL (and in distilled water at 100 μg/mL). Images were taken on a Gatan

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**Table 1. Supplier information and DLS results.**

| NP                  | Supplier name          | NP diameter (suppl) | NP diameter (TEM in 7H9) | Appearance | Surface area (m²/g) (suppl) | Z-average (d.nm) DLS | Zeta potential (mV) DLS | PdI DLS |
|---------------------|------------------------|---------------------|--------------------------|------------|-----------------------------|----------------------|-------------------------|---------|
| Ag (NM 300)         | JRC Repository         | <20 nm              | <50 nm                   | Suspension-Brown | 53.34 ± 3.1               | −8.77 ± 2.9          | 0.469 ± 0.02             |         |
| Cu(II)O             | Sigma-Aldrich®         | <50 nm              | Unknown*                 | Powder-Black | 386.94 ± 37.2              | −11.55 ± 0.6         | 0.359 ± 0.02             |         |
| ZnO (NM 110)        | JRC Repository         | 100 nm              | Unknown*                 | Powder-White | 241.36 ± 6.7               | −7.45 ± 0.5          | 0.307 ± 0.02             |         |

Hydrodynamic diameter (Z-average), zeta potential, and polydispersity index (PdI) of NP panel was assessed using dynamic light scattering (DLS). Data expressed as mean ± SEM (n = 3). Temp: 25°C. dispersant: middlebrooks 7H9 (100 μg/mL), pH 6.9 ± 0.2, equilibrium time: 30 seconds.

*TEM showed NP agglomeration/aggregation in 7H9 therefore an accurate diameter measurement was unobtainable from DLS.

*Supplied as a suspension.
Orius CCD camera. For all particle types, they were prepared as outlined above and 10 μL of each suspension was pipetted onto the surface of formvar/carbon-coated 200 mesh copper grid and allowed to dry at room temperature for 40 minutes prior to imaging (Figure 1a–f and Supplementary material Figure 1a–c).

Bacterial strain and growth conditions

The Map K10/GFP strain was kindly supplied by Raul Barletta, University of Nebraska, Lincoln (Harris et al., 2002). Map strain K10 was transformed with a plasmid carrying the gfp gene and a kanamycin resistance gene, thus allowing both growth and viability to be tracked with fluorescence. Map K10/GFP was cultured in Middlebrook 7H9 broth medium supplemented with 10% (vol/vol) Middlebrook ADC enrichment (albumin/dextrose/catalase [Becton Dickinson, Oxford, UK]), with 0.2% (vol/vol) glycerol, 0.05% (vol/vol) Tween 80, 2 μg/mL mycobactin J, and 25 μg/mL of kanamycin (7H9K+) at 37°C gently stirred (250 rpm) continuously with magnetic stirrer bars.

Fluorescent microplate assay

Map K10/GFP was grown in 7H9K+ until the exponential stage of growth was reached. Growth was monitored by McFarland (McF)
Fluorescence was measured using a GloMax (no bacteria, no NP) and NP and medium (no bacteria) were included. To check for NP interference, wells of medium only (250 rpm) and kept in the dark throughout the experimental procedure. To determine the interference, the data were then normalized to the slope of the control (0 μg/mL) for each particle type/salt control by dividing each value by the control value and 2* the standard error for each data point using the LINEST function in Excel (Microsoft Office Worksheet). This data was then plotted using the dose response modeling software PROAST (version 38.9 [RIVM, Bilthoven, The Netherlands]) in the R software (version 3.1.0, [R Foundation for Statistical Computing, Austria]). By setting the critical effect size (CES) to 50% it was possible to obtain the half maximal effective concentration (EC50) [the critical effect dose (CED)] and lower/upper 90% confidence intervals (CEDL/ CEDU) to allow for statistical comparison to determine if the NP differed significantly from each other, and/or their corresponding salt controls.

Results

NP characterization

DLS

The size and zeta potential of the tested NP are outlined in Table 1. DLS results suggest that agglomeration/aggregation occurred in the growth medium of Map K10/GFP (7H9), particularly for Cu(II)O and ZnO NP (Table 1). Z-average (d.nm) results show particle size >380 nm (Cu(II)O) and >235 nm (ZnO), whereas Ag particle size was found to be <55 nm (Table 1), thus suggesting the aggregation state of Cu(II)O NP and ZnO NP in 7H9.

