Chem Soc Rev

REVIEW ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Chem. Soc. Rev.*, 2014, **43**, 1501

Nanosilver-based antibacterial drugs and devices: Mechanisms, methodological drawbacks, and guidelines

Loris Rizzello and Pier Paolo Pompa*

Despite the current advancement in drug discovery and pharmaceutical biotechnology, infection diseases induced by bacteria continue to be one of the greatest health problems worldwide, afflicting millions of people annually. Almost all microorganisms have, in fact, an intrinsic outstanding ability to flout many therapeutic interventions, thanks to their fast and easy-to-occur evolutionary genetic mechanisms. At the same time, big pharmaceutical companies are losing interest in new antibiotics development, shifting their capital investments in much more profitable research and development fields. New smart solutions are, thus, required to overcome such concerns, and should combine the feasibility of industrial production processes with cheapness and effectiveness. In this framework, nanotechnology-based solutions, and in particular silver nanoparticles (AgNPs), have recently emerged as promising candidates in the market as new antibacterial agents. AgNPs display, in fact, enhanced broad-range antibacterial/antiviral properties, and their synthesis procedures are quite cost effective. However, despite their increasing impact on the market, many relevant issues are still open. These include the molecular mechanisms governing the AgNPs–bacteria interactions, the physico-chemical parameters underlying their toxicity to prokaryotes, the lack of standardized methods and materials, and the uncertainty in the definition of general strategies to develop smart antibacterial drugs and devices based on nanosilver. In this review, we analyze the experimental data on the bactericidal effects of AgNPs, discussing the complex scenario and presenting the potential drawbacks and limitations in the techniques and methods employed. Moreover, after analyzing in depth the main mechanisms involved, we provide some general strategies/procedures to perform antibacterial tests of AgNPs, and propose some general guidelines for the design of antibacterial nanosystems and devices based on silver/nanosilver.

Received 28th June 2013

DOI: 10.1039/c3cs60218d

www.rsc.org/csr

Introduction

The evolutionary mechanisms of humans and their symbiotic bacteria have been shared for thousands of years, resulting in the selection of interactions in the form of mutualism and/or commensalism.[1–5] When such symbiosis turns out to a parasitic relationship, typically due to ecological or genetic/physiological changes, infections may occur within the host organisms. In this framework, bacteria were recognized to be the cause of several human diseases since the late 1800s; starting from that period, significant efforts have been pursued on many fronts to achieve solutions to this serious concern, including vaccination, improvement of hygienic conditions, and antibiotics development. Since their discovery, antimicrobial drugs have, in particular, saved millions of people and eased several patients suffering from chronic infections. Table 1 summarizes the history and chronological steps of the approval of some important antibacterial compounds by the Food and Drug Administration (representative data from 1935 to 2004).[6]

Albeit in the past the medical community optimistically dubbed antimicrobial agents as "the miracle drugs", subsequent evidences highlighted their strong limitations.[7–11] It should be, in fact, mentioned that, over time, bacteria evolved several resistance mechanisms against antibiotics, thus making their infection treatment extremely difficult.[12–15] As an example, penicillin was introduced in the early 1940s for the extensive treatment of *Staphylococcus aureus* related infections, and the first penicillin resistant *S. aureus* strains were identified in 1942. Fig. 1 shows the timescale evolution of the approval of some important antibiotics, along with the evidences of the rise of bacterial resistance. It is clear that, upon commercialization of a new compound, resistance is observed even a few years later (typically, between 1 and 3 years).[16–18]

Istituto Italiano di Tecnologia (IIT), Center for Bio-Molecular Nanotechnologies@UniLe, Via Barsanti, 73010 Arnesano (Lecce), Italy. E-mail: pierpaolo.pompa@iit.it; Fax: +39-0832-1816230; Tel: +39-0832-1816214

Table 1 The history of discovery and approval of principal antibiotics.[6] Adapted from J. H. Powers, *Clin. Microbiol. Infect.*, 2004, **10**(S4), 23–31

Year introduced	Class of drug
1935	Sulfonamides
1941	Penicillins
1944	Aminoglycosides
1945	Cephalosporins
1949	Cloramphenicol
1950	Tetracyclines
1952	Macrolides/lincosamides/streptogramins
1956	Glycopeptides
1957	Rifamicins
1959	Nitroimidiazoles
1962	Quinolons
1968	Trimethoprim
2000	Oxazolidinones
2003	Lipopeptides

In this framework, the treatment of infectious diseases has been estimated to cost more than 120 billion dollars per year to the U.S. society as direct healthcare expenses (Fig. 2A). Yet, this represents a considerable underestimation because it neglects the disease-associated overheads (*e.g.*, the long-term care or the treatment of chronic infections). Moreover, the healthcare costs associated with the treatment of resistant pathogens consists of *ca.* \$5 billion annually (Fig. 2A).[16] However, this estimation is also expected to rise, due to the constant and dramatic increase in antibiotic resistant bacterial strains (Fig. 2B). On the other side, although the infection treatment could represent an alluring opportunity for drug discovery companies, with an esteemed market of *ca.* \$25.5 billion per year (Fig. 2A),[19,20] the major pharmaceutical corporations are losing interest in antibiotics research and development. This is because such drugs are not so rewarding, in terms of long-term profits, as compared to drugs used for the treatment of chronic diseases (that require long-period therapies). The development of antibiotics is indeed expensive (*ca.* \$1 billion is required to have a new drug in the market), time consuming and risky (the investments require more than 10 years), and is also unattractive because of their too short lifecycle (due to bacterial resistance). Moreover, the nature of the market is fairly mature, and the actual clinical trials have become highly discriminating.[19] All of these factors have led big pharma to spend their research investments in much more productive ways (about 70% of the largest pharmaceutical companies have fully abandoned their R&D antibiotics sectors since 1999 [19,20]), while the "pipeline" of new antimicrobial-based therapies is significantly drying up (see Fig. 2C). By taking into account all these considerations, it is clear that the constant rise in antibiotic-resistance bacteria, combined with a significant decrease of antibacterial agents approval in the last decades is creating great concern worldwide, and bacterial infections represent, again, one of the greatest health challenges.[8,17,18,21,22]

Hence, new longer-term solutions for successful control of such diseases, which could integrate biological methods with the currently available nanotechnology tools, are absolutely required. Among all the recent non-traditional antibacterial agents, silver

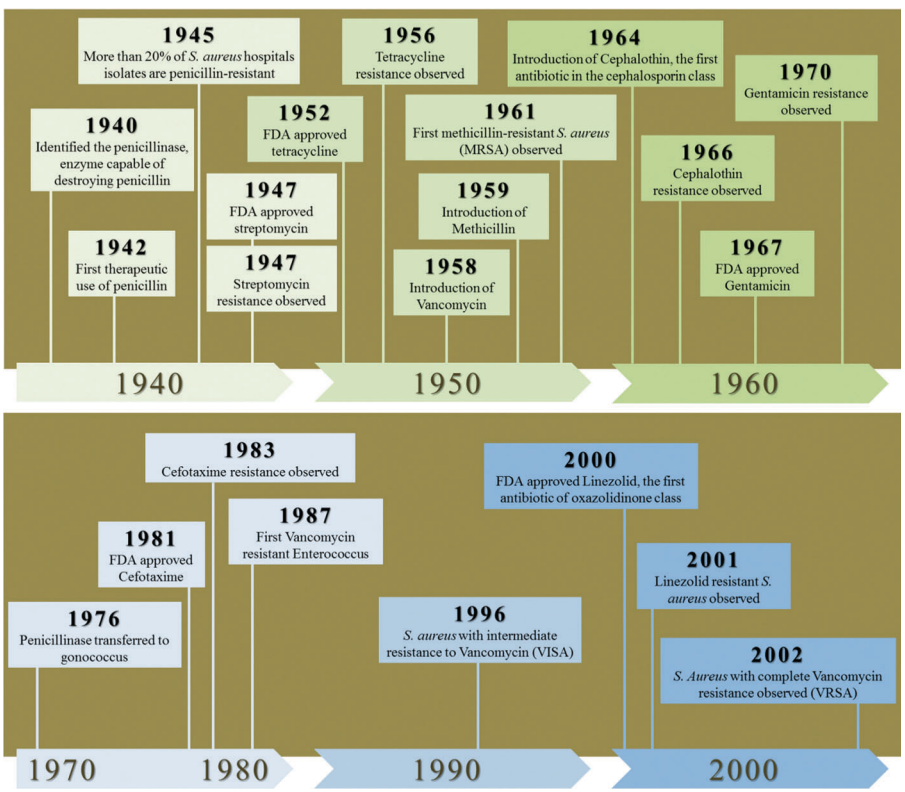

Fig. 1 Timescale of the milestones related to some drug approvals and drug resistance development.[18] From G. Taubes, *Science*, 2008, **321**, 356–361. Reprinted with permission from the AAAS.

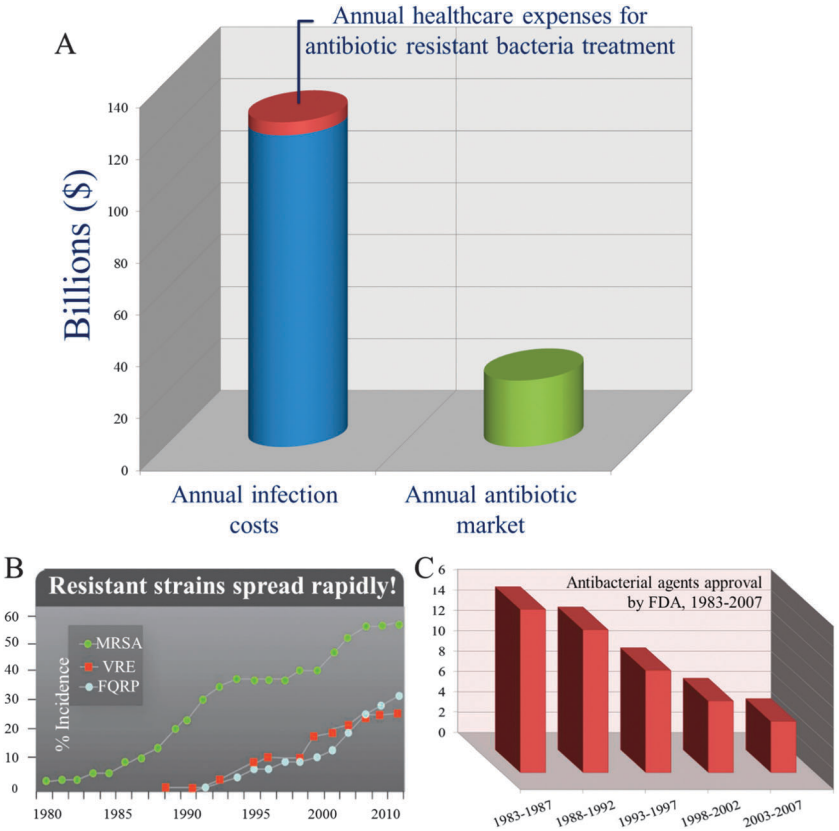

Fig. 2 (A) Direct annual costs in the U.S. related to infectious diseases and antibiotic resistant bacteria (left). Annual market related to antibiotics (right). Data from http://www.niaid.nih.gov/about/whoweare/planningpriorities/strategicplan/Pages/intro.aspx, and from the Infection Disease Society of America. (B) Graph showing the rate of resistance of three bacteria raising great concern to public health: methicillin-resistant *Staphylococcus aureus* (MRSA, green circles), vancomycin-resistant enterococci (VRE, red squares), and fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP, blue circles). Source: centers for Disease Control and Prevention (CDC), and ref. 18. From G. Taubes, *Science*, 2008, **321**, 356–361. Reprinted with permission from the AAAS. (C) Negative trend of new systemic (*i.e.*, nontopical) antibacterial molecules approved by the U.S. Food and Drug Administration (FDA), per 5-year period.[8,17,21,22]

nanoparticles (AgNPs) have been recognized as optimal candidates for defeating pathologies previously treated with conventional antibiotics, because of their strong and broad-spectrum antimicrobial characteristics. Albeit silver itself has been known for its bactericidal nature since ancient times,[23–26] the recent improvements of ''bottom-up'' approaches in nanofabrication techniques enabled the design of several type of AgNPs having different and tunable physico-chemical properties (*e.g.*, size, shape, and surface chemistry).[27] This is confirmed by the huge and constantly increasing amount of literature data available on the synthesis and use of AgNPs (Fig. 3A), and their application as antimicrobial agents (Fig. 3B). This latter constitutes about 10% of all the commercial/research uses of AgNPs or silver-based nanocomposites, which leads to an annual worldwide production of nanosilver of *ca.* 320 tons.[28,29] Nowadays, in fact, many retail products exploit AgNPs and silver nanocomposites as antimicrobial agents, ranging from clothing to household water filters, cosmetics, contraceptives, and even childrens toys.[29,30] In addition, several biomedical fields are also exploiting nanosilver as a potent antibacterial agent, including dentistry,[31] drug delivery,[32] eye care,[33] orthopedics,[34–36] pharmaceutics,[37–41] and surgery.[42–44] However, in spite of such a huge and quite indiscriminate use of Ag nanoproducts, a clear and definitive knowledge of the effects of AgNPs on microorganisms is still lacking. The key-point is the absence of NP standard assays and of a definitive explanation of their molecular mechanisms of action. Very recently, some important works elucidated previously unexplored aspects of this topic. In particular, Eckhardt *et al.* provided extensive analyses from a chemical viewpoint of the interaction of silver at molecular and cellular levels (with a specific focus on the binding with aminoacids, peptides, proteins, and DNA), as well as detailed discussion on the biocompatibility of silver for medical devices.[45] Also, Chernousova *et al.* and Hajipour *et al.* accurately reviewed the biocidal effects of silver in its different forms (namely, as a metal, salt, and nanoparticle),[46,47] while Lemire *et al.* focused on the mechanisms and molecular targets of metals.[48]

