

Nanosilver and the Microbiological Activity of the Particulate Solids versus the Leached Soluble Silver

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3 4 5 6 7 8 9 10	organic complexes. The work also highlights the unique oxidative stress- independent bacterial toxicity of silver salt. Taken together, the findings advocate that future enquiries on the antimicrobial potency and also importantly, the environmental and clinical impact of Ag NPs use, should pay attention to the potential bacterial toxicological responses to the undissolved Ag particulates, rather than just to the leaching of soluble silver. The findings also put into question the common use of silver salt as model material for evaluating bacterial toxicity of Ag NPs.
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37	still unresolved cytotoxicity origins. To date, research efforts have mostly described the antimicrobial
38	contribution from the leaching of soluble silver, while the undissolved solid Ag particulates are often
39	considered as being microbiologically inert, serving only as source of the cytotoxic Ag ions. Here, we
40	show the rapid stimulation of lethal cellular oxidative stress in bacteria by the presence of the undissolved
41	Ag particulates. The cytotoxicity characteristics are distinct from those arising from the leached soluble
42	Ag, the latter being locked in organic complexes. The work also highlights the unique oxidative stress-
43	independent bacterial toxicity of silver salt. Taken together, the findings advocate that future enquiries on
44	the antimicrobial potency and also importantly, the environmental and clinical impact of Ag NPs use,
45	should pay attention to the potential bacterial toxicological responses to the undissolved Ag particulates,
46	rather than just to the leaching of soluble silver. The findings also put into question the common use of
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Introduction

The rapid development in nanotechnology has seen inorganic nanomaterials such as nanosilver, copper oxide and zinc oxide, subjected to advanced physicochemical manipulation to exhibit powerful antimicrobial activity (Gunawan et al. 2009, 2011, 2013a, Hajipour et al. 2012). Among these materials, nanosilver (silver nanoparticles, Ag NPs) is currently one of the most commercialized due to its potent and broad-spectrum antimicrobial characteristics (Consumer Products Inventory – Project on Emerging Nanotechnologies). Along with applications as core or co-antimicrobial ingredients in wound dressings and internal catheters (Ge et al. 2014), Ag NPs have also been incorporated in an increasing array of consumer products (Deardorff 2014), ranging from personal care products, textiles and household appliances to food and beverages and even children's products (Benn et al. 2010, Quadros et al. 2013). The widespread use is despite the ill-defined antimicrobial mechanisms of Ag NPs, in particular the lack of knowledge regarding the origins of cytotoxicity. The controversy has been at least one of the underlying reasons for regulatory bodies to still classify and regulate Ag NPs as regular bulk silver. Therefore, the nanoparticles are subjected to the same reporting requirements, threshold levels and toxicity tests as bulk silver, despite the mounting evidence indicating differences in their antimicrobial potency and properties (Faunce and Watal 2010). The antimicrobial activity of Ag NPs is influenced by the particles' physicochemical characteristics (e.g. size, shape, surface functional groups) as well as interactions with the particles' environment. In real-world settings of Ag NPs antimicrobial applications, the almost inevitable contact of the nanoparticles with aqueous environments, including those in the environment and in the human body, will lead to leaching of soluble silver species through oxidative dissolution of the silver metal (Trop et al. 2006, Benn and Westerhoff 2008, Liu et al. 2012, Sotiriou et al. 2012). Considerable research efforts have described the cytotoxic activity of the leached soluble silver on bacteria, even in their various forms, such as the soluble Ag(I)-chloride anionic complexes (Levard et al. 2013) and organo complexes (Gunawan et al. 2009), as a result of potential interactions of the released