TEM

Micrographs from TEM analysis suggest that Ag NP were well dispersed in 7H9 at both concentrations imaged (Figure 1a and b). Limited agglomeration of Ag particles was observed, which was more apparent at the highest concentration tested (100 μg/mL), and the spherical shape of the particles was clearly visible (Figure 1a and b). Primary particle size was found to be <50 nm (Figure 1a). Cu(II)O and ZnO NP were agglomerated/aggregated in 7H9 at both concentrations tested (Figure 1c–f). As mainly agglomerates/aggregates were visible for both of these NP types, it was not possible to measure their primary particle size in 7H9 using TEM. Neither NP types were spherical but instead quite angular and irregular in shape, particularly the ZnO NP where miscellaneous shapes and sizes were visible (Figure 1e and f). When the NP were dispersed in 2% FCS as above, then serial dilutions of NP were made to give final concentrations per well ranging from 0 to 100 μg/mL and were added to the wells containing Map K10/GFP giving a final volume of 200 μL per well. The samples were incubated at 37°C with medium agitation (250 rpm) and kept in the dark throughout the experimental procedure. To check for NP interference, wells of medium only (no bacteria, no NP) and NP and medium (no bacteria) were included. Fluorescence was measured using a GloMax Multi plate reader (Promega, Southampton, UK) at an excitation of 490 nm and emission of 510–570 nm, the plate was shaken for 0.3 minutes in an orbital motion, before each reading. Bacterial fluorescence was monitored immediately after NP addition (day 0), then at the same time each day for 7 days. The same steps were taken for the three salt controls. All experiments were performed in triplelicate under sterile conditions.

Data analysis

Results expressed in arbitrary fluorescent units (AFU) against time (days) were transformed and normalized to obtain the usual dose response relationship, to compare the effects of the NP and salt controls on the growth of the mycobacteria. The exponential growth of the bacteria was calculated: $G = G_0 \exp(\beta t)$ (where $G =$ fluorescent units, $G_0 =$ bacterial growth at time 0, $\beta =$ growth rate (per day), and $t =$ time (days)). To estimate $\beta$, first the data were transformed by taking the log of $G$: $\log G = \log G_0 + \beta t$, allowing $\beta$ to be estimated by linear regression. The data were then normalized to the slope of the control (0 μg/mL) for each particle type/salt control by dividing each value by the control value and 2* the standard error for each data point using the LINEST function in Excel (Microsoft Office Worksheet). This data was then plotted using the dose response modeling software PROAST (version 38.9 [RIVM, Bilthoven, The Netherlands]) in the R software (version 3.1.0, [R Foundation for Statistical Computing, Austria]). By setting the critical effect size (CES) to 50% it was possible to obtain the half maximal effective concentration (EC50) [the critical effect dose (CED)] and lower/upper 90% confidence intervals (CEDL/ CEDU) to allow for statistical comparison to determine if the NP differed significantly from each other, and/or their corresponding salt controls.

Bacterial growth: monitored by McF and fluorescence

The McF standard of 10 mL liquid cultures of Map K10/GFP was plotted against time to generate a growth curve over a period of 37

Figure 2. (a) Biphasic growth of Map K10/GFP over 37 days in 10 mL cultures in 7H9K* medium (using McFarland [McF] standards). The curve displays biphasic growth of the mycobacteria, where the organism is switching between carbon sources in the 7H9K* medium. The first exponential growth phase is between days 5 and 11, the second exponential growth phase begins at approximately day 16. Map K10/GFP was taken between days 9 and 11 for the fluorescent assay. (b) Map K10/GFP grown in 200 μL of 7H9K* monitored by fluorescence, in a microplate format over a period of 7 days, with each point displaying 2* standard error of the mean (SEM) ($n = 6$) (AFU = arbitrary fluorescent units).