In this review, we first analyze the biocidal effects of AgNPs, based on the data available; then, we discuss several open issues regarding the mechanisms of action of nanosilver, the lack of standardized tests, and the limits/drawbacks in the techniques and methods employed. We also suggest some strategies to overcome possible experimental artifacts, which

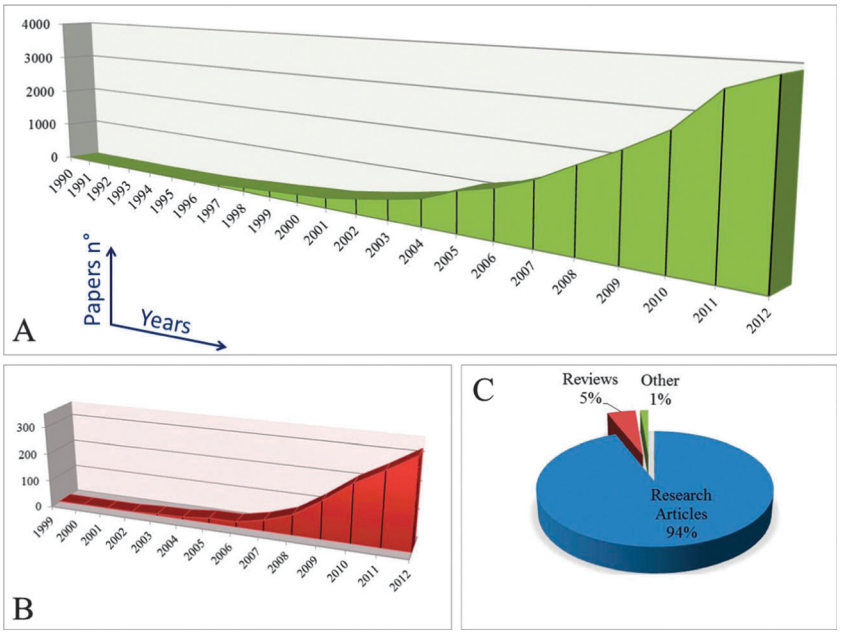

Fig. 3 Trend of scientific literature data on AgNPs and their application as antimicrobial agents. (A) Number of papers, over time, dealing with synthesis and use of AgNPs (source: Web of Science®, keywords: "Silver nanoparticles"). (B) Scientific articles on the application of AgNPs as antimicrobial tools (source: Web of Science®, keywords: "Silver nanoparticles" and "Bacteria"). The bactericidal effects of AgNPs represent *ca.* 10% of all the applications of AgNPs. As reported in (C), most of the papers in (B) belong to research articles (about 94%), while only 5% represent review discussions.

are at the basis of the current discrepancies in the literature. Finally, we provide some guidelines for the design and development of nanosilver-based devices.

Bactericidal effects of silver nanoparticles

In this section, we report and discuss some important studies on the bactericidal properties of AgNPs, giving particular attention to the role played by their physico-chemical characteristics (*i.e.*, size, shape, and surface characteristics), to their action mechanisms, as well as to their dose.

A size-dependence study of the bactericidal effects of AgNPs, in the range of 1–100 nm, against several GRAM negative bacteria (namely, *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhi*, and *Pseudomonas aeruginosa*), was carried out by Morones *et al.*[49] They demonstrated that 75 μg mL^{-1} of nanosilver was the cutoff value inhibiting all the bacterial strains tested, irrespective of the NPs size. By exploiting high angle annular dark field scanning transmission electron microscopy (HADDF-STEM), they found that AgNPs in the range of ~1–10 nm attach to the surface of the cell membrane with higher affinity, as compared to bigger nanoparticles, drastically perturbing the membrane functions. They ascribed such behavior to the larger surface area available in smaller AgNPs. In particular, the AgNPs–membrane interaction was reported to induce local membrane poration, with consequent internalization of NPs, which cause further damage, due to their interaction with both intracellular proteins (especially sulfur rich-proteins) and DNA. The authors also found that the Ag^+ ions, released from the particles surface, provide an additional contribution to the bactericidal effect, with similar mechanisms (namely, a massive binding to membrane proteins and induction of local holes). Although the exact cause of membrane damage is still debated in terms of physico-chemical interaction dynamics and the intracellular molecular targets of AgNPs or ions have not been yet identified, these data suggest that the bactericidal effects are due to both NPs and ions, which share similar mechanisms of action.

The issues of size-dependent effects and action mechanisms were also tackled by Choi and Hu.[50] The authors synthesized AgNPs in the range of 5–21 nm, and examined the correlation between size, intracellular Reactive Oxygen Species (ROS) generation, and nitrification inhibition in nitrifying microorganisms. First, they carried out inhibition growth experiments, using AgNPs, AgCl colloids, and Ag^+ ions, finding out that AgNPs were the most efficient (the EC_{50} was 0.14, 0.25, and 0.27 mg L^{-1}, respectively). Concerning AgNPs, they specifically observed that the smallest AgNPs have stronger efficacy, as compared to bigger ones. Second, they showed that AgNPs, AgCl-based colloids and Ag^+ ions all induced the generation and similar intracellular accumulation of ROS, indicating that ROS concentration mainly correlated with the final concentration of silver (and not to its form). Third, the authors carried out membrane integrity assays (by means of bacterial live/dead fluorescence based tests), finding that, in contrast with previous findings,[49] 1 mg L^{-1} of silver (in all the forms tested) did not compromise cell membrane integrity. This study suggested that the toxicity of AgNPs is strictly related to a ROS-mediated cell death, and that the final dose of silver is the crucial parameter to elicit specific effects. However, as also stated by the authors, a direct proof of ROS-related inhibition was not provided, and it is not

clear whether AgNPs or Ag^+ ions are more effective for ROS production. The same authors carried out additional studies in order to assess the bactericidal effect of AgNPs, AgCl colloids, and Ag^+, using two different approaches, namely a combination of respirometry and automatic microtiter fluorescence assay.[51] In this case, albeit AgNPs were found to elicit a stronger inhibition of the respiration of autotrophic nitrifying organisms, as compared to the other silver forms (all at a concentration of 1 mg L^{-1} of silver), the prolonged microtiter assay demonstrated that Ag^+ ions were the most efficient (*ca.* 100%) in hindering the growth of GFP-codifying *Escherichia coli*, as compared to AgNPs and AgCl (all at a concentration of 0.5 mg L^{-1}). Such data highlighted the discrepancies regarding the effectiveness of AgNPs and/or Ag^+ ions in eliciting antibacterial effects. In particular, it is evident that the results are strongly dependent on the method/technique used to carry out the biological assays, suggesting the need for more standardized approaches.

This concept is even more evident in the work of Sondi and collaborators.[52] The authors carried out, in fact, inhibition assays of *E. coli*, upon incubating nanoparticles with both solid and liquid media, finding out that AgNPs are less efficient when they are dispersed in liquid, as compared to the solid medium (at the same concentration). In this case, the discrepancy may be explained with the effective final dose of silver available. By a combination of TEM and SEM investigations, the authors also observed that AgNPs, with an average size of *ca.* 25 nm, induced the formation of several ''pits'' in the cell wall, confirming the finding of NPs-based membrane damage.

The possible dependence of membrane damage on NPs physico-chemical properties was also studied by El Badawy and collaborators.[53] The authors explored the toxicity of AgNPs having various surface charges, ranging from highly negative to highly positive values, against Bacillus species. The AgNPs used in this work were uncoated ($\zeta = -22$ mV), citrate coated ($\zeta = -40$ mV), polyvinylpyrrolidone coated ($\zeta = -12$ mV), and branched polyethyleneimine coated ($\zeta = +39$ mV). The experimental data demonstrated a direct correlation between the antimicrobial activity of AgNPs and their surface charge. Specifically, the more negative AgNPs were the least toxic, while the positively charged NPs were the most effective. The authors ascribed this phenomenon to a stronger electrostatic interaction between positively charged-AgNPs and bacterial membrane (the Bacillus spp. displayed a ζ of -37 mV under test conditions), which lead to membrane disruption and, in turns, to significant bactericidal effects (representative TEM images of polyethyleneimine coated-AgNPs interacting with bacterial membrane are shown in Fig. 4). The surface charge of NPs may likely promote their interaction with bacterial membrane, with a consequent increase of their effective dose.

In addition to the surface charge, other works analyzed the influence of AgNPs shape in eliciting the biocidal effects. In particular, Pal and collaborators synthesized spherical, elongated (rod-shape), and truncated triangular silver nanoplates (having a {111} lattice plane), and investigated their antibacterial properties both in liquid system (nutrient broth) and on agar plates.[54] The results showed that the truncated nanotriangles displayed the strongest biocidal activity against *E. coli* cells, compared to spherical and rod-shape NPs, and to silver ions (in the form of $AgNO_3$). The authors suggested that the {111} lattice plane enhance the activity of silver at the nanoscale level. Moreover, energy-filtering transmission electron microscopy (EFTEM) images revealed considerable damage in the bacterial membrane upon NPs treatment, in agreement with the previous studies.[49,52] Although the authors were not able to provide a definite explanation on the role of NPs shape on the killing activity, they speculated that the superior antibacterial characteristic of triangular nanoplates was also related to their positive surface charge that enhanced electrostatic interactions with bacterial cells.

While all these studies tried to correlate the physico-chemical properties of AgNPs with the bactericidal effects, Lok and collaborators investigated the molecular mechanisms of action of AgNPs by a proteomic approach, using *E. coli* as a model system.[55] The authors performed parallel proteomic investigations (bi-dimensional electrophoresis, MS identification, and immunoblot analyses) on 9 nm AgNPs and Ag^+ ions, revealing that short exposure of *E. coli* cells to nano-Ag or Ag^+ ions resulted in alterations (up-regulation) in the expression of a panel of envelope protein precursors (*i.e.*, OmpA, OmpC, OmpF, OppA, MetQ), which is a direct evidence of dissipation of proton motive force. Also heat shock proteins (IbpA, IbpB, and 30S ribosomal subunit S6), which have chaperone functions against stress-induced protein denaturation, were found to be differentially regulated upon AgNPs incubation. Consistent with the proteomic investigations, the authors demonstrated that AgNPs were able to destabilize the outer membrane of bacteria, to collapse their membrane potential, and deplete the levels of intracellular ATP. The authors concluded that the molecular mechanism of action of AgNPs and Ag^+ ions was almost similar. These findings are summarized in Fig. 5.

Many other research efforts have been made to understand of the role of AgNPs size and the mechanisms of action in eliciting antimicrobial properties,[39,56–73] overall confirming the previous findings. In particular, smaller NPs were shown to induce a stronger inhibition of microorganisms growth with respect to bigger ones (although it should be noted that, at the same dose in mass, smaller AgNPs are much more numerous with respect to bigger ones), while the biocidal effect was mainly ascribed to direct membrane damage, ROS production, and block of respiration, induced by both AgNPs and Ag^+ ions, which seem to share similar mechanisms.

However, despite the high number of important studies, there was still a high level of uncertainty concerning the mechanism of toxicity, particularly regarding the role played by the nanoparticle and/or by the Ag^+ ions, which may be released from the NP surfaces. This ''ions or NPs'' question, which has been debated for decades, seems to be solved only very recently. Xiu and collaborators, in fact, proposed that the antibacterial activity of AgNPs is entirely due to the release of Ag^+ ions in the medium rather than to NPs themselves, whose contribute to toxicity is negligible.[74] In particular, the authors synthesized PEG-coated AgNPs of 5 and 11 nm in diameter and stored them under anaerobic conditions, in which the ions release is strongly prevented.