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84	silver with the ubiquitous presence of halides (Cl ⁻ , Br ⁻ , I ⁻) and biomolecules in the environment
85	and in body fluids (Silver 2003, Liu et al. 2012, Eckhardt et al. 2013). Uncertainty however, still
86	lingers as to the bacterial toxicological responses to the undissolved Ag residue (Gunawan et al.
87	2009, Sotiriou and Pratsinis 2010, Xiu et al. 2012), that remains after leaching of silver. The
88	solid Ag particulates have been indicated to physically interact with cellular membranes of
89	bacteria (Sondi and Salopek-Sondi 2004, Mirzajani et al. 2011), but otherwise are often regarded
90	as being inert, indirectly contributing to the antimicrobial activity as a source of the cytotoxic Ag
91	ions. This view is inclusive of the hypothesized Trojan-horse type of Ag NPs cytotoxicity,
92	whereby leaching occurs intracellularly following uptake of particles, or, the suggested cell-
93	particle contact to cause additional leaching at the cell-particle interface and in turn, increasing
94	the uptake of Ag ions by bacteria (Lemire et al. 2013, Bondarenko et al. 2013). The elucidation
95	of the source of Ag NPs cytotoxicity will not only clarify the nanoparticles' 'true' antimicrobial
96	potency in real-world applications, but will also contribute to more accurate assessments of their
97	long-term impact on the environment and human health.
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99 Here, we investigated the origins of Ag NPs cytotoxicity through detailed investigations of 100 bacterial toxicological responses to the 'overall' presence of nanosilver (*i.e.* both leached soluble 101 Ag and Ag particulate residue are present in the systems), as compared to those of the 102 corresponding pre-leached filtered Ag leachate samples. Nanosilver in products can be in the forms of nano-sized Ag(I) or metallic Ag⁰ coated on or impregnated in support materials 103 104 (Gunawan et al. 2017). As model material, the current work used nanosilver in the form of nanosized Ag₂O deposits ($d_{TEM} = 2 \text{ nm}$ (Gunawan et al. 2009)) homogenously dispersed on the 105 106 surface of inert TiO₂ support (d_{TEM} = 30 nm (Gunawan et al. 2009)). It is noteworthy to point out that studies have observed discrepancies on the leaching behaviour as well as capability of 107 cellular oxidative stress stimulation of Ag(I) versus Ag⁰ nanoparticles (Gunawan et al. 2009, 108 109 Gunawan et al. 2013b). Nonetheless, the generated knowledge of cellular responses to the two 4

fundamental forms of nanosilver-derived microbiologically active components, that is, the leached soluble silver and the solid Ag particulates in the present study, is relevant to the countless nanosilver design with variation in the particle's properties (e.g. size, shape and oxidation states). This facile approach enables unambiguous elucidation of the source of nanoparticulate cytotoxicity without the need to employ simulation materials, such as soluble Ag salt (Gunawan et al. 2009, Sotiriou and Pratsinis 2010, Gunawan et al. 2011, Bondarenko et al. 2013, Ivask et al. 2014), which, as also shown in the current work, exhibits different cytotoxicity characteristics. We report cytotoxic activity of the solid Ag particulates on bacteria, distinct from the leached soluble silver.

120 Methods

121 Synthesis of Ag NPs and Preparation of Ag leachate from NPs

The 5 at% Ag/TiO₂ nanoparticles as finely dispersed Ag₂O on inert TiO₂ support were
synthesized using the flame spray pyrolysis (FSP) technique as earlier described (Gunawan et al.

124 2013b, note that at% refers to the percentage of Ag atom relative to the total number of atoms in

125 the particle). TEM images of the particles and XPS spectra that confirm the presence of silver (I)

126 oxide are available (Gunawan et al. 2009). The Ag-leachate was prepared by aseptically pre-

127 dissolving known amounts of Ag NPs (3, 6, 8, 10 mg Ag L⁻¹) in sterile Luria Bertani (LB) broth

128 (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl in deionized water) at 37°C, 280 rpm under

129 dark conditions for 6 h, unsonicated. The undissolved particulates (mean aggregate size = $1.09 \pm$

130 0.03 μm by dynamic light scattering (Gunawan et al. 2009)) were removed by centrifugation

- 131 (5,000 rpm) followed by filtration of the leachate with 0.22 μm polyethersulfone membrane
- 132 (Millipore Express). Comparable light scattering intensity of the filtered Ag leachate to that of
- 133 the filtered LB medium confirmed the removal of the solid Ag residue (data not shown). The
- 134 concentration of soluble silver in the filtered Ag leachate was determined by inductively coupled