Standards using a Densimat® (Figure 2a) (Peñuelas-Urquides et al., 2013). Centrifugation at 3080 x g at 20°C for 20 minutes was used to pellet 3 mL of the bacterial suspension in the exponential phase of growth. The pellet was re-suspended in the appropriate volume of 7H9 to give a McF standard 2, approximating to 3.2 x 10^8 colony-forming units (CFU) per milliliter. Map K10/GFP was further diluted (1/16 in 7H9) and cells were seeded in a 96-well, sterile, black plate (2 x 10^5 CFU/well) (Nunc, Themo Fisher, Loughborough, UK) in a volume of 100 μL per well. The outer wells were filled with 200 μL of distilled water to prevent dehydration of the experimental wells. Serial dilutions of NP were made to give final concentrations per well ranging from 0 to 100 μg/mL and were added to the wells containing Map K10/GFP giving a final volume of 200 μL per well. The samples were incubated at 37°C with medium agitation (250 rpm) and kept in the dark throughout the experimental procedure. To check for NP interference, wells of medium only (no bacteria, no NP) and NP and medium (no bacteria) were included. Fluorescence was measured using a GloMax Multi plate reader (Promega, Southampton, UK) at an excitation of 490 nm and emission of 510–570 nm, the plate was shaken for 0.3 minutes in an orbital motion, before each reading. Bacterial fluorescence was monitored immediately after NP addition (day 0), then at the same time each day for 7 days. The same steps were taken for the three salt controls. All experiments were performed in triplelicate under sterile conditions.

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Bacterial growth: monitored by McF and fluorescence

The McF standard of 10 mL liquid cultures of Map K10/GFP was plotted against time to generate a growth curve over a period of 37
Figure 3. (a) Viability Testing: Growth of Map K10/GFP with Ag NP. Map K10/GFP grown in 200 μL of 7H9 in a microplate format, with Ag NP at different concentrations (6.25–100 μg/mL) over a period of 7 days, represented in the form: G = G0 \exp(βt) (where \( β \) represents the change in fluorescence [representing mycobacterial growth] per day) using the raw data with each point displaying 2* the SEM (\( n = 6 \)). (b) Data transformation: growth of Map K10/GFP with Ag NP normalized to the control (where \( β \) represents the change in fluorescence [representing mycobacterial growth] per day). Normalized, transformed data showing the growth of Map K10/GFP in 200 μL of 7H9, in a microplate format, with Ag NP at different concentrations (6.25–100 μg/mL), normalized to the slope of the control. \( β \) was calculated through linear regression analysis of the log data (Table 2, Figure 3a). Each point displaying 2* the standard error displayed, using LINEST function Excel (Microsoft Office Word) (\( n = 6 \)).

Figure 4. (a) NP versus salt efficacy: comparison between NP and salt controls on the growth of Map K10/GFPNP normalized to the control. Map K10/GFP grown in 200 μL of 7H9 in a microplate format, with salts or NP at different concentrations (6.25–100 μg/mL). \( β \) data obtained as described previously (Figure 3a and b) and all compounds plotted using PROAST 38.9 to allow comparison between NP and salts. (b) The half maximal effective concentration (EC50) for each compound with the upper and lower 90% confidence intervals. Plot of the confidence intervals of the EC50 (on the common logarithmic scale) on the rate of mycobacterial growth for each compound. When the confidence intervals do not overlap we can conclude with reasonable confidence that the compounds are significantly different (at slightly less than 90% due to multiple comparisons).

Anti-mycobacterial properties of NP

Following exposure to Ag, Cu(II)O, or ZnO NP, the growth of Map K10/GFP was inhibited in a dose- and time-dependent manner. The different stages of the data analysis are presented within the manuscript for Ag (Figure 3a and b, Table 2) and in the supplementary material for ZnO and Cu(II)O (Supplementary material Figures 2 and 3). The results are first illustrated as AFU against time obtained on the GloMax® Multi+ (Promega, Southampton, UK) and presented using Excel (Microsoft Office Worksheet) as G = G0 \exp(βt) (Figure 3a). The control well values (medium plus NP but no bacteria) were subtracted from the experimental well values to exclude any NP interference in fluorescence readings. There is a clear dose-dependent inhibition of the Map K10/GFP growth, observable within 6–7 days of exposure at concentrations as low as 6.25 μg/mL. This is also reflected in the log data, which displays the linear relationship from which \( β \) was calculated (LogG = LogG0 + βt) (Table 2). Figure 3(b) depicts the transformed and normalized change in fluorescence data for Ag NP, with 2* the standard error displayed.
allowing development of a dose response curve for Ag NP in this case, but that can be replicated to allow comparison between NP. Figure 4(a) illustrates the relative growth rate of the mycobacteria (the β data plotted in PROAST (38.9)) on treatment with each of the NP (and salts) and that there is a dose-dependent inhibition of growth, with an obvious difference in efficacy between the different NP. Figure 4(b) shows the EC_{50} values with confidence intervals for each NP/salt control. Comparing the confidence intervals for the 3 NP it is clear that they do not overlap, therefore we can be reasonably confident that there is a significant difference between the 3 NP tested (Figure 4b, Table 3). There is no significant difference between the Ag/ZnO NP and their corresponding salts however, which were remarkably similar in terms of their effect on the growth of Map K10/GFP (Figure 4a and b). Cu(II)O NP and CuSO_{4} were significantly different, with the copper salt being more toxic (Figure 4a and b). Using this analysis, the toxicity of the different NP can be ranked in the following order: Ag > Cu(II)O > ZnO. Assessment of colony growth after incubation for 12 weeks on agar confirmed the ranking obtained using the fluorescence assay (Supplementary material Figure 4).