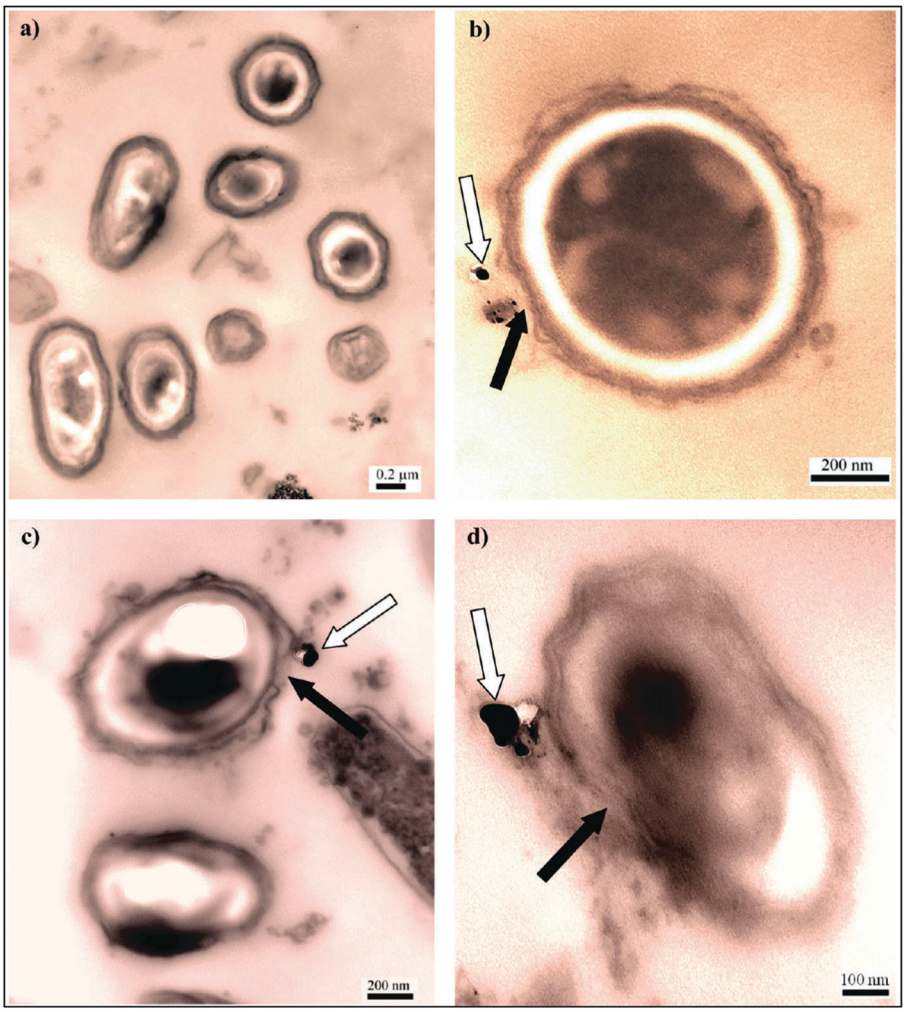

Fig. 4 Representative transmission electron micrographs (TEM) of (A) control cells compared to (B–D) cells exposed to polyethyleneimine coated-AgNPs. White arrows highlight the AgNPs and black arrows their impact on the cellular membranes.[53] Reprinted with permission from A. M. El Badawy *et al., Environ. Sci. Technol.*, 2011, **45**, 283–287. Copyright (2011) American Chemical Society.

In fact, the release of Ag^+ ions can be induced by exposing silver to oxygen, as explained by the following equations:[75]

$$4Ag(0) + O_2 \rightarrow 2Ag_2O \quad (1)$$

$$2Ag_2O + 4H^+ \rightarrow 4Ag^+ + 2H_2O \quad (2)$$

It is clear that oxygen molecules promote the formation of silver oxide; this latter is then the main cause of Ag^+ ions release, through interaction with H^+ ions. Acidic conditions, thus, induce an overall enhanced rate of release with respect to neutral pH conditions. Moreover, the authors chose *E. coli* as a model candidate for antimicrobial experiments, because it is a facultative microorganism that exhibits similar susceptibility to Ag^+ ions under both aerobic and anaerobic conditions. Interestingly, the viability assay showed that, under anaerobic conditions (in which there is no AgNPs dissolution), the NPs have no detectable effects on microorganisms, up to NP concentrations that were thousands of times higher than their minimum lethal concentration (MLC) found under aerobic conditions (Fig. 6). This indicates that the silver ions released form the NP surface are the main responsible for the biocidal activity. The authors concluded that the AgNPs physico-chemical properties (size, shape, and charge) affect the toxicity only indirectly, namely thorough mechanisms that influence the rate, location, and extent of Ag^+ release from the nanoparticle surfaces. As an example, very small AgNPs typically exert more pronounced toxicity because of their higher surface area and associated faster rate of Ag^+ release, compared to bigger AgNPs. These findings elucidated some previously uncharacterized aspects of AgNPs bacterial toxicity. However, there are still several open issues concerning the mechanisms of ions damage to bacteria, numerous experimental and methodological limits, and the lack of standardized protocols and reference AgNP materials, which we discuss in the following section.

Open issues

Despite the massive use of AgNPs in commercial applications and the numerous studies regarding their bactericidal properties,

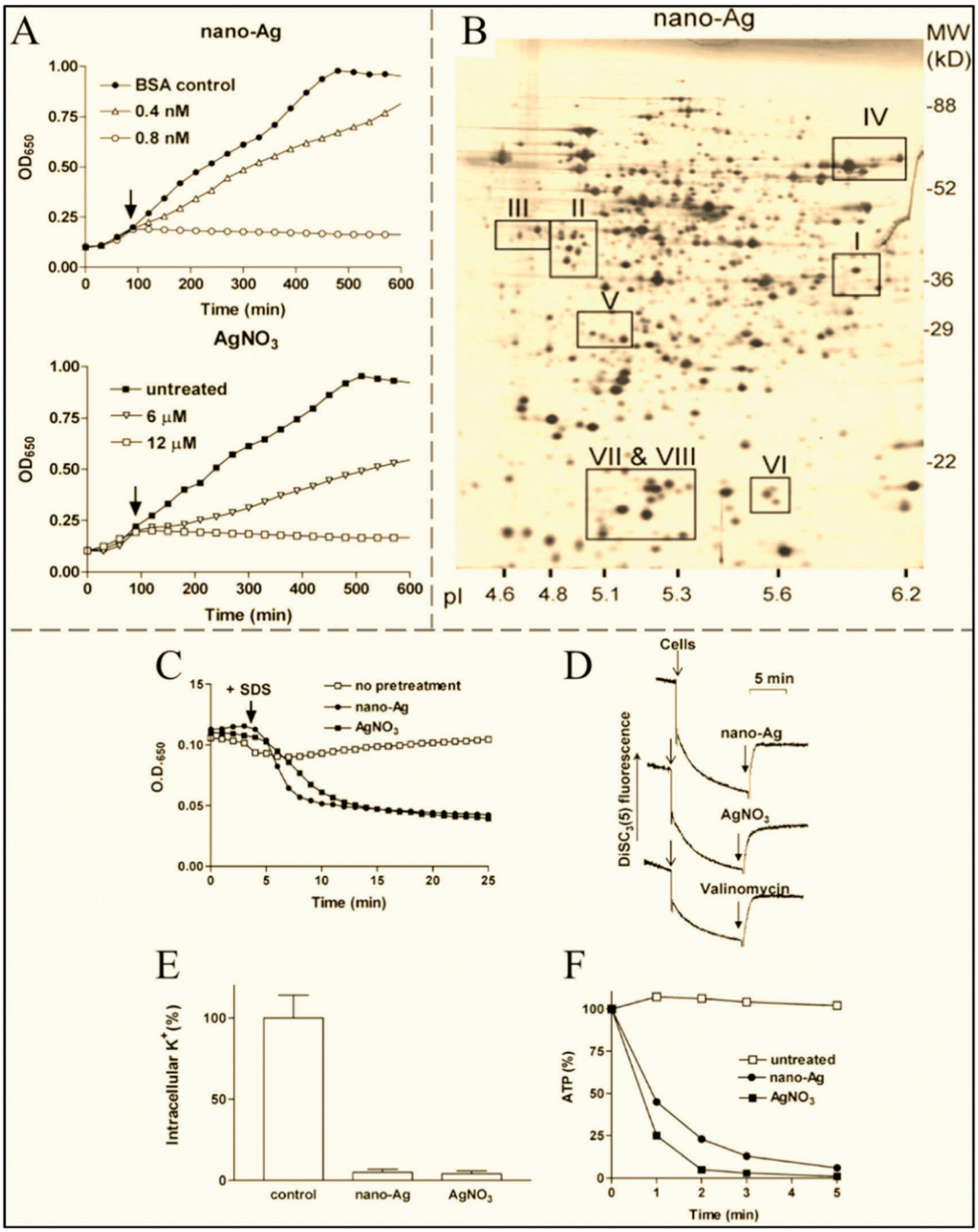

Fig. 5 (A) Antibacterial activity of AgNPs and $AgNO_3$. *E. coli* cells were grown at 35 °C to the early exponential phase (OD_{650} = 0.15) in M9 defined medium. AgNPs (0.4 and 0.8 nM, stabilized with BSA) or $AgNO_3$ (6 and 12 μM) were added at the time indicated by the arrows and the OD_{650} was continuously monitored. (B) 2D gel images of *E. coli* cells treated with AgNPs. (C) SDS-based outer membrane destabilization assays confirmed the similar behavior of Ag^+ and AgNPs. (D) Membrane potential assays confirmed that Ag^+ and AgNPs collapse membrane potential, in a similar way to valinomycin. (E) Cellular potassium content assays revealed an almost complete loss of intracellular potassium upon incubation with silver ions and AgNPs (confirming the collapse of membrane potential). (F) Ag^+ ions and AgNPs decreased, in a similar way, the cellular ATP levels, due to the collapse of membrane potential and to a possible over-stimulation of hydrolysis of residual ATP.[55] Reprinted with permission from C. N. Lok *et al.*, *J. Proteome Res.*, 2006, **5**, 916–924. Copyright (2006) American Chemical Society.

there is still a significant level of controversy/uncertainty. In the following, we discuss such points, giving particular attention to the drawbacks and limits of some methods, and providing some suggestions to overcome them. We also suggest some guidelines for the efficient design of antibacterial devices based on nanosilver. In particular, we discuss when AgNPs or Ag^+

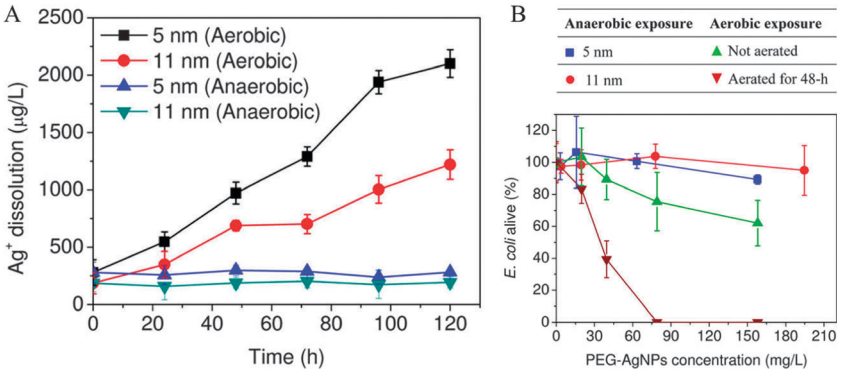

Fig. 6 (A) PEG-AgNPs (5 nm and 11 nm) dissolution under aerobic and anaerobic conditions. Dissolved Ag^+ concentration increased with air exposure time for both PEG-5 nm and PEG-11 nm nanoparticles under aerobic conditions, while no silver ions were detected under anaerobic conditions. (B) Viability assays show no statistically significant toxicity with concentrations up to 158 (for 5 nm AgNPs) and 195 mg L^{-1} (for 11 nm AgNPs), which are, respectively, 6224 and 7665 times higher than MLC for Ag^+. Antibacterial assays (6 h exposure) with the same 5 nm PEG-AgNPs under aerobic conditions (conducted immediately after transferring the particles out of the anaerobic chamber) showed toxicity. Storage in an aerobic atmosphere (48 h with magnetic stirring to increase oxygen exposure) resulted in higher Ag^+ release and higher toxicity. AgNPs may thus serve as a vehicle to deliver Ag^+ more effectively to the bacteria cytoplasm and membrane, whose proton motive force would decrease the local pH (as low as pH 3.0) and enhance Ag^+ release. Reprinted with permission from Z. M. Xiu *et al.*, *Nano Lett.*, 2012, **12**, 4271–4275. Copyright (2012) American Chemical Society.

should be exploited to achieve the desired antibacterial effects, depending on the specific purpose of the devices.

Mechanism of antibacterial action of AgNPs

In the previous section it has been discussed that AgNPs elicit bactericidal effects thanks to the aerobic release of silver ions, which are the primary cause of toxicity to microorganisms. However, from a typical molecular microbiology point of view, the action mechanism of silver ions is still not completely understood. There are some hypothesized mechanisms, mainly regarding direct Ag^+-induced membrane damage, Ag^+-related ROS production, and cellular uptake of Ag^+ ions (or even NPs, due to membrane poration), with consequent disruption of ATP production and hindering of DNA replication activities.

