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Antimicrobial properties of nano-silver: A cautionary approach to ionic interference

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ABSTRACT

Hypothesis: Metallic nanoparticles such as nano-silver have found many applications as alternative antimicrobials in recent years. However methods for determining their proposed antimicrobial activity have received little attention to date. The disk diffusion assay is commonly used as a demonstration of antimicrobial properties and is a regular feature in synthetic nanoparticle papers. The aim of this study was to assess its effectiveness in demonstrating the "nanoparticle specific" antimicrobial properties in the absence of ionic contributions from unreacted reducing agents and or impurities.
Experiments: The disk diffusion assay was carried out on a range of silver nanoparticles, both in-house synthesised and commercially available, using *Escherichia coli* ATCC 25922 as a model organism.
Results: Capped and purified nanoparticles show no antimicrobial activity despite claims to the contrary for this assay. Results will be discussed in terms of the need for researchers without a background in microbiology to understand the mechanism of antimicrobial action before choosing an assay. Also discussed is the importance understanding the physiochemical characteristics of when interpreting results. Finally the relevance of the results in terms establishing protocols for method development for 'nanoparticle specific' antimicrobial properties will also be considered.

1. Introduction

The evolution of antibiotic resistant bacteria such as MRSA has resulted in a significant body of research focused on finding alternative antimicrobial agents capable of eradicating these superbugs [11]. One promising alternative which has been proposed is nano-silver [23]. Metallic nanoparticles in general have been reported to have broad spectrum activity against both Gram-positive and Gram-negative bacteria including antibiotic resistant bacteria like MRSA and MRSE [2,18,20].

The mechanism of the antimicrobial properties of metallic nanoparticles is still under debate with the consensus being that it is due to ionic contributions rather the any "nanoparticle specific" activity [30]. Nevertheless there has been a significant upsurge in reports of nanomaterials exhibiting antimicrobial properties [25,21,1,15,9]. A deeper investigation reveals that many of these reports merely append a simplistic disk diffusion assay or a variation of this assay to essentially a material synthesis report [26,10]. Often such studies fail to take account of the physicochemical properties of the nanoparticle which have been crucially important in helping to standardise approaches for the assessment of nanoparticle toxicity and are likewise expected to contribute significantly to understanding the antimicrobial properties of nanomaterials [19,28]. In addition, limited statistical analysis is often carried out in the aforementioned work with the inhibition zone being taken as conclusive evidence that the nanomaterial under study is antimicrobial with no regard to impurities, particle coatings, competing processes or known mechanisms of interaction [25,1,17].

The choice of the disk diffusion assay in these circumstances is usually based on the fact that it is a relatively economical and easy assay to perform, in principle. Indeed it is a common method for testing the sensitivity of bacteria to antibiotics, which are soluble molecules with minimal impurities. In the disk diffusion assay a micro-organism is classed as sensitive or resistant to a material based on the radius of the zone of inhibition which forms around a small filter paper disk containing the test material. In comparison to antibiotics, the properties of nanomaterials are less consistent, with significant variations between batches in terms of particle size distribution, bioactivity, purity and surface chemistry [28,19]. Furthermore nano-metals such as nano-silver are often insoluble in aqueous solution and have a tendency to agglomerate in certain environments such as nutrient broth [19] making them unsuitable for many assays other than the disk diffusion assay. In addition nano-metals are also particularly efficient at forming

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metal ions in solution which are well known to exhibit antimicrobial properties [6]. Ion formation must be taken into account when selecting an assay and in the interpretation of subsequent results to distinguish the nano-particle specific effect from the ionic effect. This is particularly true when considering particles which have been stabilised in aqueous solvents (using coating/capping agents such as polyvinylpyrolidone (PVP), citrate buffers or surfactants) which will have a reduced capacity to form ions, and for 'as-produced' unpurified nanoparticles which will have unreacted reducing agents present from the synthesis [25]. Considering these aspects, a key question that this paper will address is whether or not the disk diffusion assay is a suitable method for assessing the antimicrobial nature of nano-metals. The paper will examine the most appropriate approach to differentiate between the particle and the ionic effect thereby helping to interpret the results of the disk diffusion assay for a range of nano-silver particles, coated and uncoated. It will also identify several pitfalls which researchers should be aware of with respect to the processing of nanomaterials prior to testing. The results reported reaffirm the need to characterise the physicochemical properties of the nanoparticle prior to assessing any potential antimicrobial properties analogous to the approach taken in the emerging area of nanotoxicology [28]. Finally the relevance of the results in terms establishing protocols for method development for 'nanoparticle specific' antimicrobial properties will be discussed.