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135	plasma mass spectrometry (ICP-MS) (Nexion 300D, PerkinElmer). ICP-MS analysis was also
136	performed on the undissolved Ag residue (3-4 h digestion with 70% (v/v) HNO ₃ to dissolve the
137	Ag solid). This Ag solid concentration (no cells) reflected, at least in approximation, the
138	presence of the undissolved Ag fraction in the nanoparticle-bacteria exposure systems (note the
139	comparable leaching of Ag NPs in the presence and absence of bacteria, Figure 1 and S1,
140	Supplementary Data). The ICP-MS analysis of the (digested) solid Ag residue and the
141	corresponding Ag leachate fractions (undigested) found that their concentrations added up
142	(within 10-15%) to the nominal total Ag concentrations of the nanoparticles (Figure S1). Finally,
143	the ICP-MS analysis of digested leachate samples found comparable Ag concentrations before
144	and after digestion, which further validated the removal of the solid Ag residue. Suspended
145	Ag/TiO ₂ particulates in the growth medium is expressed as mg L^{-1} to reflect their heterogeneous
146	presence, while the homogeneous nature of soluble Ag is referred to in ppm.

147

148 Bacterial Growth Studies with Ag NPs, Ag leachate and AgNO₃ salt

149 The growth experiments on Bacillus subtilis strain UNSW 448700 were carried out in triplicate 150 in LB culture medium at 37°C, 280 rpm under dark conditions for 6 h. To prepare the bacterial 151 inoculum, a single agar plate colony was cultured overnight at 30°C, 220 rpm in LB broth. A 152 measured volume of 1-2 mL of the overnight culture (typical OD₆₀₀ of 6-8) was transferred into 153 50 mL fresh LB broth for a further 0.5-1 h conditioning at 37°C, 280 rpm. For the Ag NPs and 154 AgNO₃ exposure, pre-weighed Ag NPs (1.1x of the intended dosage) and 0.5 mL (110x 155 concentrated of the intended dosage) solution of AgNO₃ were aseptically added into 50 mL and 156 49.5 mL LB respectively. The experiments were initiated by the addition of 5 mL bacterial 157 inoculum into the 50 mL broth containing suspended Ag NPs or dissolved silver salt (OD₆₀₀ bacteria initial = 0.04, corresponding to $\sim 2 \times 10^7$ cfu mL⁻¹). For the Ag leachate exposure, 5 mL 158 159 of the bacterial inoculum was added into 50 mL LB containing 1.1x concentrated pre-leached Ag

NPs (particle-free). The growth profiles were determined by OD_{600} measurement of the biomass (UV/Vis spectrophotometer, Hitachi U-1100) and the growth inhibiting effects were assessed relative to controls with no added silver. A cell-free silver control (particulates or soluble silver) was employed as a reference to obtain the OD_{600} corresponding to the bacteria. The corresponding leaching profile of Ag NPs during the bacterial exposure was measured by ICP-MS (Nexion 300D, PerkinElmer). For this purpose, a measured volume was sampled from the NPs-exposed culture, centrifuged (5,000 rpm) then filtered with the 0.22 μ m membrane to remove the bacteria and Ag solid. The resulting solution was 100x diluted in deionized water and subjected to the ICP-MS analysis.

170 Detection of Intracellular ROS and Cell Viability

The measurement of cellular ROS generation was performed using the cell permeable oxidative reporter dye H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Sigma-Aldrich). Following its uptake, cellular esterases cleave the diacetate moieties of H₂DCFDA to form H₂DCF, which readily transforms to the fluorescent DCF when reacts with ROS. The cell viability assay was based on the fluorescent nucleic acid dye propidium iodide (Sigma-Aldrich) staining. PI enters cells with damaged cytoplasmic membrane, while being excluded by healthy cells. Following removal of the culture medium by centrifugation, samples from the Ag NPs, Ag leachate and AgNO₃ exposure systems (and the silver-free controls) were washed and re-suspended in sterile saline (8 g L⁻¹ NaCl, 0.2 g L⁻¹ KCl) at 2.5 x 10⁸ CFU mL⁻¹. Independent cellular ROS and cell viability assays were carried out with 10 µM H₂DCFDA and 30 µM PI for 1 h and 15 min respectively, at room temperature under dark conditions. The stained cells were washed with saline and analysed by flow cytometry (FACSCanto[™] II, BD Bioscience) at 488 nm excitation with 530 nm and 670 nm emission filter settings for the detection of DCF and PI fluorescence respectively. DCF fluorescence was also measured using a microplate reader (EnsightTM) Multimode, Perkin Elmer) at 492 nm and 520 nm excitation and emission filter settings