The direct membrane damage by Ag^+ ions has been proposed in several works,[49,51,52,57,58,62] where imaging investigations, mostly based on TEM analyses, revealed pits or even large holes within the bacterial membrane. Silver ions may interact with sulfur containing membrane proteins[49] (*e.g.*, with the thiol groups of respiratory chain proteins), causing physical damage to the membrane. In particular, according to the hard-soft acid-base theory (HSAB), the thiol moiety is a soft (polarizable) ligand, namely with a quite large and diffuse distribution of electrons, and with HOMO (highest occupied molecular orbitals) of high energy. Thiol group may, therefore, bind with high affinity soft cations, such as Ag^+, having a LUMO (low unoccupied molecular orbital) of low energy.[76,77] Because of the large size and overall low charge of the atoms involved in the coordination, and of the small HOMO–LUMO separations between them, a quasi-covalent bond is favorable as compared to ionic bond. Apart from sulfur containing membrane peptides and proteins, Ag^+ may be also involved in Ag–N and Ag–O bonds,[78–80] with preferential linear coordination geometry around the Ag^+ ion.[81–83] Several other coordination modes of Ag^+–aminoacids/peptides have been proposed from a theoretical[80,84,85] and experimental viewpoint,[86–89] showing, for instance, that histidine has much more affinity to silver compared to cysteine and methionine (usually considered as the best candidates for binding silver). In this scenario, the recent and elegant work of Mirolo and coworkers shed light on the coordination of Ag^+ ions by histidine.[90] In particular, they examined a specific histidine-rich periplasmic silver-binding protein, SilE, which is responsible for silver resistance (for more details, see the "silver resistance" section below). After solving the crystal structures of histidine–Ag complexes (at various pH), the authors concluded that the imidazole ring on the histidine side-chain is the exclusive silver-binding moiety of the ligand, and that the Ag^+ binding is stronger under neutral rather than acidic conditions (the protonation of the imidazole rings displace the Ag^+ from the coordination site). Further calculations based on the hybrid density functional theory (DFT) enabled the development of a model for the action mode of SilE.

All these Ag^+–membrane proteins interactions may lead, in turn, to a drastic change in membrane permeability, by a progressive release of lipopolysaccharides (LPS) and membrane proteins,[52,91] resulting in the dissipation of proton motive force and depletion of intracellular ATP levels.[55] This may also elicit the intracellular accumulation of Ag^+ ions (and, in principle, also of some NPs, although this latter evidence has been seldom reported). In particular, intracellular silver ions may bind proteins of the respiratory chains,[92,93] consequently uncoupling the respiration (namely, the electron transport through the membrane proteins) from the oxidative phosphorylation pathway (that uses energy released by the oxidation of nutrients to produce ATP).[55,94,95] Upon entrance, Ag^+ ions were also proposed to increase the frequency of DNA mutation. In particular, investigations based on a combination of FTIR spectroscopy and capillary electrophoresis revealed that guanine N7 and adenine N7 are optimal binding sites, in DNA, for Ag^+.[96] Additionally, silver ions may induce cytoplasmic shrinkage, DNA condensation phenomena, and

detachment of cell-wall membrane.[97–100] Another mechanism of Ag poisoning may be based on site-specific enzyme inhibition and, in particular, ionic mimicry. In this latter case, Ag^+ has been demonstrated to displace both Cu and Zn from their coordination with the superoxide dismutase enzyme (Cu–Zn SOD), with its consequent inactivation.[101] In this context, the surface charge of AgNPs may play an important role, as it can affect the possibility of NPs binding to bacteria, due to electrostatic interactions.[57] For instance, positive surface charge of NPs may promote their binding to bacterial membrane, leading to a higher effective dose available, with a consequent higher local ions release. At the same time, silver ions release is strongly promoted in the close proximity of the external membrane of bacteria, because of the proton motive force that induces a local strong decrease of pH (down to values of 3).[74,102] However, this does not mean that positively charged AgNPs are universally more effective against bacteria, as the real surface charge of NPs in bacterial medium (including protein corona effects) governs the NPs–bacteria interactions.

The toxicity effects of silver on microorganisms have been also ascribed to Ag^+ ions-related ROS production.[56,103–105] The excess of ROS leads, in fact, to oxidative stress, due to additional generation of free radicals that may damage both lipids and DNA.[106,107] In particular, Ag^+ ions, in combination with dissolved oxygen molecules, may act as a catalyst, generating high levels of ROS. Furthermore, the free radicals may arise from direct impairing of the respiratory chain enzymes, carried out by silver,[103] they can be photocatalytically induced,[50] or by Ag-promoted Fenton reactions.[108] In this latter case, Ag^+ may target and destroy the [4Fe–4S] clusters of proteins,[109–111] (usually present in *E. coli* in a concentration of *ca.* 20 μM as Fenton-active form)[112] leading to additional cytoplasmic release of Fenton-active Fe and, thus, increased ROS production. However, many research data on the bactericidal effects of both ROS and also reactive nitrogen species (RSN) remain controversial. Microorganisms have, in fact, several molecular strategies to subvert the ROS- and RSN-mediated stress, including direct detoxification carried out by enzymes, such as catalase, superoxide dismutase, and peroxidase (for ROS elimination), and NO reductase, *S*-nitrosogluthathione reductase and peroxynitrite reductase (for RNS detoxification).[113] In addition, bacteria actively respond to both oxidative and nitrosative stress at transcriptional level, by regulating the expression of several proteins (such as OxyR, SoxRS, PerR, OhrR, BosR, and NorR), which enable bacteria a high survival probability against such kind of stress.[113] By taking in consideration all the current knowledge, we developed a general scheme in Fig. 7, which describes all the proposed effects of AgNPs to microorganisms.

Role of the physico-chemical properties of the AgNPs

Significant efforts have been dedicated to correlate the physico-chemical properties of AgNPs with their antibacterial effects. Unfortunately, while the surface charge seems to play an important role (see above), by examining the data available in literature on NPs size and shape, a large disagreement is evident. This is mainly due to the lack of AgNP standards, along with the absence of standardized protocols and procedures in microbiology assays. Concerning the first point, considerable issues have to be overcome in both synthesis processes and nanoparticles characterization approaches. The fabrication of AgNPs with well-controlled sizes and size distributions in high yield represented, in fact, a big challenge also in the recent past. Their chemical synthesis is, in fact, influenced by various thermodynamic and kinetic factors, and considerable difficulty remains in capturing the distinct stages of nucleation and growth of AgNPs.[114–116] Only very recently some good results have been achieved.[114,117–121] In particular, it has been demonstrated that specific peptides can be exploited as catalysts and templates for the (green) synthesis of AgNPs, obtaining highly controlled NP dimensions.[119] For instance, an elegant approach employed a colorimetric on-bead screening of split and mix libraries, of both natural and unnatural amino acids, to test the formation of controlled AgNPs (Fig. 8A and B).[120] Unlike some previous combinatorial approaches for the identification of suitable peptides,[122–124] this colorimetric screening represented a powerful tool to identify peptides that induce the formation of high quality AgNPs, revealing the specific peptide motifs responsible for tuning the AgNPs size. In another work, Upert *et al.* exploited oligoprolines for the synthesis of AgNPs with controlled size. In particular, the authors used aldehyde-functionalized oligoprolines of different lengths, combined with a Tollens reaction for Ag^+ reduction, finding out that the molecular dimensions of the rigid oligoprolines are directly related to the increase of AgNPs size (Fig. 8C and D).[121] Compared to more standard synthesis processes, where the stabilizing agents are usually polymers,[125,126] citric acid,[127] tyrosine,[128,129] and thiols,[130] AgNP–peptide hybrid materials have been demonstrated as optimal candidates for applications in medicine, biotechnology, and optical devices.[131]

However, it should be considered that most of the literature available on the bactericidal effects of AgNPs is based on the use of NPs with largely uncontrolled properties (*e.g.*, highly polydisperse in terms of size and shape, and/or aggregated). Moreover, several research works employed such nanomaterials without carrying out any physico-chemical characterizations, thus exacerbating the discrepancies in the observed results. A crucial point for reproducible and standardized assays is, therefore, the characterization of NPs before any antibacterial tests: NPs should be deeply characterized both after the synthesis processes (*e.g.*, in aqueous solution), and, most importantly, *in situ* (*e.g.*, after incubation with the bacterial culture media). The assessment of the NPs physico-chemical properties in biological fluids is not trivial, as bacteriological media may lead to significant changes of the original properties of NPs, resulting in the generation of new nano-objects having completely different characteristics.[132–136] For instance, NPs may have larger size, different surface charge and coating (depending on the adsorption of specific proteins and other small molecules onto their surfaces) compared to the as-synthesized NPs, and they may undergo significant agglomeration/aggregation phenomena. Most of the AgNPs, in fact, are not stable in bacterial culture media, thus severely compromising the

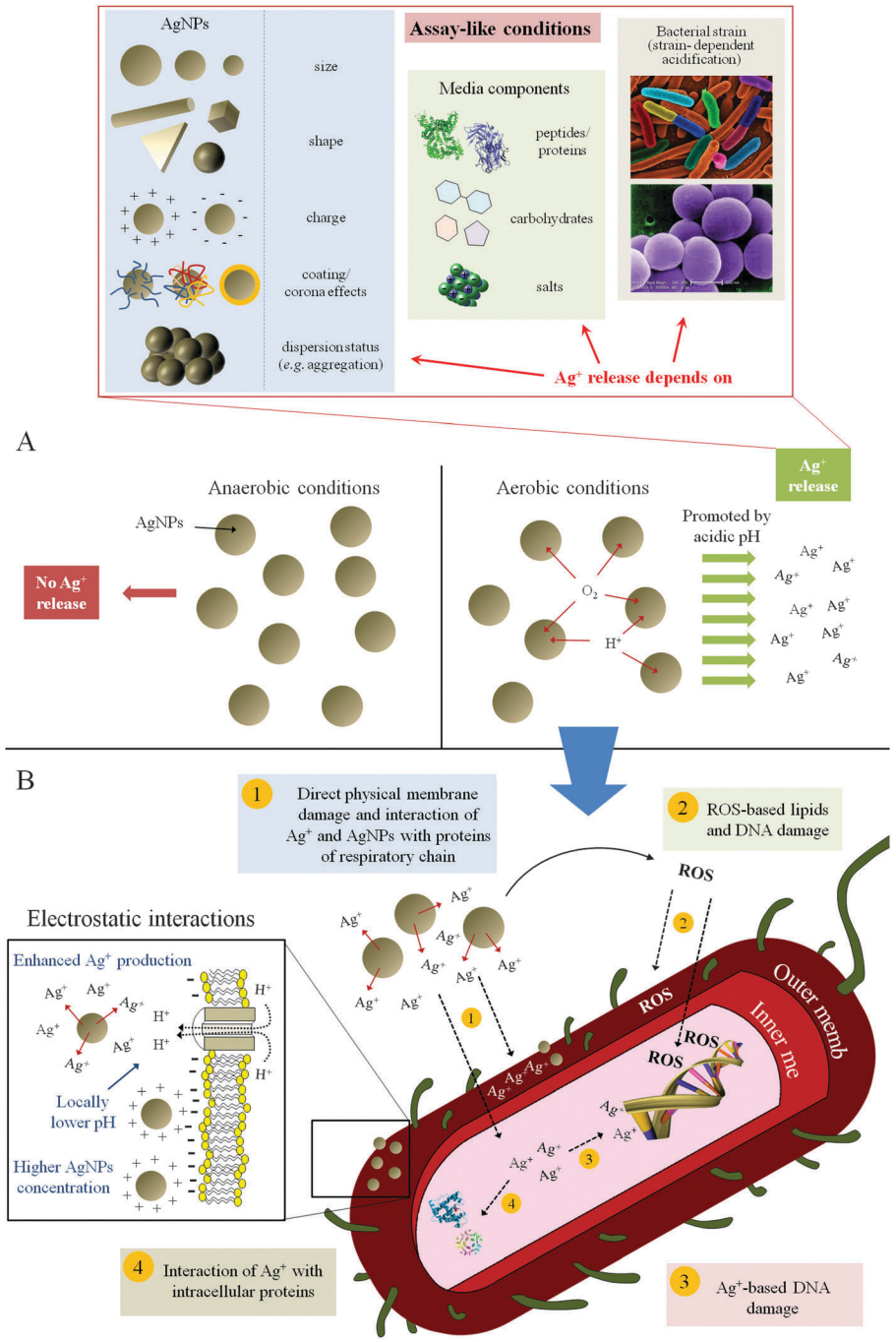

Fig. 7 (A) Silver ions release is promoted by acidic and aerobic environment.[65] In the inset, the parameters affecting the NPs dissolution in real, assay-like conditions are reported. Top picture: photo from a public domain, retrieved from Wikimedia Commons and bottom picture: courtesy: CDC. (B) Proposed mechanisms of AgNPs-related toxicity. Silver ions may directly damage bacterial membrane, by blocking the respiratory chain, collapsing the membrane potential and stopping ATP production (1). Additionally, they may promote the formation of ROS, which then damage both the membrane lipids and DNA (2). Ag^+ ions may bind intracellular proteins and the bacterial chromosome, upon entering the cytosol, thus influencing the metabolic activity and replication (3–4). Ag^+ uptake can be promoted by membrane damage (although they might enter also through membrane channels). Inset: positively charged AgNPs may be attracted by negatively charged bacterial membrane leading to higher local dose of NPs. Here, the proton motive force takes place, causing a local decrease of pH. This can further promote the dissolution of AgNPs, resulting in a local higher Ag^+ concentration. In this picture, a GRAM negative bacterium has been taken as model microorganism.

observed bactericidal effects. In addition, all these factors may strongly influence the dynamics of ions release, and thus the effective dose of Ag^+, causing significant irreproducibility in the results.