2. Materials and methods

2.1. Nanoparticles

PVP-coated silver nanopowder, particle size <100 nm was purchased from Sigma Aldrich, Ireland (product number 576832). A 20 ppm stock solution of 20 nm citrate stabilisedzed nano-silver solution, particle size <20 nm was purchased from Sigma Aldrich Ireland (product number 730793). The above nanoparticle products were used as standards, due to their high quality, monodispersity and purity. As well as these, a third set of uncoated particles were synthesised by a sodium citrate reduction of silver nitrate, as described by Fang et al. [8]. A stock 10^{-3} M $AgNO_3$ aqueous solution (Sigma Aldrich, Ireland) and a stock 1% trisodium citrate solution (Sigma Aldrich, Ireland) were prepared. 500 ml of $AgNO_3$ aqueous solution was heated to boiling, and to this solution 10 ml of trisodium citrate solution was added drop by drop with vigorous magnetic stirring. Heating and stirring were continued for 30 min until a clear yellow liquid (silver nanoparticle solution) was observed in the flask. The nanoparticle presence was confirmed using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer to indentify the silver plasmon as discussed in previous studies [7,26]. These in-house synthesised and commercial nanoparticles were then characterised using multiple techniques detailed below to estimate particle size.

2.2. Culture media

Nutrient broth was purchased from Sigma Aldrich, Ireland (product number 70122). 25 g of nutrient broth was mixed with 1 L of deionised water before being autoclaved. Nutrient agar was prepared by adding 15 g of bacteriological agar (Sigma Aldrich product number A5306) to 25 g of nutrient broth before adding 1 L of deionised water and autoclaving.

2.3. Bacterial strains

The majority of researchers use either/or *Escherichia coli* and *Staphylococcus aureus* [25,31,21]. Therefore as an initial representative strain, *E. coli* ATTC strain 25,922 was chosen and purchased from ATCC. *E. coli* was taken from frozen stocks and subcultured onto nutrient agar before being incubated at 37 °C for 24 h. This was then subcultured twice more in the same manner before being used in experiments.

2.4. Nanoparticle characterisation

As an initial step in all nanoparticle work, it is crucial to perform a series of physiochemical characterisation steps to establish the nature of the particle under study [19,28]. Nanoparticles can aggregate over time and in different environments, forming larger species than may be expected (>100 nm) thereby reducing "nanoparticle-specific" effects. As a result it has become good practice in nanomaterial laboratories to physically confirm specifications of particles prior to use [22]. While the type of characterisation required may vary for different particles and studies, it is expected that at a minimum the particle size range or distribution would be determined. All particles used in this study where characterised for size/distribution using two methods; Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM). In addition particle purity and coatings (where applicable) were confirmed using UV–Visible absorption spectroscopy [5], X-ray diffraction spectroscopy (XRD) [4], and atomic absorption spectroscopy (AAS) [13]. Colloidal stability was assessed using zeta potential analysis [27]. For the purpose of clarity for this paper only details of methods pertaining to particle size distribution and were appropriate purity or concentration will be presented and discussed.

2.4.1. Particle size distribution

Dynamic Light Scattering (DLS), also known as Photon Correlation Spectroscopy, or Quasi-Electrical Light Scattering, is a non invasive technique used to measure particle size distributions in solvated environments, typically in the nanometer size range. The parameter that is measured by Dynamic Light Scattering is the hydrodynamic radius, which refers to how a particle diffuses in a fluid [16]. Applications of DLS are typically the measurement of particles in a liquid, e.g. proteins, polymers, micelles, nanoparticles, colloidal suspensions, and emulsions [16,19].

DLS was used to establish particle size of both commercially available and in house synthesised nano-metal samples. A Malvern Nano series ZS Zetasizer, operating of version 7.10 of the DTS Nano Software, was employed for all analysis. All samples were made up to a 20 ppm concentration in deionised, ultra-pure water and measured in triplicate.

2.4.2. Particle stability

Zeta potential is the measure of the stability of a colloid system. When particles are suspended in a media of different phase (e.g. solids suspended in liquid) their interaction with the media surrounding them determines whether the particles will coagulate or stay dispersed. In this case, the stability of the nanoparticles were measured both in water and in nutrient broth.

A Malvern Nano series ZS Zetasizer, operating of version 7.10 of the DTS Nano Software, was employed for all analysis. All samples were made up to a 20 ppm concentration in deionised, ultra-pure water/nutrient broth and measured in triplicate.

2.4.3. Particle size and shape

Scanning Electron Microscopy (SEM) was used to determine particle size and shape. There have been reports associating nanoparticle shape with different biological responses and so it was deemed important to ensure that all particles in this study were approximately the same shape (for example spherical) [24]. For the SEM characterisation all nanoparticle samples were diluted in ethanol, spin coated onto a cleaned silicon wafer and imaged using