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2 3	186	respectively. The stained cells were also visualized with a BX51WI fluorescence microscope
4 5	187	(Olympus) with 460–490 nm excitation filter settings.
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9 10	189	Results and discussion
11 12	190	Bacterial growth inhibition: Activity of the solid Ag particulates, the leached soluble Ag and
13 14	191	silver salt
15 16	192	To distinguish the cytotoxicity or antimicrobial contribution of the leached soluble Ag and the
17 18	193	undissolved Ag particulates, we exposed a model bacteria <i>B. subtilis</i> UNSW 448700 to $0 - 10$
19 20 21	194	mg Ag L^{-1} NPs (Ag/TiO ₂) and compared the bacterial growth to that of the corresponding
22 23	195	leachate-only systems, as a function of soluble silver detected in the exposure systems. The
24 25	196	leachate samples were prepared by aseptically pre-dissolving Ag NPs in culture medium
26 27	197	followed by removal of the solid Ag residue. Firstly, as shown in Figure 1a, the dose-response
28 29	198	growth inhibiting effects of the Ag NPs correlates with the increasing concentration of soluble
30 31	199	silver that leached from the NPs. The extent of growth of <i>B. subtilis</i> was reduced to ~80% upon
32 33 34	200	exposure to 3 mg Ag L ⁻¹ NPs (1.3 ppm silver leached into the culture medium at equilibrium)
35 36	201	relative to silver-free control cultures after 6 h. The control cultures were characterized by a
37 38	202	relatively short lag phase of 1 h, followed by 3 to 4 h active exponential growth phase before
39 40	203	entering the stationary phase at 6 h (Figure 1b). Increasing the NPs dosage to 6 mg Ag L^{-1} (2.7
41 42	204	ppm leached Ag) saw 50% bacterial growth, while almost complete growth suppression was
43 44	205	observed at MIC ₉₅ 10 mg Ag L ⁻¹ NPs exposure (4 ppm leached Ag, see Figure 1b for growth
45 46 47	206	profile, MIC ₉₅ is minimum inhibitory concentration that cause 5% growth relative to the control).
48 49	207	At all of the tested Ag NPs loading, leaching of Ag from NPs was rapid, with detection of ~70%
50 51	208	soluble Ag (relative to the leached Ag concentration detected at equilibrium) within 5 min of the
52 53	209	Ag NPs-bacterial exposure (see Figure 1c inset for leaching profile of 10 mg Ag L ⁻¹ NPs).
54 55	210	Equilibrium was reached in 1 h with the soluble Ag concentration remained constant afterwards,
56 57 58	211	indicating absence of the Ostwald ripening phenomenon that refers to re-deposition of the $\frac{8}{8}$
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212 leached Ag on larger particulates (Sotiriou et al. 2012). Increasing the Ag NPs loading saw 213 detection of elevated soluble Ag concentration at equilibrium, with the extent of leaching 214 essentially comparable at 38 - 40% relative to the total added Ag (Figure 1c). This is consistent 215 to earlier studies under comparable conditions (Gunawan et al. 2009, Sotiriou and Pratsinis 216 2010) with the relatively high degree of leaching was due to, at least in part, the presence of 217 organics in the culture medium as shown later in this study. Note that at all of the tested Ag NPs 218 loadings, similar extent of leaching were observed in the absence of bacteria, therefore excluding 219 the possibility of microbial-induced leaching of Ag (Figure S1).