An important point is also that the ion release kinetic may be strongly affected by the presence of bacteria. Microorganisms, in fact, typically reduce the pH of the culture media, thus eliciting an increase of the rate of ion release from

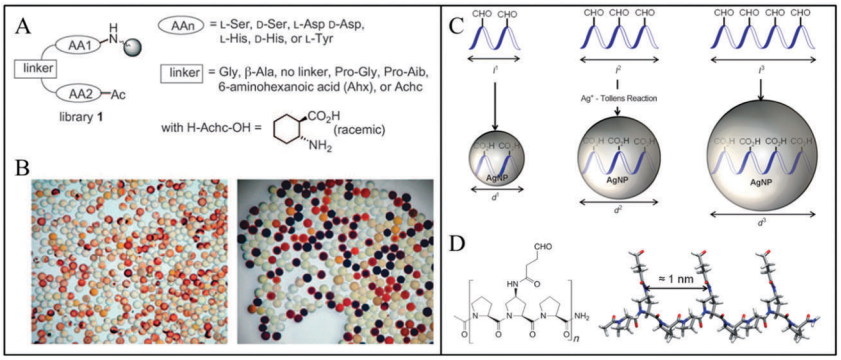

Fig. 8 Peptides mediated synthesis of AgNPs. (A) General structure of peptides library useful for screening the synthesis conditions. (B) AgNPs formation within the combinatorial assay of peptide library 1 complexed to Ag^+ ions, followed by treatment with light (left) or sodium ascorbate (right). Red beads contained, for instance, Ac-His-Ahx-Asp-R and AgNPs were of ~50 nm; orange beads contained, for instance, Ac-Ser-Ahx-Tyr-R and AgNPs were of ~10 nm. Reprinted with permission from K. Belser *et al.*, *Angew. Chem., Int. Ed.*, 2009, **48**, 3661–3664. (C) Metallization of aldehyde-functionalized oligoproline helices. (D) General structure and model of oligoprolines functionalized with aldehydes in every third position. Reprinted with permission from G. Upert *et al.*, *Angew. Chem., Int. Ed.*, 2012, **51**, 4231–4234.

the NPs surface. The acidification of the environment can be both strain- and medium-dependent (*e.g.*, function of bacterial metabolic pathways and/or presence of specific molecules in the medium), and may cause a significant lowering of the pH of the assay, with clear consequences on the toxicity outcomes of AgNPs. This means that the same AgNPs might display different anti-bacterial efficiency against two strains, just because of the different acidification of the two media. For instance, the lactic acid bacteria (LAB, *e.g.*, the *genera Lactobacillus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, and *Lactococcus*) produce lactic acid (derived from pyruvate, the end product of glycolysis), useful for acidifying the environment and inhibiting the growth of competitors.[137] Media acidification is also carried out by other microorganisms, such as the acetic acid bacteria (*e.g.*, *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*). These latter have a characteristic membrane-bound enzyme, the pyrroquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH), involved in the acetic acid fermentation by oxidizing ethanol to acetaldehyde.[138] In this framework, it is thus crucial to characterize the NPs dissolution behavior exactly in the conditions used for the antibacterial tests, namely directly in the culture medium and in the presence of the specific bacterial strain.

Another cause of test variability is represented by the huge amount of different bacterial culture media available. Although their basic chemical composition is quite common (namely, a combination of proteins source and salts), there is a high variability and heterogeneity in the media exploited for the antimicrobial tests. Hence, diverse media differently impact the physico-chemical properties of nanomaterials in an unpredictable way. This means that the same AgNPs may give different antibacterial results if tested in two different media. At the same time, the media composition also influences the final dose of Ag^+, since free ions will be partly hijacked depending on their affinity with the specific proteins and salts present. For these reasons, a correct procedure to guarantee the reproducibility of results should take into account that AgNPs have to be deeply characterized in the same culture medium used for the antibacterial assays (in terms of both colloidal stability and kinetics of ions release), and the used medium should be highlighted as an important parameter of the assay.

Another fundamental issue to be considered is the method to probe the NPs dissolution dynamics in relevant media. In this respect, several approaches have been exploited in order to characterize the Ag^+ release from the NPs surface. The most common techniques are the inductively coupled plasma spectrometry-based techniques (*i.e.*, optical emission or the mass-spectrometry, ICP-OES and ICP-MS, respectively), which have the advantage to be rather sensitive. However, these methods may suffer from some drawbacks mainly because Ag^+ ions have to be physically separated from the NPs prior to the ICP quantification, potentially leading to experimental artifacts. For instance, while centrifugation may not achieve complete sedimentation of the smallest NPs, ultrafiltration may lead to ion adsorption onto the membrane, and is also time consuming (in this case, AgNPs may also dissolve during the long separation process). Therefore, alternative methods have been proposed, such as UV-vis analyses. An interesting work, in fact, recently suggested that the most appropriate methodological approach to investigate the AgNPs dissolution (even in complex biological and environmental matrices) is the characterization of their surface plasmon absorption band.[139] The authors explained that, unlike other approaches, the absorbance method is the most accurate to correctly quantify the amount of silver in the form of ions or NPs, even in biological and environmental fluids that typically contain chloride (with consequent formation of AgCl precipitates, not detected by ICP-based approaches). However, although this method allows to precisely monitoring AgNPs degradation (provided that nanoparticles remain stable and monodispersed), it does not offer reliable information regarding the real Ag^+ bio-availability, since ions could be sequestered by medium proteins and salts, or form precipitates. The absorbance peak, in fact, does not detect the formation of AgCl clusters and/or Ag–proteins complexes, which both decrease the final effective dose of Ag^+

(which are the primary agents determining bactericidal toxicity). Hence, a combination of both UV-vis and ICP techniques should be exploited, in order to have accurate (though not exact) information about the dissolution state and dynamics of AgNPs. In any case, as also discussed above, the AgNPs dissolution should be characterized in the presence of bacteria, because of the microorganism-related acidification of the medium.

The colloidal stability of AgNPs in bacterial media is another fundamental aspect to be assessed (aggregation phenomena can sometimes be detected even by naked eye, thanks to the color change of the suspension). A possible solution to overcome such typical instability is surface passivation of AgNPs with specific capping agents, such as bovine serum albumin (BSA), as also suggested by MacCuspie.[140] In this work, the author exploited a variety of instrumental techniques (including atomic force microscopy, dynamic light scattering, and UV-vis spectroscopy), finding out that BSA capping provides better stability of AgNPs in bacteriological medium, as compared to purely electrostatic stabilization, such as citrate.[140] However, while this method represents to date the most precise procedure to perform standardized tests, it should be mentioned that such conditions are quite far from real situations, both *in vitro* and *in vivo*, where BSA may induce, for instance, immunogenic phenomena. Moreover, this stabilization process may change the original surface charge of NPs and influence the rate of ions release in uncontrollable way, since several protein layers may cover the NP surfaces. Hence, the issue of standardized assays remains still open, as a definitive, reliable procedure to precisely control the colloidal stability of AgNPs has still to be developed.

Another crucial aspect is represented by possible interferences in the biological assays employed for the evaluation of antibacterial activity. One of the most used approaches is, in fact, the spectrophotometric analyses of the optical density, or turbidity, of bacterial suspensions (typically at a λ = 600 nm), that enables to measure the cell concentration. However, while the advantage of turbidity measurements is its execution simplicity, one drawback is that AgNPs themselves may give significant contribution to the optical density of the sample, due to their large extinction coefficient (especially if the AgNPs are agglomerated, with consequent red-shift of the plasmon band and overlap with the read-out window of the bacterial concentration).[141] In addition, the fluorescent and colorimetric assays employed to understand the live/dead bacterial ratio, upon AgNPs treatment, also suffer from potential artifacts, because AgNPs might interact and interfere with the components of the commercial kits, leading to false positive/negative results (commercial kits, in fact, have not been designed to test NPs).[142] Indeed, particular attention and accurate control experiments are required for viability and metabolic assays, to avoid NPs-induced artifacts.

Hence, all the above issues suggest the need for more standardized tests, which should take into account all the possible limitations of each technique and method. In particular, the agglomeration state of AgNPs, the rate of ions release, and the changes in NPs physico-chemical properties upon incubation with bacteriological media are crucial parameters to be assessed, in order to obtain reliable biological outcomes.

AgNPs or silver ions for antimicrobial devices?

In the previous sections, we explained that the mechanisms of bactericidal action of AgNPs are mostly due to the silver ions released from their surface. A direct consequence of this concept is that AgNPs are less effective against microorganisms than silver ions (at the same silver dose). This is because, in the case of AgNPs, there are significantly less ions immediately available for eliciting the bactericidal effects. It is strongly unlikely, in fact, to have an immediate and abundant dissolution of AgNPs in ions, in the biological environment of the assay, to produce the same amount of free ions available in the case of Ag salts. Such different efficiency is confirmed by directly comparing the effects of AgNPs (freshly synthesized and extensively washed) and Ag^+ ions, at the same dose, in hindering the growth of *E. coli* cells. As shown in Fig. 9, silver ions are much more effective against bacterial growth, as compared to the same amount of silver in the form of nanoparticles.[143] Different information can be deduced from these outcomes. First, the higher toxicity of silver nitrate can be ascribed to the immediate and more abundant source of antibacterial compound available in the culture medium to bacteria. AgNPs require, in fact, a certain time to release Ag^+ ions, and the initial dose of AgNPs-derived silver ions is typically quite low (at least in the case of freshly prepared and washed AgNPs). Second, an important guideline arises from the above considerations, namely that all the antibacterial experiments with AgNPs have to be performed with freshly prepared or washed AgNPs suspensions. This is necessary to avoid data irreproducibility, due to the variable presence of Ag^+ ions in the starting solution, since ions concentration would be a function of samples ageing. This means that, an older AgNPs batch may be more effective against bacteria than a freshly prepared sample, as also demonstrated by Kittler and collaborators,[144] thus dramatically exacerbating the issue of batch-to-batch variability. Another important guideline, based on the same argument, deals with the general design of antibacterial device,

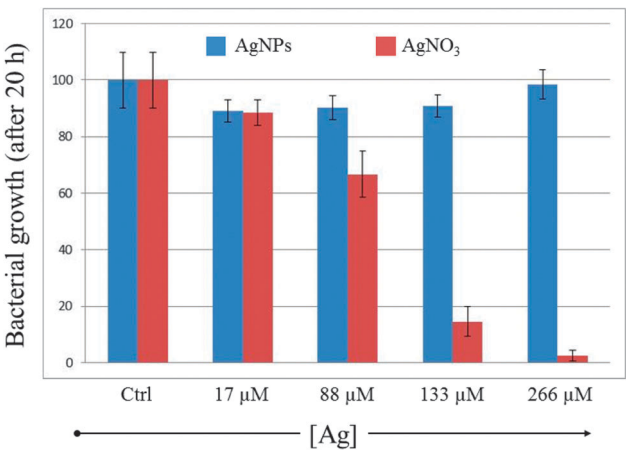

Fig. 9 Growth assays of *Escherichia coli* incubated with 20 ± 3 nm AgNPs (blue) and $AgNO_3$ (red).[143] 17, 88, 133, and 266 μM of silver correspond to 0.1, 0.5, 0.75, and 1 nM of 20 nm AgNPs. AgNPs were synthesized according to the method of Dadosh.[114]

exploiting silver or nanosilver. When projecting a specific application-tailored device, in fact, the required time-scale efficacy of antibacterial effects should be taken in strong consideration. The applications requiring an immediate high dose of antibacterial compounds should be designed with silver salts, which enable to strongly hinder the fast growth of microorganisms at the early stage. Alternatively, for a controlled long-term release of Ag^+ ions, AgNPs are preferential candidates, also because they can be finely engineered (by means of specific surface functionalization) in order to tune the kinetics of ion release. In this framework, AgNPs represent a sort of silver ions "pool" that can be delivered within precise body compartments and even within intracellular organelles (*e.g.*, vacuole containing pathogens, where several intracellular microorganisms proliferate), thus paving the way to their exploitation for the treatment of chronic infections related to persistent microorganisms. Furthermore, some specific medical devices, *e.g.*, for implantology, may significantly improve their performance by combining the two silver forms, namely ions and NPs. Such hybrid tools may have, in fact, the advantage of (i) an immediate source of Ag^+, which may, for instance, hinder the adhesion and colonization of bacteria (thus avoiding the formation and development of severe biofilm-related infections), and (ii) a slow and controlled long-term delivery of small amount of silver ions, from the NPs. Furthermore, it should be mentioned that AgNPs have some intrinsic positive characteristics that Ag^+ miss, thus making them good candidates for the development of innovative antibacterial drugs. AgNPs possess, in fact, a significant Trojan-horse behavior, which leads to a stronger internalization within (infected) cells and organisms with respect to salts/ions. At the same time, they may have the additional advantage of precise cellular targeting, upon surface functionalization. Moreover, unlike the classical tests which are carried out in solution (*i.e.*, model assays), in real conditions (*i.e.*, in *in vivo* experiments) NPs lead to higher effective dose as compared to Ag^+ ions.