Fluorescent microplate assay optimization

During the microplate assay development, a number of variables were manipulated, such as plate choice (black plates were chosen in order to minimize the background fluorescence), sample mixing time, number of CFU/well, medium volume, evaporation/ dehydration rates during incubation, stage of cell growth, etc. (Supplementary material Figure 5a and b). The changes made were informed by published literature (Collins et al., 1998), experimental observations and knowledge of the exceptionally fastidious nature of this microorganism.

Discussion

A new protocol for screening the efficacy of anti-mycobacterial substances

Reliable, rapid, and sustainable testing of new compounds with anti-mycobacterial properties is required to identify new agents, which may replace or accompany existing antibiotics. In this study we adapted and refined an assay that uses a GFP-modified Map strain to allow a quick, low-cost screening of NP toxicity against mycobacteria. We demonstrated that Map K10/GFP can be used to screen the relative toxicity of different NP within just 7 days, representing a significant saving of both time and money. Additionally, we developed a novel method for analysis of the data to permit easy comparisons between data sets and therefore assess the relative potency of different anti-mycobacterial agents. Adoption of the technical and data analysis protocols will allow greater numbers of potential anti-mycobacterial agents to be screened. The growth stage of the mycobacteria, plate type, and cell number per well were found to be important factors for optimal performance. The main advantage of this assay is the utilization of the reporter molecule GFP to monitor growth and viability of Map K10. GFP is intrinsically fluorescent and only requires oxygen for stimulation (Collins et al., 1998). It exhibits very low toxicity, is extremely stable, and allows for easy visualization/imaging (Chalifre et al., 1994; Collins et al., 1998). Only viable mycobacteria fluoresce. GFP has been used as a reporter for many organisms (including Mtb), making it a suitable choice for this assay (Changsen et al., 2003; Collins et al., 1998). While this paper has focused on metal/metal oxide NP, the same protocol could be used to test a range of therapeutic types.

Comparing NP anti-mycobacterial efficacy

The anti-microbial properties of NP, resulting from characteristics such as their small size (i.e. high surface area to volume ratio), surface reactivity, reactive oxygen species (ROS) production, and ion release are well documented (Pandey & Khuller, 2006; Park et al., 2009). However, this knowledge base does not include the impact of metal/metal oxide NP on pathogenic mycobacteria. The toxicity of NP tested in this study varied in their toxicity against Map K10/GFP and could be ranked in the following order: Ag > Cu(II)O > ZnO. The mechanisms underlying NP toxicity to other bacterial species has been shown to involve; disruption of the cell wall through direct NP contact, plasma membrane leakage and production of ROS (Kim et al., 2007; Pasquet et al., 2014; Ruparelia et al., 2007; Theophel et al., 2014). The mechanisms of toxicity were not investigated in this study, but we would hypothesize them to be similar.