220

221 Despite the correlation between Ag NPs growth inhibiting effects and Ag leaching, a 222 comparison with bacterial growth in the corresponding leachate-only systems yields an 223 interesting observation. Exposure of *B. subtilis* to the pre-leached soluble Ag in fact resulted in much less growth inhibition when compared to those of the corresponding Ag NPs samples 224 225 (Figure 1a). The presence of \sim 1.3 ppm Ag leachate for example, was benign to the cultures as 226 they grew to a similar extent as the silver-free control cultures after 6 h. This was in contrast to the ~20% growth reduction of the bacteria when exposed to the corresponding 3 mg Ag L^{-1} NPs 227 228 with comparable leached soluble Ag content. At higher exposure, the bacterial growth in 4 ppm 229 Ag leachate system was ~85% relative to the control cultures (refer to Figure 1b for growth 230 profile), in contrast to the near complete growth suppression observed in the corresponding 10 mg Ag L⁻¹ NPs system. Even doubling the concentration of Ag leachate to 8.3 ppm only slightly 231 232 reduced the bacterial growth to \sim 75%. The findings suggest predominant cytotoxicity 233 contribution from the undissolved Ag particulates, rather than that arising from the leached 234 soluble Ag. Further antimicrobial simulation with an equivalent concentration of soluble Ag 235 from AgNO₃ salt as shown in Figure 1a, saw more severe growth inhibiting activity of the salt. In 236 the presence of 4 ppm soluble Ag from AgNO₃ for example, $\sim 25\%$ B. subtilis growth was

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2 3	237	observed relative to the control cultures after 6 h (growth profile is shown in Figure 1b), in
4 5	238	contrast to the ~85% growth in the leachate system with comparable Ag concentration. Such
7 8	239	differences in cytotoxicity may arise from unique cellular physiological responses to the
9 10	240	different silver species; the leached soluble Ag and the undissolved Ag particulates from Ag
11 12	241	NPs, and the soluble silver from silver salt, as investigated in the following.
13 14	242	
15 16	243	Dynamic stimulation of cellular oxidative stress and cell death
17 18 19	244	We carried out dynamic tracking of intracellular reactive oxygen species (ROS) generation
20 21	245	(measured by H ₂ DCFDA assay) and cell viability (measured by propidium iodide assay,
22 23	246	whereby PI enters cells with damaged cytoplasmic membrane, which is indicative of cell death)
24 25	247	over the 6 h growth course of <i>B. subtilis</i> in the presence of the various forms of silver; the Ag
26 27	248	NPs (MIC ₉₅ 10 mg Ag L ⁻¹ as reference point, contained 4 ppm leached Ag), its corresponding
28 29	249	Ag leachate system (4 ppm Ag) and the equivalent AgNO ₃ system (4 ppm Ag).
30 31 22	250	
33 34	251	The solid Ag particulates and the leached soluble Ag
35 36	252	At 5 min exposure to 10 mg Ag L^{-1} NPs, a 3-fold higher cellular ROS level was detected in <i>B</i> .
37 38	253	subtilis relative to the basal ROS levels of the silver-free control cultures, which are by-products
39 40	254	of aerobic metabolism in bacteria (Choi and Hu 2008, Gunawan et al. 2011, Eckhardt et al.
41 42	255	2013) (Figure 2a, 2b, 4a). Within 30 min of Ag NPs exposure, the cellular ROS level doubled to
43 44 45	256	~6-fold of the control. A secondary oxidative stress response, the cellular ROS stimulation has
46 47	257	been increasingly realized as one of the major cellular toxicological responses to Ag NPs in
48 49	258	bacteria (Choi and Hu 2008, Hwang et al. 2008, Lemire et al. 2013, Gunawan et al. 2013b). The
50 51	259	ROS generation is thought to result from destruction of the iron-sulfur [4Fe-4S] clusters of
52 53	260	proteins by Ag metal (Xu and Imlay 2012, Lemire et al. 2013) and in turn, releasing the Fenton-
54 55	261	active free Fe into the cytoplasm for subsequent reaction with cellular H_2O_2 to produce hydroxyl
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262	radicals (OH [•]) (Imlay et al. 1988). Alternatively, indirect destruction of the iron-sulfur clusters
263	could result from inhibition of respiratory enzymes by Ag NPs in bacteria (Li et al. 2010, 2011).
264	The resulting premature leakage of electrons to oxygen will generate superoxide radicals (O_2^{\bullet})
265	(Imlay 2003) that in turn again, induces the release of free Fe from iron-sulfur clusters in
266	proteins (Kohanski et al. 2007). Indeed, there have been reports on the cytoplasmic presence of
267	the solid Ag particulates upon bacterial exposure to Ag NPs, as well as the presence of the solids
268	within the bacterial membrane layers (Morones et al. 2005, Grigor'eva et al. 2013, Pal et al.
269	2007). Here, 75-90% PI-positive non-viable bacteria had been detected within 5 to 30 min
270	exposure to Ag NPs, then close to 100% bactericidal or cell death toxicity at as early as 1 h
271	exposure (Figure 2a, 2b, 4b), which indicates cytoplasmic membrane as one of the target
272	destruction sites of the Ag NPs-stimulated cellular ROS (1-8% non-viable cells were detected in
273	the control cultures over the 6 h growth course) (D'Autreaux et al. 2007, Lemire et al. 2013). As
274	expected, the levels of cellular ROS drastically dropped following the rapid high level
275	stimulation, with the majority if not all of the bacterial population were already killed (Sintubin
276	et al. 2011, Gunawan et al. 2013b). Up to this stage, the data suggest that the generation of high
277	levels of cellular ROS and associated bacteria killing was likely to be responsible for the near
278	complete suppression of <i>B. subtilis</i> growth (Figure 1a, 1b).
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Interestingly, such cellular ROS stimulation was absent in the bacteria when studied in the corresponding 4 ppm Ag leachate system. Over the 6 h growth course, only basal ROS levels, comparable to those of the silver-free control cultures were detected (Figure 2a, 3a, 4a) and not surprisingly, the little to no changes in the fraction of non-viable cells relative to the control (Figure 2a, 3a, 4b). The stimulation of lethal levels of cellular oxidative stress by the presence of solid Ag particulates therefore suggests their substantial contribution to the cytotoxicity effects observed in the growth studies. Recalling the observed ~15% growth inhibition of the bacteria in