Bacterial resistance to silver: a new worrying topic?

In the Introduction we mentioned that the indiscriminate use of antibiotics in the last decades has led to a drastic increase in bacterial antibiotic resistance. Similarly, bacteria are likely developing molecular strategies to resist also to silver/nanosilver, since it is increasingly used in a great number of commercial and medical tools. Usually, the bacterial resistance mechanisms to toxicants are encoded by specific DNA sequences that are present in non-chromosomal genetic material, named plasmids. Surprisingly, microbiologists revealed that some particular strains of *E. coli* (*i.e.*, K-12, and O157:H7) display a specific chromosomal gene cluster, named *sil*, which codifies for several proteins responsible for heavy metal resistance, and particularly for silver.[145–147] The *sil* cluster consists of nine genes encoding for two efflux system proteins, SilCBA and SilP, whose molecular action is combined with two other periplasmic silver binding proteins, namely SilE and SilF.[146] In particular, the SilCBA proteins complex (consisting of the outer membrane SilC, the inner membrane SilA, and the SilB, that links SilC and SilA together), acts as an antiporter: it pumps Ag^+ ions from the cytoplasm out of the cell, while pumping a H^+ from outside to inside the cell. The SilC proteins, at the same time, directly transport Ag^+ ions within the periplasmic space, thus acting as a P-type ATPase. Herein, the silver binding proteins SilE and SilF (a sort of molecular chaperones) complex the free Ag^+ ions, and transport them up to the SilCBA complex, that continues the ejection process.[146] However, it should be noted that both these resistance mechanisms are mainly devoted to counteract the action of intracellular silver ions, whilst they cannot prevent or repair direct membrane damage by Ag^+. On the other side, experimental evidences underlined that the bacterial resistance and sensitivity to silver are strictly dependent on the overall bioavailability of Ag^+. Hence, changes in environmental conditions may alter the ions availability and, in turn, the bacterial resistance/sensitivity. Gupta and coworkers demonstrated, in fact, that the chloride (Cl^-), bromide (Br^-), and iodide (I^-) halide anions may complex the Ag^+ ions in different ways, in both liquid and solid culture media, leading to the formation of silver salts (in the form of precipitates/clusters) or ionic water-soluble complexes, depending on the halides concentration.[148] Thus, when the main product is the water insoluble AgCl, an overall decrease in the bioavailability of free Ag^+ was found, which resulted in an increase in silver resistance.[148] On the other side, when the culture medium is composed of high amount of halides, the formation of different water soluble complexes, such as AgX_2^- and AgX_3^{2-} (where X is Cl, Br, or I) occurs. As a consequence, the water-soluble Ag–halide ionic complexes might have improved access to the cell membrane, resulting in an increased bioavailability of Ag^+, which finally increases the sensitivity of both Ag-resistant and Ag-sensitive bacteria.[148] In this framework, any large-scale synthesis approach of AgNPs as antimicrobial agents should take into account the possibility of a chemical conjugation with specific molecular inhibitors of the Ag^+ pump proteins, akin to the strategies already developed for some commercial antibacterial agents, as in the case of the combined action of amoxicillin (β-lactam antibiotic) and clavulanic acid (inhibitor of the bacterial β-lactamase that degrade the β-lactam nucleus).

Additionally, we would like to mention that the silver resistance phenomena may be transferred from the Enterobacteriaceae (the first microorganisms demonstrated to exhibit silver resistance) to other more hazardous families, such as the Neisseriaceae or Staphylococcaceae. Such perspective could represent a serious epidemiologic concern, especially for hospitalized patients, where the opportunistic pathogens related infections are one of the first causes of death.

Potential issues to humans and the environment

As discussed in the introduction section, the current worldwide production of nanosilver for commercial applications is *ca.* 320 tons per year. Hence, also the release of silver in the environment (in the form of ions, clusters, and micro/nanoparticulate) is constantly rising, and it was esteemed to be *ca.* 20 tons per year.[149] However, also in this framework, contrasting opinions on the potential risks have been reported.[150–156] For instance, functional eco-toxicogenomic

investigations on the nematode *Caenorhabditis elegans* highlighted the strong toxicity of AgNPs when released in the soil environment. In this study, the authors demonstrated that AgNPs dramatically decreased the reproduction potential of *C. elegans*, and increased the expression of the superoxide dismutases-3 (Sod-3), a marker for oxidative stress.[157] Similar results were obtained with other organisms, such as the green alga *Chlamydomonas reinhardtii*,[67] or zebrafish models.[158] However, the question whether Ag^+ ions or AgNPs represent serious concerns for ecological niches (including algae, plants, and fungi) remains to be fully elucidated. Ecotoxicity investigations have been, in fact, typically carried out in laboratory-like conditions, which are quite far from real situations, where the physicochemical properties of silver-based materials are almost unpredictable. For instance, it should be considered that the NPs surface characteristics and dispersion status may be strongly affected upon release in the various environmental matrices, and that after dispersion in the sea, river, or soil, silver may be transformed and stored as AgCl or Ag_2S precipitates.[159–161] Therefore, ecotoxicology assessment of the potential impact of nanosilver may be even more difficult to explore, due to the intrinsic variability of the materials when released in the environment. In addition, the rising concentration of silver as environmental pollutant is also increasing the chance for exposure to humans, especially by dermal contact and inhalation. Hence, also the nanotoxicity assessment of AgNPs is gaining great interest.[162,163] After absorption, in fact, AgNPs may accumulate in tissue and organs such as the skin, liver, lung, kidney, and the bloodstream, causing adverse effects.[164–168] In particular, AgNPs have been demonstrated to induce cell death and oxidative stress in human skin carcinoma and fibrosarcoma cells, and to cause DNA damage and apoptosis in fibroblasts and liver cells.[168,169] A quite commonly accepted molecular mechanism of AgNPs toxicity to eukaryotic cells includes reduced mitochondrial function, increased LDH leakage, depletion of GSH level, apoptosis, ROS generation and DNA fragmentation.[169–178] AgNPs may interact and unfold/inactivate, like in the case of microorganisms, sulfur-containing proteins, and especially thioredoxin, superoxide dismutases, and GSH. However, also in this case, the available data cannot lead to definitive conclusions about the nanosilver toxicology potential and related molecular mechanisms, as experiments suffer from several limitations, such as data variability/irreproducibility along with lack of NP reference materials and standardized protocols and assays (*e.g.*, standard operating procedures, SOPs, for NPs characterization and dispersion in biological media for *in vitro* tests). This also results in the unfeasibility to achieve a comprehensive risk assessment. As a consequence, there are also important regulation problems: for instance, in late 2011 the European Commission asked SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks) to provide a conclusive report about AgNPs toxicity,[179] despite some researchers strongly asking for prompt regulatory measures by EU rather than further research and analyses.[150] In this framework, it is worth mentioning that some problems also originate from difficulties in giving a correct definition of nanoparticles by regulatory bodies,[180] which is a fundamental aspect to be considered in future research efforts. The exploitation of standardized nanomaterials and assays are probably pivotal for correctly relating the physicochemical properties of NPs with their biological outcomes and, thus, with any consequent adverse effects.

Concluding remarks

In conclusion, the results on the bactericidal effects of AgNPs are certainly suggesting their further exploitation as a new class of antibacterial agents. The available data, in fact, demonstrated that nanosilver has an enhanced broad-range activity against bacteria, representing a promising opportunity for pharma and nanotech industries. The biocidal properties of AgNPs have been proposed to differently depend on their physico-chemical properties, namely their size, shape, and surface charge. However, very recent findings disclosed that the most important factor is the nanoparticles capability to release silver ions, which have been deemed as the real cause of toxicity to bacteria. In this framework, the possibility to engineer AgNPs in order to finely tune the Ag^+ release phenomena, as well as to control the delivery process, may represent a powerful route to fabricate innovative antibacterial drugs and hybrid nanocomposites. On the other side, many crucial issues have not been yet solved, and much effort should be focused towards the definition of standardized procedures and materials, and a comprehensive understanding of how AgNPs interact with bacteria at a molecular level. Beyond the above considerations, it should be mentioned that nanosilver may represent a source of toxicity to humans and the environment, and specific nanoregulation, as well as clinical and ecological monitoring, should be developed. At the same time, the potential bacterial resistance to silver deserves serious attention.

References

1 E. M. Bik, P. B. Eckburg, S. R. Gill, K. E. Nelson, E. A. Purdom, F. Francois, G. Perez-Perez, M. J. Blaser and D. A. Relman, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 732–737.
2 L. Dethlefsen, M. McFall-Ngai and D. A. Relman, *Nature*, 2007, **449**, 811–818.
3 Z. Pei, E. J. Bini, L. Yang, M. Zhou, F. Francois and M. J. Blaser, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 4250–4255.
4 M. J. Blaser and D. Kirschner, *Nature*, 2007, **449**, 843–849.
5 L. V. Hooper, *Nat. Rev. Microbiol.*, 2009, **7**, 367–374.
6 J. H. Powers, *Clin. Microbiol. Infect.*, 2004, **10**(S4), 23–31.
7 B. Spellberg, *Clin. Infect. Dis.*, 2008, **47**, 294.
8 B. Spellberg, R. Guidos, D. Gilbert, J. Bradley, H. W. Boucher, W. M. Scheld, J. G. Bartlett and J. Edwards Jr, *Clin. Infect. Dis.*, 2008, **46**, 155–164.
9 A. S. Fauci, *Clin. Infect. Dis.*, 2001, **32**, 675–685.
10 A. S. Fauci, *Acad. Med.*, 2005, **80**, 1079–1085.
11 D. M. Morens, G. K. Folkers and A. S. Fauci, *Nature*, 2004, **430**, 242–249.

