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Ligands affecting silver antimicrobial efficacy on *Listeria monocytogenes* and *Salmonella enterica*

Antonio Martínez-Abad [a], Gloria Sánchez [a], Jose M. Lagaron [a], Maria J. Ocio [a,b,*]

[a] Novel Materials and Nanotechnology Group, IATA, CSIC, Av. Agustin Escardino 7, 46980 Paterna, Valencia, Spain
[b] Dpto. Medicina Preventiva, Facultad de Farmacia, Universidad de Valencia, Vicente Andrés Estellés, s/n, 46100 Burjassot, Valencia, Spain

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ABSTRACT

Although silver is being extensively used in food or other applications as the key component to control microbial proliferation, many factors affecting its real potential are still unknown. In the present work, the presence of specific ligands or the contents in organic matter was correlated with silver speciation and its antibacterial performance. Silver was found to be only active in form of free silver ions (FSI). The presence of chloride ions produced an equilibrium of stable silver chloride complexes which were void of antimicrobial efficacy. However, even at relatively high concentrations of chlorides, a small fraction of FSI may still be present, producing a bactericidal effect with concentrations at the nanomolar level under optimum conditions. Low concentrations of thiol groups completely inactivated silver, while methylsulphur groups only affected its efficacy at very high concentrations. Antibacterial performance revealed differences of about 1000-fold between results for environments with high organic matter content and results for aqueous salt buffers. Thiol groups were nonetheless not found directly associated with the decrease in antimicrobial performance in a nutrient rich environment. These results point out the complexity of the antimicrobial systems based on silver and can have relevance in food or other applications of silver as an antimicrobial.

1. Introduction

In the last decade, the demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products has globally increased which has encouraged the industry to develop new technologies as an alternative to food-thermal technologies. These new alternative technologies such as lower thermal or high pressure treatments may in some instances allow pathogenic bacterial growth (Valero & Francés, 2006). However, even if foodborne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the post-processing steps, when the risk of cross-contamination is elevated. As a result, a reduction of food shelf-life is observed and the risk of foodborne illnesses is greatly increased. Therefore, new preservation techniques, such as incorporation of antibacterial substances to the food products in order to extend its preservation is currently being investigated and applied.

The use of silver as antimicrobial for food-related applications has been recognised since silver pottery and cutlery where used in antiquity (Klasen, 2000). Although the mechanism remains disputed (Dibrov, Dzioba, Gosink, & Häse, 2002; Texter, Ziemer, Rhoades, & Clemans, 2007), it is generally accepted that free silver ions (FSI) bind to membrane constituents, destabilising the membrane potential and causing proton leakage (Liau, Read, Pugh, Furr, & Russell, 1997; Matsumura, Yoshikata, Kunisaki, & Tsuchido, 2003) and it also interferes with DNA replication and ion transport across the respiratory chain (Feng et al., 2000; Semeykina & Skulachev, 1990), all of which eventually lead to cell death. Due to this combination of unspecific mechanisms, silver ions are not likely to develop any resistances and are active against a very broad spectrum of bacteria, yeasts, fungi and even viruses in tiny concentrations (Thomas & McCubbin, 2003), remaining nontoxic to human cells (Russell & Hugo, 1994; Williams, Doherty, Vince, Grashoff, & Williams, 1989).

Therefore, its use has become more and more popular in the past few years. Apart from the medical field, silver is nowadays incorporated as the key component to control microbial proliferation in a wide variety of materials used in our daily life like textile clothing, coatings in home appliances and food related applications like water treatment units or a great variety of food-contact materials (see Bosetti, Massè, Tobin, & Cannas, 2002; Chen & Schluesener, 2008; Gupta & Silver, 1998; Li et al., 2008; Rai, Yadav, & Gade, 2009 for review). In most of these materials, the antimicrobial effect relies on the leaking of silver ions based on ion-exchange from mineral carriers, like montmorillonites (Busolo, Fernandez, Ocio, & Lagaron, 2010; Malachová, Praus, Pavlíčková, & Turicová, 2009), tobermorites (Coleman, 2009) and most predominantly

* Corresponding author at: Novel Materials and Nanotechnology Group, IATA, CSIC, Av. Agustin Escardino 7, 46980 Paterna, Valencia, Spain. Tel.: +34 963900022.
E-mail address: ajo@iata.csic.es (M.J. Ocio).

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zeolites (Cowan, Abshire, Houk, & Evans, 2003; Galeano, Korff, & Nicholson, 2003; Nakane et al., 2006). The versatility and cost-effectiveness of these materials have made silver the most widely used polymer additive for food applications (Appendini & Hotchkiss, 2002; Quintavalla & Vicini, 2002).

However, despite its widespread use, there is still much to be learnt about the chemical interactions taking place between the active silver species, the different bacteria and the matrix with which they interact. Most studies focus on the characterisation of silver particles and the release rates from different materials, neglecting the crucial effect that the chemical environment of action may have on their antimicrobial performance. Looking at the final concentrations achieved in solution that have been reported to exhibit antibacterial properties, these values go from the ppb range (Bjarnsholt et al., 2007; Hwang, Katayama, & Ohgaki, 2007; Kim et al., 1998) to hundreds of μg/ml (Hamilton-Miller & Shah, 1996; Nomiya et al., 2004; Ruparelia, Chatterjee, Duttagupta, & Mukherji, 2008; Sondi & Salopek-Sondi, 2004; Thomas, Yallapu, Sreedhar, & Bajpai, 2007) (4 orders of magnitude difference). Highlighting that, standardization of silver ion biocidal tests is difficult, as many solubility issues affecting speciation and bioavailability of silver are still unknown (Chopra, 2007).