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the presence of 4 ppm Ag leachate (Figure 1a, 1b), it would be reasonable to deduce that the exposure only resulted in sub-lethal cytotoxicity, causing a minor fraction of the viable cells uncultivable or slowly proliferating, as further indicated by our growth prediction based on the fraction of viable cells (Figure S2). Indeed, doubling the Ag leachate concentration to 8.3 ppm still saw typical cellular ROS (Figure 3c, 4a inset) and dead cells (Figure 3c, 4b inset) detection as those of the control cultures, despite the slightly higher growth suppression, at ~25% (Figure 293 1a).

295 The leached soluble Ag and silver salt

The minimal cellular ROS stimulation was also seen upon exposure of *B. subtilis* to the equivalent 4 ppm soluble Ag from AgNO₃. Similar to the 4 ppm Ag leachate system, no elevated level of cellular ROS was observed over the 6 h growth course relative to the control cultures (Figure 2a, 3b, 4a). Unlike the leachate samples however, up to $\sim 40\%$ non-viable cells were detected in the salt system (Figure 3b, 4b), indicating attacks on cytoplasmic membrane (Eckhardt et al. 2013). Considering the comparable Ag content, such discrepancies in cytotoxicity are most likely to result from differences in the chemical speciation of the soluble silver, as herein described. Our Ag NPs leaching study (at the MIC₉₅ 10 mg Ag L⁻¹ NPs) in the individual culture medium components revealed a characteristic trend of complexation-assisted dissolution of nanoparticles (Gunawan et al. 2011), with higher extent of Ag leaching in the peptide-rich components, in particular tryptone (90% leaching relative to the total added Ag), compared to those in the deionized water (60% leaching) or NaCl (10% leaching) (Figure 4c). A soft Lewis acid, Ag(I) forms silver-peptide complexes upon its release from NPs (Bolea et al. 2014), which is most likely to result from its strong affinity to the NH_x donor groups of histidine (NH^+) , arginine $(-NH_2^+)$ and lysine $(-NH_3^+)$ amino acids and also to the thiol $(-S^-)$ donor groups of cysteine and methionine amino acids (Eckhardt et al. 2013). Silver-peptide complexes also form with $AgNO_3$ (Bolea et al. 2014), with a fraction of silver is thought to remain as free ions in