12 D. I. Andersson and D. Hughes, *Nat. Rev. Microbiol.*, 2010, **8**, 260–271.
13 B. R. Levin and D. E. Rozen, *Nat. Rev. Microbiol.*, 2006, **4**, 556–562.
14 M. J. Schwaber, T. De-Medina and Y. Carmeli, *Nat. Rev. Microbiol.*, 2004, **2**, 979–983.
15 S. B. Levy and B. Marshall, *Nat. Med.*, 2004, **10**, S122–S129.
16 H. W. Boucher, *Clin. Infect. Dis.*, 2010, **50**, S4–S9.
17 H. W. Boucher, G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg and J. Bartlett, *Clin. Infect. Dis.*, 2009, **48**, 1–12.
18 G. Taubes, *Science*, 2008, **321**, 356–361.
19 H. Kresse, M. J. Belsey and H. Rovini, *Nat. Rev. Drug Discovery*, 2007, **6**, 19–20.
20 S. J. Projan and D. M. Shlaes, *Clin. Microbiol. Infect.*, 2004, **10**, 18–22.
21 B. Spellberg, J. H. Powers, E. P. Brass, L. G. Miller and J. E. Edwards Jr, *Clin. Infect. Dis.*, 2004, **38**, 1279–1286.
22 L. B. Rice, *J. Infect. Dis.*, 2008, **197**, 1079–1081.
23 S. Y. Liau, D. C. Read, W. J. Pugh, J. R. Furr and A. D. Russell, *Lett. Appl. Microbiol.*, 1997, **25**, 279–283.
24 H. J. Klasen, *Burns*, 2000, **26**, 131–138.
25 J. J. Buckley, A. F. Lee, L. Olivi and K. Wilson, *J. Mater. Chem.*, 2010, **20**, 8056–8063.
26 E. Fee, *Science*, 1990, **249**, 305.
27 J. A. Dahl, B. L. Maddux and J. E. Hutchison, *Chem. Rev.*, 2007, **107**, 2228–2269.
28 B. Nowack, H. F. Krug and M. Height, *Environ. Sci. Technol.*, 2011, **45**, 1177–1183.
29 A. Kumar, P. K. Vemula, P. M. Ajayan and G. John, *Nat. Mater.*, 2008, **7**, 236–241.
30 C. Marambio-Jones and E. M. V. Hoek, *J. Nanopart. Res.*, 2010, **12**, 1531–1551.
31 H. S. Jia, W. S. Hou, L. Q. Wei, B. S. Xu and X. G. Liu, *Dent. Mater.*, 2008, **24**, 244–249.
32 A. G. Skirtach, A. M. Javier, O. Kreft, K. Kohler, A. P. Alberola, H. Mohwald, W. J. Parak and G. B. Sukhorukov, *Angew. Chem., Int. Ed.*, 2006, **45**, 4612–4617.
33 R. E. Weisbarth, *Optometry Vision Sci.*, 2007, **84**, 2–3.
34 P. Podsiadlo, S. Paternel, J. M. Rouillard, Z. F. Zhang, J. Lee, J. W. Lee, L. Gulari and N. A. Kotov, *Langmuir*, 2005, **21**, 11915–11921.
35 V. Alt, T. Bechert, P. Steinrucke, M. Wagener, P. Seidel, E. Dingeldein, E. Domann and R. Schnettler, *Biomaterials*, 2004, **25**, 4383–4391.
36 W. Chen, Y. Liu, H. S. Courtney, M. Bettenga, C. M. Agrawal, J. D. Bumgardner and J. L. Ong, *Biomaterials*, 2006, **27**, 5512–5517.
37 K. C. Bhol, J. Alroy and P. J. Schechter, *Clin. Exp. Dermatol.*, 2004, **29**, 282–287.
38 K. C. Bhol and P. J. Schechter, *Br. J. Dermatol.*, 2005, **152**, 1235–1242.
39 J. L. Elechiguerra, J. L. Burt, J. R. Morones, A. Camacho-Bragado, X. Gao, H. H. Lara and M. J. Yacaman, *J. Nanobiotechnol.*, 2005, **3**, 6.
40 K. C. Bhol and P. J. Schechter, *Dig. Dis Sci.*, 2007, **52**, 2732–2742.
41 R. W. Sun, R. Chen, N. P. Chung, C. M. Ho, C. L. Lin and C. M. Che, *Chem. Commun.*, 2005, 5059–5061.
42 Y. Li, P. Leung, L. Yao, Q. W. Song and E. Newton, *J. Hosp. Infect.*, 2006, **62**, 58–63.
43 R. Bayston, W. Ashraf and L. Fisher, *J. Hosp. Infect.*, 2007, **65**(Suppl 2), 39–42.
44 K. Galiano, C. Pleifer, K. Engelhardt, G. Brossner, P. Lackner, C. Huck, C. Lass-Florl and A. Obwegeser, *Neurol. Res.*, 2008, **30**, 285–287.
45 S. Eckhardt, P. S. Brunetto, J. Gagnon, M. Priebe, B. Giese and K. M. Fromm, *Chem. Rev.*, 2013, **113**, 4708–4754.
46 S. Chernousova and M. Epple, *Angew. Chem., Int. Ed.*, 2013, **52**, 1636–1653.
47 M. J. Hajipour, K. M. Fromm, A. A. Ashkarran, D. J. de Aberasturi, I. R. de Larramendi, T. Rojo, V. Serpooshan, W. J. Parak and M. Mahmoudi, *Trends Biotechnol.*, 2012, **30**, 499–511.
48 J. A. Lemire, J. J. Harrison and R. J. Turner, *Nat. Rev. Microbiol.*, 2013, **11**, 371–384.
49 J. R. Morones, J. L. Elechiguerra, A. Camacho, K. Holt, J. B. Kouri, J. T. Ramirez and M. J. Yacaman, *Nanotechnology*, 2005, **16**, 2346–2353.
50 O. Choi and Z. Q. Hu, *Environ. Sci. Technol.*, 2008, **42**, 4583–4588.
51 O. Choi, K. K. Deng, N. J. Kim, L. Ross, R. Y. Surampalli and Z. Q. Hu, *Water Res.*, 2008, **42**, 3066–3074.
52 I. Sondi and B. Salopek-Sondi, *J. Colloid Interface Sci.*, 2004, **275**, 177–182.
53 A. M. El Badawy, R. G. Silva, B. Morris, K. G. Scheckel, M. T. Suidan and T. M. Tolaymat, *Environ. Sci. Technol.*, 2011, **45**, 283–287.
54 S. Pal, Y. K. Tak and J. M. Song, *Appl. Environ. Microbiol.*, 2007, **73**, 1712–1720.
55 C. N. Lok, C. M. Ho, R. Chen, Q. Y. He, W. Y. Yu, H. Z. Sun, P. K. H. Tam, J. F. Chiu and C. M. Che, *J. Proteome Res.*, 2006, **5**, 916–924.
56 J. S. Kim, E. Kuk, K. N. Yu, J. H. Kim, S. J. Park, H. J. Lee, S. H. Kim, Y. K. Park, Y. H. Park, C. Y. Hwang, Y. K. Kim, Y. S. Lee, D. H. Jeong and M. H. Cho, *J. Nanomed. Nanotechnol.*, 2007, **3**, 95–101.
57 M. Raffi, F. Hussain, T. M. Bhatti, J. I. Akhter, A. Hameed and M. M. Hasan, *J. Mater. Sci. Technol.*, 2008, **24**, 192–196.
58 A. B. Smetana, K. J. Klabunde, G. R. Marchin and C. M. Sorensen, *Langmuir*, 2008, **24**, 7457–7464.
59 L. Kvitek, A. Panacek, J. Soukupova, M. Kolar, R. Vecerova, R. Prucek, M. Holecova and R. Zboril, *J. Phys. Chem. C*, 2008, **112**, 5825–5834.
60 S. Mohanty, S. Mishra, P. Jena, B. Jacob, B. Sarkar and A. Sonawane, *Nanomedicine*, 2012, **8**, 916–924.
61 K. P. Bankura, D. Maity, M. M. R. Mollick, D. Mondal, B. Bhowmick, M. K. Bain, A. Chakraborty, J. Sarkar, K. Acharya and D. Chattopadhyay, *Carbohydr. Polym.*, 2012, **89**, 1159–1165.

62 G. K. Vertelov, Y. A. Krutyakov, O. V. Efremenkova, A. Y. Olenin and G. V. Lisichkin, *Nanotechnology*, 2008, **19**, 355707.
63 K. J. Kim, W. S. Sung, S. K. Moon, J. S. Choi, J. G. Kim and D. G. Lee, *J. Microbiol. Biotechnol.*, 2008, **18**, 1482–1484.
64 K. J. Kim, W. S. Sung, B. K. Suh, S. K. Moon, J. S. Choi, J. Kim and D. G. Lee, *Biometals*, 2009, **22**, 235–242.
65 J. Kim, J. Lee, S. Kwon and S. Jeong, *J. Nanosci. Nanotechnol.*, 2009, **9**, 1098–1102.
66 L. Sun, A. K. Singh, K. Vig, S. R. Pillai and S. R. Singh, *J. Biomed. Nanotechnol.*, 2008, **4**, 149–158.
67 E. Navarro, F. Piccapietra, B. Wagner, F. Marconi, R. Kaegi, N. Odzak, L. Sigg and R. Behra, *Environ. Sci. Technol.*, 2008, **42**, 8959–8964.
68 L. Lu, R. W. Y. Sun, R. Chen, C. K. Hui, C. M. Ho, J. M. Luk, G. K. K. Lau and C. M. Che, *Antiviral Ther.*, 2008, **13**, 253–262.
69 C. Baker, A. Pradhan, L. Pakstis, D. J. Pochan and S. I. Shah, *J. Nanosci. Nanotechnol.*, 2005, **5**, 244–249.
70 A. Panáček, L. Kvítek, R. Prucek, M. Kolář, R. Večeřová, N. Pizúrová, V. K. Sharma, T. j. Nevěčná and R. Zbořil, *J. Phys. Chem. B*, 2006, **110**, 16248–16253.
71 W. R. Li, X. B. Xie, Q. S. Shi, S. S. Duan, Y. S. Ouyang and Y. B. Chen, *Biometals*, 2011, **24**, 135–141.
72 S. Sadhasivam, P. Shanmugam and K. Yun, *Colloids Surf., B*, 2010, **81**, 358–362.
73 K. Kathiresan, N. M. Alikunhi, S. Pathmanaban, A. Nabikhan and S. Kandasamy, *Can. J. Microbiol.*, 2010, **56**, 1050–1059.
74 Z. M. Xiu, Q. B. Zhang, H. L. Puppala, V. L. Colvin and P. J. J. Alvarez, *Nano Lett.*, 2012, **12**, 4271–4275.
75 J. Y. Liu and R. H. Hurt, *Environ. Sci. Technol.*, 2010, **44**, 2169–2175.
76 R. G. Pearson, *J. Am. Chem. Soc.*, 1963, **85**, 3533–3539.
77 R. G. Parr and R. G. Pearson, *J. Am. Chem. Soc.*, 1983, **105**, 7512–7516.
78 N. C. Kasuga, Y. Takagi, S.-i. Tsuruta, W. Kuwana, R. Yoshikawa and K. Nomiya, *Inorg. Chim. Acta*, 2011, **368**, 44–48.
79 A. G. Orpen, L. Brammer, F. H. Allen, O. Kennard, D. G. Watson and R. Taylor, *J. Chem. Soc., Dalton Trans.*, 1989, S1–S83.
80 T. Shoeib, K. W. M. Siu and A. C. Hopkinson, *J. Phys. Chem. A*, 2002, **106**, 6121–6128.
81 M. E. Kamwaya, E. Papavinasam, S. G. Teoh and R. K. Rajaram, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 1984, **40**, 1318–1320.
82 K. Nomiya, S. Takahashi, R. Noguchi, S. Nemoto, T. Takayama and M. Oda, *Inorg. Chem.*, 2000, **39**, 3301–3311.
83 K. Nomiya and H. Yokoyama, J. Chem. Soc., *Dalton Trans.*, 2002, 2483–2490.
84 J. Jover, R. Bosque and J. Sales, *Dalton Trans.*, 2008, 6441–6453.
85 J. s. Jover, R. n. Bosque and J. Sales, *J. Phys. Chem. A*, 2009, **113**, 3703–3708.
86 V. W. M. Lee, H. Li, T.-C. Lau, R. Guevremont and K. W. M. Siu, *J. Am. Soc. Mass Spectrom.*, 1998, **9**, 760–766.
87 N. C. Kasuga, R. Yoshikawa, Y. Sakai and K. Nomiya, *Inorg. Chem.*, 2012, **51**, 1640–1647.
88 N. C. Kasuga, M. Sato, A. Amano, A. Hara, S. Tsuruta, A. Sugie and K. Nomiya, *Inorg. Chim. Acta*, 2008, **361**, 1267–1273.
89 C. B. Acland and H. C. Freeman, *J. Chem. Soc. D*, 1971, 1016–1017.
90 L. Mirolo, T. Schmidt, S. Eckhardt, M. Meuwly and K. M. Fromm, *Chem.–Eur. J.*, 2013, **19**, 1754–1761.
91 N. A. Amro, L. P. Kotra, K. Wadu-Mesthrige, A. Bulychev, S. Mobashery and G. Y. Liu, *Langmuir*, 2000, **16**, 2789–2796.
92 S. Y. Liau, D. C. Read, W. J. Pugh, J. R. Furr and A. D. Russell, *Lett. Appl. Microbiol.*, 1997, **25**, 279–283.
93 H. G. Petering, *Pharmacol. Ther., Part A*, 1976, **1**, 127–130.
94 P. Dibrov, J. Dzioba, K. K. Gosink and C. C. Hase, *Antimicrob. Agents Chemother.*, 2002, **46**, 2668–2670.
95 K. B. Holt and A. J. Bard, *Biochemistry*, 2005, **44**, 13214–13223.
96 H. Arakawa, J. F. Neault and H. A. Tajmir-Riahi, *Biophys. J.*, 2001, **81**, 1580–1587.
97 W. J. Yang, C. C. Shen, Q. L. Ji, H. J. An, J. J. Wang, Q. D. Liu and Z. Z. Zhang, *Nanotechnology*, 2009, **20**, 085102.
98 Q. L. Feng, J. Wu, G. Q. Chen, F. Z. Cui, T. N. Kim and J. O. Kim, *J. Biomed. Mater. Res.*, 2000, **52**, 662–668.
99 W. K. Jung, H. C. Koo, K. W. Kim, S. Shin, S. H. Kim and Y. H. Park, *Appl. Environ. Microbiol.*, 2008, **74**, 2171–2178.
100 E. T. Hwang, J. H. Lee, Y. J. Chae, Y. S. Kim, B. C. Kim, B. I. Sang and M. B. Gu, *Small*, 2008, **4**, 746–750.
101 M. R. Ciriolo, P. Civitareale, M. T. Carrì, A. De Martino, F. Galiazzo and G. Rotilio, *J. Biol. Chem.*, 1994, **269**, 25783–25787.
102 P. V. AshaRani, G. L. K. Mun, M. P. Hande and S. Valiyaveettil, *ACS Nano*, 2009, **3**, 279–290.
103 H. J. Park, J. Y. Kim, J. Kim, J. H. Lee, J. S. Hahn, M. B. Gu and J. Yoon, *Water Res.*, 2009, **43**, 1027–1032.
104 Y. Inoue, M. Hoshino, H. Takahashi, T. Noguchi, T. Murata, Y. Kanzaki, H. Hamashima and M. Sasatsu, *J. Inorg. Biochem.*, 2002, **92**, 37–42.
105 H. Le Pape, F. Solano-Serena, P. Contini, C. Devillers, A. Maftah and P. Leprat, *J. Inorg. Biochem.*, 2004, **98**, 1054–1060.
106 A. Nel, T. Xia, L. Madler and N. Li, *Science*, 2006, **311**, 622–627.
107 S. J. Stohs and D. Bagchi, *Free Radical Biol. Med.*, 1995, **18**, 321–336.
108 J. Imlay, S. Chin and S. Linn, *Science*, 1988, **240**, 640–642.
109 W. V. Sweeney and J. C. Rabinowitz, *Annu. Rev. Biochem.*, 1980, **49**, 139–161.
110 N. Khoroshilova, C. Popescu, E. Munck, H. Beinert and P. J. Kiley, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 6087–6092.
111 D. Mitra, S. J. George, Y. Guo, S. Kamali, S. Keable, J. W. Peters, V. Pelmenschikov, D. A. Case and S. P. Cramer, *J. Am. Chem. Soc.*, 2013, **135**, 2530–2543.