In this respect, some studies in the branch of environmental toxicology have been dealing with the effect of ligands on the toxicity of silver to fish and algae. Computational modelling has also been used to predict silver chloride complexes available at different salinities (Ward & Kramer, 2002) and the influence of organic matter or food in the bioavailability and toxicity of silver to these organisms has been investigated (Glover, Sharma, & Wood, 2005; Kolts, Boese, & Meyer, 2006; Nichols, Brown, Wood, Walsh, & Playle, 2006; VanGenderen, Ryan, Tomasso, & Klaine, 2003).

However, the bioavailability of silver in these cases is more influenced by the uptake mechanism and the nature of the studied organisms than by silver speciation itself (Bielmyer, Brix, & Grosell, 2008; Lee, Fortin, & Campbell, 2005). Accordingly, controversy arises when deciding how much natural organic matter and which silver chloride complex are responsible for toxicity or protection against silver and to the best of our knowledge, no literature has yet been published about these effects on foodborne pathogenic bacteria.

As the antibacterial mechanism of silver seems to imply different unspecific pathways, and thus probable sublethal damage (Junghanns & Müller, 2008), it is crucial to elucidate how exposure to ligands present in complex matrices like food may alter the speciation of silver and how this speciation is correlated with the bactericidal effect.

In the present work, antimicrobial assays in different growth media were performed against two of the most relevant foodborne pathogenic bacteria, i.e. *Salmonella* and *Listeria monocytogenes*. The results were correlated to the FSI concentrations, as measured by anodic stripping voltammetry (ASV) (Joyce-Wöhrmann & Münstedt, 1999; Ward & Kramer, 2002). The impact of various ligands on silver speciation was also examined.

2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

L. monocytogenes CECT 5672 and *Salmonella enterica* CECT 554 were obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain). These strains were stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at −80 °C until needed. For experimental use, the stock cultures were maintained by monthly subculture to agar Tryptone Soy Agar (TSA) slants at 4 °C. Previous to each study, a loopful of bacteria was transferred to 10 ml of TSB and incubated at 37 °C overnight. A 100 μl aliquot from the overnight culture was again transferred to TSB and grown at 37 °C to the mid-exponential phase of growth. This culture served as the inoculum for antimicrobial assays starting with approximately 5×10^5 CFU/ml. These CFU counts were accurately and reproducibly obtained by inoculation into 10 ml growth medium of 0.1 ml of a culture having an absorbance value of 0.20 for *S. enterica* and 0.15 for *L. monocytogenes* as determined by optical density at 600 nm by ultraviolet visible (UV) spectroscopy (SP-2000 UV, Spectrum Instruments, Shanghai, China).

2.2. Chemical preparations

Experimental tests were performed using different aqueous silver nitrate solutions (from now on referred to as silver) prepared daily from serial dilutions of silver nitrate powder (Sigma–Aldrich, Germany) as the antimicrobial compound. Ultrapure water (Traceselect ultra, Fluka) was used as a base for the preparation of the different chemical environments. Potassium chloride, ammonium chloride and sodium chloride (Panreac, Barcelona, Spain) were used as a source of chloride ions. L-cysteine and L-methionine (Panreac) were used as a source of thiol (–SH) and methylsulphur ($-SCH_3$) groups, respectively. For the simulation of complex environments, the bacterial growth media TSB and M9 minimal medium (Sigma–Aldrich) alone, or supplemented with 0.1 mg/ml Methionine (Panreac) (M9-Met) were selected.

2.3. Silver ions quantification

Silver ions in free ionic form were quantified by means of voltammetric analysis. Samples were prepared dissolving a silver ion solution in the sample medium to achieve a final concentration of 100 μg/ml (approx. 0.6 mM) silver, then incubated at 37 °C and finally measured for free ions by differential pulse anodic stripping voltammetry (ASV) with an Autolab III potentiostat setup (EcoChemie, Switzerland) under conditions stated in Metrohm Application Bulletin No. 207/2e "Analysis of silver by stripping voltammetry". As the addition of the different components and substances in the concentrations used in the study did not affect the technique response, a calibration curve in high purity water was prepared daily for each set of measurements. The FSI working range was 0.004–0.4 μg/ml. All experiments were carried out in duplicate.

2.4. Antimicrobial tests

Antimicrobial capacity of silver under these various conditions was performed according to the broth macrodillution technique (M-26A) described by the Clinical and Laboratory Standards Institute (CLSI) with modifications. Briefly, a bacterial suspension in mid-exponential phase was inoculated into 10 ml of the selected environment of growth (ultrapure water supplemented with increasing concentrations of chlorides or sulphur groups, M9 medium or TSB) with silver concentrations of 0.1 μg/ml (0.59 μM) in M9 and 50 or 100 μg/ml (0.59 mM) in TSB as to achieve approximately 10^5 CFU/ml and incubated at 37 °C for 20–24 h. Cells suspensions were serially diluted in buffered peptone water (Scharlab S.L, Barcelona, Spain) and 100 μl spread on TSA. Colonies were counted after incubation at 37 °C for 24 h. Each of the experiments was performed in triplicate.

2.5. Effect of centrifugation

Samples with silver concentrations of 100 μg/ml (0.58 mM) in ultrapure water, M9-Met and TSB were incubated 24 h at 37 °C with and without the presence of increasing concentrations of bac-