313	the organic-rich medium (Percival et al. 2005). Thermodynamically feasible, the co-existence of
314	free metal ions and organo metal complexes has been reported for the chemical speciation of
315	soluble copper salts, also a soft Lewis acid metal, in similar culture medium as that used here
316	(Gunawan et al. 2011) (note that the current technology for elemental analysis does not
317	differentiate free Ag ions to those locked in organo complexes (Eckhardt et al. 2013)). When
318	compared to free Ag ions, the hindered transport of the bulkier silver-peptide complexes into
319	bacteria (Solioz and Odermatt 1995) is thought to be at least in part, responsible for the
320	passivated, in this case, sub-lethal cytotoxicity of the Ag leachate. Unlike free Ag ions, research
321	indicates that soluble organo Ag complexes are not recognized by the P-type ATPase transporter
322	present in bacteria (Luoma 2008). As also observed in the current study with the AgNO ₃ systems,
323	exposure of bacteria to Ag ions has been reported to suppress their proliferation, which was
324	indicated to result from a ROS-independent inhibition of metabolic enzymes (dehydratases) (Xu
325	and Imlay 2012), the lack of cellular ROS stimulation also apparent in this work. Further,
326	complete suppression of <i>B. subtilis</i> growth was seen at 8.3 ppm Ag from AgNO ₃ (Figure 1a),
327	despite there being no change in the fraction of non-viable cells when compared to the 4 ppm Ag
328	exposure (Figure 3d, 4b inset). Our growth prediction based on the fraction of viable cells
329	indicates major presence of non- or slowly proliferating viable cells with the AgNO ₃ exposure
330	(Figure S2). This loss in replication could also result from the known interactions of Ag ions
331	with DNA in bacteria (most likely with the phosphorus moieties) causing DNA condensation
332	(Feng et al. 2000). The seemingly higher cytotoxic effects of Ag ions as compared to the organo
333	Ag complexes are in agreement with other bacterial studies, whereby extracellular presence of
334	thiol-containing reduced gluthathione (GSH) as silver complexing agent lowered the
335	antimicrobial activity of Ag ions on the Gram-positive Staphylococcus aureus and the Gram-
336	negative Escherichia coli and Pseudomonas aeruginosa (Mulley et al. 2014). Finally, the
337	detection of only basal cellular ROS levels in the AgNO3 exposure systems, even at the double
338	8.3 ppm Ag (Figure 3d, 4a inset), rules out the oxidative stress stimulation as the main
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3	339	mechanisms of AgNO ₃ cytotoxicity. Indeed, studies have found no differences in the
4 5 6	340	antimicrobial activity of Ag ions under aerobic and anaerobic conditions on bacteria (Sintubin et
7 8	341	al. 2011).
9 10	342	
11 12 12	343	Conclusions
13 14 15	344	Here, we report multiple cytotoxicity origins of Ag NPs towards bacteria. Presence of
16 17	345	undissolved Ag particulates in a biological environment is not inert. In their presence, rapid
18 19	346	generation of lethal cellular ROS levels were detected in bacteria, while the corresponding
20 21	347	leached soluble Ag, being locked in organo complexes, only imparts sub-lethal cytotoxicity. The
22	348	observed differences in bacterial toxicological responses to the solid versus soluble Ag
24 25 26	349	corroborate earlier reports on the distinct extent of growth inhibiting activity of the Ag NPs'
27 28	350	soluble and solid components (Gunawan et al. 2009, Sotiriou and Pratsinis 2010). With regard to
29 30	351	the widespread use of Ag NPs, the resolved unique toxicological responses are expected to result
31 32	352	in better recognition of the antimicrobial potency of the nanoparticles in real-world settings and
33 34	353	importantly, the long-term impact. Research inquiries have shown elevated and persistent
35 36 27	354	presence of silver in wounds, bladder and even in sewage and estuaries, being associated with
38 39	355	the intended or in some cases, accidental release from nanosilver applications; the use of wound
40 41	356	dressings, pesticides and washing machines are among the examples (Chen et al. 2004, Trop et al.
42 43	357	2006, Reidy et al. 2013, Donner et al. 2015, Beddow et al. 2017). The current findings imply
44 45	358	bacterial toxicological responses to not only the leached soluble Ag, but also the Ag particulates
46 47	359	in the microbial habitats. Indeed, studies have observed disruptions in the dynamic and balance
48 49	360	of microbial communities from natural aquatic waters upon exposure to nanosilver (Das et al.
50 51 52	361	2012, Beddow et al. 2017), with the work also detecting presence of soluble Ag and aggregates
53 54	362	of Ag from nanosilver in these environmental samples (Beddow et al. 2017). The resolved
55 56	363	toxicological responses is key to the elucidation of the recently discovered bacterial potential for
57 58 59	364	adaptation to Ag NPs cytotoxicity (Das et al. 2012, Gunawan et al. 2013b). Finally, the work 14