112 K. Keyer and J. A. Imlay, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 13635–13640.
113 F. C. Fang, *Nat. Rev. Microbiol.*, 2004, **2**, 820–832.
114 T. Dadosh, *Mater. Lett.*, 2009, **63**, 2236–2238.
115 C. Burda, X. B. Chen, R. Narayanan and M. A. El-Sayed, *Chem. Rev.*, 2005, **105**, 1025–1102.
116 M. A. Perez, R. Moiraghi, E. A. Coronado and V. A. Macagno, *Cryst. Growth Des.*, 2008, **8**, 1377–1383.
117 B. Chen, X. L. Jiao and D. R. Chen, *Cryst. Growth Des.*, 2010, **10**, 3378–3386.
118 H. Y. Liang, W. Z. Wang, Y. Z. Huang, S. P. Zhang, H. Wei and H. X. Xu, *J. Phys. Chem. C*, 2010, **114**, 7427–7431.
119 H. Wennemers, *J. Pept. Sci.*, 2012, **18**, 437–441.
120 K. Belser, T. Vig Slenters, C. Pfumbidzai, G. Upert, L. Mirolo, K. M. Fromm and H. Wennemers, *Angew. Chem., Int. Ed.*, 2009, **48**, 3661–3664.
121 G. Upert, F. Bouillère and H. Wennemers, *Angew. Chem., Int. Ed.*, 2012, **51**, 4231–4234.
122 R. R. Naik, S. J. Stringer, G. Agarwal, S. E. Jones and M. O. Stone, *Nat. Mater.*, 2002, **1**, 169–172.
123 A. R. Bassindale, A. Codina-Barrios, N. Frascione and P. G. Taylor, *Chem. Commun.*, 2007, 2956–2958.
124 R. Lévy, N. T. K. Thanh, R. C. Doty, I. Hussain, R. J. Nichols, D. J. Schiffrin, M. Brust and D. G. Fernig, *J. Am. Chem. Soc.*, 2004, **126**, 10076–10084.
125 A. H. Latham and M. E. Williams, *Langmuir*, 2006, **22**, 4319–4326.
126 S.-W. Kim, S. Kim, J. B. Tracy, A. Jasanoff and M. G. Bawendi, *J. Am. Chem. Soc.*, 2005, **127**, 4556–4557.
127 J. A. Dahl, B. L. S. Maddux and J. E. Hutchison, *Chem. Rev.*, 2007, **107**, 2228–2269.
128 S. Ray, A. K. Das and A. Banerjee, *Chem. Commun.*, 2006, 2816–2818.
129 P. Kshirsagar, S. S. Sangaru, M. A. Malvindi, L. Martiradonna, R. Cingolani and P. P. Pompa, *Colloids Surf., A*, 2011, **392**, 264–270.
130 D. I. Gittins and F. Caruso, *ChemPhysChem*, 2002, **3**, 110–113.
131 P. Graf, A. Mantion, A. Foelske, A. Shkilnyy, A. Mašić, A. F. Thünemann and A. Taubert, *Chem.–Eur. J.*, 2009, **15**, 5831–5844.
132 D. Walczyk, F. B. Bombelli, M. P. Monopoli, I. Lynch and K. A. Dawson, *J. Am. Chem. Soc.*, 2010, **132**, 5761–5768.
133 A. Lesniak, A. Campbell, M. P. Monopoli, I. Lynch, A. Salvati and K. A. Dawson, *Biomaterials*, 2010, **31**, 9511–9518.
134 M. P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. Baldelli Bombelli and K. A. Dawson, *J. Am. Chem. Soc.*, 2011, **133**, 2525–2534.
135 A. Lesniak, F. Fenaroli, M. P. Monopoli, C. Åberg, K. A. Dawson and A. Salvati, *ACS Nano*, 2012, **6**, 5845–5857.
136 G. Maiorano, S. Sabella, B. Sorce, V. Brunetti, M. A. Malvindi, R. Cingolani and P. P. Pompa, *ACS Nano*, 2010, **4**, 7481–7491.
137 J. Martinussen, C. Solem, A. K. Holm and P. R. Jensen, *Curr. Opin. Biotechnol.*, 2013, **24**, 124–129.
138 T. Yakushi and K. Matsushita, *Appl. Microbiol. Biotechnol.*, 2010, **86**, 1257–1265.
139 J. M. Zook, S. E. Long, D. Cleveland, C. L. A. Geronimo and R. I. MacCuspie, *Anal. Bioanal. Chem.*, 2011, **401**, 1993–2002.
140 R. I. MacCuspie, *J. Nanopart. Res.*, 2011, **13**, 2893–2908.
141 J. T. Seil and T. J. Webster, *Int. J. Nanomed.*, 2012, **7**, 2767–2781.
142 A. L. Holder, R. Goth-Goldstein, D. Lucas and C. P. Koshland, *Chem. Res. Toxicol.*, 2012, **25**, 1885–1892.
143 L. Rizzello and P. P. Pompa, unpublished data.
144 S. Kittler, C. Greulich, J. Diendorf, M. Köller and M. Epple, *Chem. Mater.*, 2010, **22**, 4548–4554.
145 S. Silver, L. T. Phung and G. Silver, *J. Ind. Microbiol. Biotechnol.*, 2006, **33**, 627–634.
146 A. Gupta, K. Matsui, J. F. Lo and S. Silver, *Nat. Med.*, 1999, **5**, 183–188.
147 A. Gupta, L. T. Phung, D. E. Taylor and S. Silver, *Microbiology*, 2001, **147**, 3393–3402.
148 A. Gupta, M. Maynes and S. Silver, *Appl. Environ. Microbiol.*, 1998, **64**, 5042–5045.
149 F. Gottschalk, T. Sonderer, R. W. Scholz and B. Nowack, *Environ. Sci. Technol.*, 2009, **43**, 9216–9222.
150 S. F. Hansen and A. Baun, *Nat. Nanotechnol.*, 2012, **7**, 409–411.
151 N. R. Panyala, E. M. Pena-Mendez and J. Havel, *J. Appl. Biomed.*, 2008, **6**, 117–129.
152 S. A. Blaser, M. Scheringer, M. MacLeod and K. Hungerbühler, *Sci. Total Environ.*, 2008, **390**, 396–409.
153 N. Musee, M. Thwala and N. Nota, *J. Environ. Monit.*, 2011, **13**, 1164–1183.
154 B. Nowack, J. F. Ranville, S. Diamond, J. A. Gallego-Urrea, C. Metcalfe, J. Rose, N. Horne, A. A. Koelmans and S. J. Klaine, *Environ. Toxicol. Chem.*, 2012, **31**, 50–59.
155 Y. Teow, P. V. Asharani, M. P. Hande and S. Valiyaveettil, *Chem. Commun.*, 2011, **47**, 7025–7038.
156 E. Navarro, A. Baun, R. Behra, N. B. Hartmann, J. Filser, A. J. Miao, A. Quigg, P. H. Santschi and L. Sigg, *Ecotoxicology*, 2008, **17**, 372–386.
157 J. Y. Roh, S. J. Sim, J. Yi, K. Park, K. H. Chung, D. Y. Ryu and J. Choi, *Environ. Sci. Technol.*, 2009, **43**, 3933–3940.
158 P. V. Asharani, Y. L. Wu, Z. Y. Gong and S. Valiyaveettil, *Nanotechnology*, 2008, **19**, 255102.
159 R. Kaegi, A. Voegelin, B. Sinnet, S. Zuleeg, H. Hagendorfer, M. Burkhardt and H. Siegrist, *Environ. Sci. Technol.*, 2011, **45**, 3902–3908.
160 B. Kim, C.-S. Park, M. Murayama and M. F. Hochella, *Environ. Sci. Technol.*, 2010, **44**, 7509–7514.
161 C. Levard, B. C. Reinsch, F. M. Michel, C. Oumahi, G. V. Lowry and G. E. Brown, *Environ. Sci. Technol.*, 2011, **45**, 5260–5266.
162 F. M. Christensen, H. J. Johnston, V. Stone, R. J. Aitken, S. Hankin, S. Peters and K. Aschberger, *Nanotoxicology*, 2010, **4**, 284–295.
163 M. Ahamed, M. S. AlSalhi and M. K. J. Siddiqui, *Clin. Chim. Acta*, 2010, **411**, 1841–1848.
164 Y.-M. Sue, J. Yu-Yun Lee, M.-C. Wang, T.-K. Lin, J.-M. Sung and J.-J. Huang, *Am. J. Kidney Dis.*, 2001, **37**, 1048–1051.

165 A. Wadhera and M. Fung, *Dermatol. Online J.*, 2005, **11**, 12.
166 S. Takenaka, E. Karg, C. Roth, H. Schulz, A. Ziesenis, U. Heinzmann, P. Schramel and J. Heyder, *Environ. Health Perspect.*, 2001, **109**, 547–551.
167 J. H. Sung, J. H. Ji, J. U. Yoon, D. S. Kim, M. Y. Song, J. Jeong, B. S. Han, J. H. Han, Y. H. Chung, J. Kim, T. S. Kim, H. K. Chang, E. J. Lee, J. H. Lee and I. J. Yu, *Inhalation Toxicol.*, 2008, **20**, 567–574.
168 S. Arora, J. Jain, J. M. Rajwade and K. M. Paknikar, *Toxicol. Appl. Pharmacol.*, 2009, **236**, 310–318.
169 S. Arora, J. Jain, J. M. Rajwade and K. M. Paknikar, *Toxicol. Lett.*, 2008, **179**, 93–100.
170 S. M. Hussain, K. L. Hess, J. M. Gearhart, K. T. Geiss and J. J. Schlager, *Toxicol. In Vitro*, 2005, **19**, 975–983.
171 S. M. Hussain, A. K. Javorina, A. M. Schrand, H. M. Duhart, S. F. Ali and J. J. Schlager, *Toxicol. Sci.*, 2006, **92**, 456–463.
172 L. Braydich-Stolle, S. Hussain, J. J. Schlager and M. C. Hofmann, *Toxicol. Sci.*, 2005, **88**, 412–419.
173 Y. H. Hsin, C. F. Chena, S. Huang, T. S. Shih, P. S. Lai and P. J. Chueh, *Toxicol. Lett.*, 2008, **179**, 130–139.
174 M. Ahamed, M. Karns, M. Goodson, J. Rowe, S. M. Hussain, J. J. Schlager and Y. L. Hong, *Toxicol. Appl. Pharmacol.*, 2008, **233**, 404–410.
175 C. Carlson, S. M. Hussain, A. M. Schrand, L. K. Braydich-Stolle, K. L. Hess, R. L. Jones and J. J. Schlager, *J. Phys. Chem. B*, 2008, **112**, 13608–13619.
176 E. J. Park, J. Yi, Y. Kim, K. Choi and K. Park, *Toxicol. in Vitro*, 2010, **24**, 872–878.
177 N. Lubick, *Environ. Sci. Technol.*, 2008, **42**, 8617.
178 T. H. Kim, M. Kim, H. S. Park, U. S. Shin, M. S. Gong and H. W. Kim, *J. Biomed. Mater. Res., Part A*, 2012, **100A**, 1033–1043.
179 http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_q_027.pdf.
180 M. Auffan, J. Rose, J. Y. Bottero, G. V. Lowry, J. P. Jolivet and M. R. Wiesner, *Nat. Nanotechnol.*, 2009, **4**, 634–641.