The toxicity of NP is often related to particle size, as uptake, and distribution are size dependent (as well as surface reactivity) (Johnston et al., 2012; Ruparelia et al., 2007). The TEM images illustrate well-dispersed, spherical Ag NP in 7H9, measuring <50 nm in diameter. Imaging the Ag NP in distilled water further supported this result. The smallest particles observed in the Ag NP suspension could have been generated via precipitation of dissolved Ag\(^+\) ions as silver salts. Thus, further contributing to the toxicity exhibited by the Ag NP. In contrast to the Ag NP, TEM illustrate that both Cu(II)O NP and ZnO NP were agglomerated and/or aggregated. This could be an artifact of the drying procedure prior to TEM analysis, however this observation was supported further by the DLS measurements of hydrodynamic diameter. Micrographs of Cu(II)O NP in distilled water display less agglomerated particles that are more visible, thus suggesting that components of the 7H9 medium caused this phenomenon, whereas ZnO were found to be highly agglomerated in both aqueous environments. One potential explanation for the relatively high toxicity of Ag NP compared to the Cu(II)O and ZnO NP, might be their better dispersion and hence smaller agglomerate size. For Cu(II)O and ZnO NP their agglomeration could reduce the interaction with mycobacteria in terms of uptake and surface area available to interact with the mycobacteria in the medium, thus being a possible contributing factor to the relatively low toxic effects exerted by these NP (particularly ZnO). When NP are in an aqueous environment, the rate of ion release is related to the pH of the medium/liquid (Mumper et al., 2013). As the pH of an aqueous solution becomes more acidic, the dissolution rate should increase (Mumper et al., 2013). The pH of 7H9 is 6.9 ± 0.2, the medium thus being a very weak acidic solution. Salt controls were run for each NP and inductively coupled plasma optical emission spectrometry (ICP-OES) analysis was attempted in the study (data not shown) to try and determine whether toxicity was ion or particle mediated, yet the methods employed did not allow the assessment of NP dissolution in 7H9, therefore this was not possible to determine. Previous studies have ascertained a solubility of the NM300 Ag NP to be less than 1% (Kermanizadeh et al., 2012), yet it would be hard to compare, as most studies prepare NP in distilled or de-ionized water and
not a complex medium such as 7H9. This warrants further investigation.

**Efficacy of Ag NP**

Silver has been used as an anti-bacterial agent for centuries and Ag NP are reported to have high anti-bacterial properties (Ansari et al., 2011; Choi et al., 2008; Kim et al., 2007; Leo et al., 2013; Rai et al., 2009; Ruparelia et al., 2007; Sharma et al., 2009; Yuan et al., 2012; Islam et al., 2013). Anti-bacterial activity of Ag is reported to increase when in the nano-form, which may be due to the ability to enter through cell membranes (Choi et al., 2008). Once inside a cell, Ag NP target proteins which protect DNA molecules thus preventing DNA replication. They can also lead to the collapse of the cell wall (Feng et al., 2000). When Ag NP come into contact with an oxygenated, aqueous environment, silver ions (Ag⁺) are released (Park et al., 2009). Ag⁺ action is assumed to be the usual mediator of toxicity by causing structural damage to bacterial cells, affecting DNA replication, interfering with membrane permeability, modulating signal transduction in bacteria, thus leading to cell death (Park et al., 2009; Rai et al., 2009). The relatively large surface area of Ag NP may further aid Ag⁺ release compared to bulk metal, contributing to the toxicity of the Ag NP. Ag⁺ binds to negatively charged parts of the bacterial cell membrane, which causes leakage of intracellular substances. Ag NP are reported to have antibacterial properties against many bacterial species (Ansari et al., 2011; Park et al., 2009) and as shown, exhibit a very potent effect on this Mycobacterium species. It seems likely that the well-dispersed Ag NP contribute to the highly toxic effect on cellular growth. These NPs were investigated as potential treatments for mycobacteria infections but as NP are already used in antiseptic hand wipes, wound dressings, sun creams, etc., the Ag NP with highly toxic anti-mycobacterial properties could potentially be exploited to be utilized as an *ex vivo* antiseptic (Lboutoumene et al., 2002) again warranting further investigation.

**Efficacy of Cu(II)O NP**

The properties of Cu NP are poorly covered in the literature, and only recently have they started to gain appropriate attention, hence little is known about their mechanism(s) of action. It is known however, that Cu effects the cell wall, by interacting with amine and carboxyl groups and that Cu ions (Cu²⁺) promote the formation of ROS and, similarly to Ag NP, they accumulate on bacterial plasma membranes, negatively effecting the proton motive forces (Chatterjee et al., 2014; Pelgrift & Friedman, 2013). CuO NP have been reported to display bactericidal properties against gram-negative species and, compared to other metals, CuO NP are considered cost-effective. Additionally, CuO NP are very stable when likened to other organic antimicrobial agents (Ananth et al., 2015; Das et al., 2013).

In this study Cu(II)O NP actually enhanced Map K10/GFP growth at the lower concentration (6.25 μg/mL) and had little effect at 12 μg/mL. This might be because, unlike Ag, Cu is a trace element in biological systems, playing a pivotal role in cell physiology. At slightly higher concentrations Cu acts as toxicant, which can be seen at concentrations of 25 μg/mL and above, where growth was inhibited. Cu(II)O NP agglomerate when in 7H9. With an average size of 386.94 nm recorded from DLS, it concurs with results obtained in a previous study, which report high rates of CuO NP agglomeration in aqueous environments (Odzak et al., 2014). This agglomeration could reduce surface area contact with the mycobacteria as aforementioned, and may contribute to the lower levels of toxicity observed for the Cu(II)O NP compared to the Ag NP.

**Efficacy of ZnO NP**

ZnO NP are widely utilized with an annual production of 550 tons (Merdzan et al., 2014) and they are reported to have strong antimicrobial properties against many bacterial species (Ramani et al., 2013). They are found in food as additives, are being used in food packaging (Prach et al., 2013; Ramani et al., 2013; Sharma et al., 2012) and have been used as a fungicide (Wong et al., 2010). ZnO NP exert bacteria toxicity through production of ROS, damage to the bacterial cell wall leading to seepage of cellular contents, and affecting membrane permeability (Moos et al., 2010; Ramani et al., 2013). Like Cu, Zn is an essential element for biological systems (Pasquet et al., 2014).

Dissolution plays a role in the toxicity of ZnO NP (Brunner et al., 2006) with ZnO usually having a very fast dissolution rate, releasing Zn ions (Zn²⁺) and leaving few to no particles detectable after 2 hours in aqueous medium (Odzak et al., 2014). ZnO are reported to have very fast agglomeration rates in aqueous environments, leading to less available surface area (Odzak et al., 2014), thus being a possible reason for the poor anti-mycobacterial activity observed. Our data shows that, ZnO NP had the weakest effect of the three NP tested against this mycobacterial species. As Zn²⁺ are essential in cellular growth, it seems that Map K10/GFP either utilized the ions, and they aided in cellular growth at the low concentrations, or Map K10/GFP was able to metabolize Zn²⁺, so no anti-mycobacterial effects were observed over the 7-day exposure period. When concentrations were increased to 50–100 μg/mL, increased toxicity occurred. Additionally, the large agglomerates formed in the 7H9 would likely reduce their toxicity.

**Conclusions**

Antibiotic resistance is a huge problem the globe over. Bacterial resistance to metals is rare and develops very slowly; therefore it should be further explored. Each of the metal/metal oxide NP tested here displayed interesting results, particularly the very high toxicity of the Ag NP and the very low toxicity displayed by ZnO NP against the *Mycobacterium*. Ag NP (and the AgNO₃ control) were the most toxic, suggesting that toxicity against *Map* K10/ GFP is both particle and ion mediated, depending on the metal type. Interestingly the ZnO NP were slightly more toxic compared to the ZnCl₂ control whereas Cu(II)O NP were significantly less toxic compared with the CuSO₄ controls.

In this study, we adapted and modified an assay that uses a GFP modified *Map* strain to allow quick, low-cost screening of metal/metal oxide NP toxicity against mycobacteria. Fluorescence is read over 7 days to give a rapid insight into the anti-mycobacterial properties of tested NP to determine if they warrant further investigation. Pathogenic mycobacteria like *Map* have very slow doubling times (>24 hours), therefore use of the traditional colony counting technique is time consuming, expensive, highly variable and challenging, as mycobacteria form clumps. Therefore, this assay offers a fast, cheaper alternative in the early stages of NP/ drug testing. In addition to developing the *in vitro* screening protocol, we also established a protocol for data analysis that allows easy comparison between the efficacies of NP.

From our results, further work is clearly required. Determining the precise dissolution rate and behavior of each NP in the complex 7H9 medium is necessary. While this paper has focused on NP, the same protocol is applicable to a range of potential therapeutic agents.

**Acknowledgements**

The authors would like to thank Dr Raul G Barletta (University of Nebraska-Lincoln, USA) for providing the *Map* K10/GFP reporter strain, Mr Stephen Mitchell (The University of Edinburgh) for TEM assistance,
Dr Craig Watkins (Moredun Research Institute), Dr Laura MacCalman (Institute of Occupational Medicine), Dr David Brown, Dr Nilesk Kanase, Mr Daniele Pantano, and Mr Corin Liddle (Heriot Watt University) for advice and support throughout this work and the ongoing project.

Declaration of interest

We alone are responsible for the content and writing of the paper. This work is funded by James Watt Scholarship (Heriot Watt University and Moredun Research Institute) and the Scottish Government Rural and Environment Science and Analytical Services Division.

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Supplementary material available online
Supplementary Figures 1-5 and Supplementary tables 1-2