3	365	highlights the unsuitability of soluble silver salt as model material for Ag NPs cytotoxicity in
5	366	biological environments, noting a distinct ROS-independent antimicrobial characteristic of
7 8	367	soluble Ag when supplied as AgNO ₃ salt.
9 10	368	
11 12	369	Acknowledgments
13 14 15	370	This work was produced with the financial assistance of the Australian Research Council under
15 16 17	371	the ARC Australian Laureate Fellowship Program and the University of Technology Sydney
18 19	372	under the Chancellor's Postdoctoral Research Fellowship Program.
20 21	373	
22 23	374	Declaration of interest
24 25	375	The authors declare no conflict of interest.
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24 25	502	
26 27	503	Supplementary material is available: Supplementary Figure S1, S2 and S3.
28 29	504	
30 31 22	505	Figure captions
32 33 34	506	Figure 1. Bacterial growth in the presence of Ag NPs, Ag NPs leachate, silver salt and leaching
35 36	507	of Ag NPs. (a) Growth of <i>B. subtilis</i> (6 h) relative to cell-only control upon exposure to Ag NPs
37 38	508	(3, 6, 8, 10 mg Ag L^{-1}), Ag leachate from NPs and AgNO ₃ as a function of soluble silver
39 40	509	detected in the bacterial exposure systems (the growth studies were performed in LB medium).
41 42	510	(b) Growth profiles of the bacteria in the presence of 10 mg Ag L ⁻¹ NPs (4 ppm Ag leached into
43 44 45	511	medium at equilibrium), 4 ppm Ag leachate from NPs and 4 ppm Ag from AgNO ₃ . Also shown
45 46 47	512	is the cell-only control growth profile (dashed line). The growth in the presence of Ag was
48 49	513	normalised to the extent of growth of the control (in colony forming units, cfu). (c) The
50 51	514	corresponding equilibrium leaching of Ag NPs in the bacterial exposure systems, shown in the
52 53	515	inset is the leaching profile for 10 mg Ag L ⁻¹ NPs. Each data point in (a), (b), (c) is the average
54 55	516	of triplicate experiments with error bars representing the maximum and minimum values
56 57 58	517	detected. The growth studies were performed under dark conditions to render the TiO_2 support
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518 photocatalytically inactive and the benign effect of the TiO_2 support on *B. subtilis* growth had 519 been confirmed (Gunawan et al. 2013b). The growth studies were reproduced on different days 520 with unique bacterial inoculum and particle preparations.

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Figure 2. Detection of cellular reactive oxygen species (ROS, H₂DCFDA staining, green cells) and cell death (PI staining, red cells) of *B. subtilis* over its growth course: (a) cell-only control and (b) in the presence of 10 mg Ag L⁻¹ NPs. All stained samples were imaged at comparable cell concentrations (scale bars = 50 μ m).

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Figure 3. Detection of cellular reactive oxygen species (ROS, H₂DCFDA staining, green cells) and cell death (PI staining, red cells) of *B. subtilis* over its growth course, in the presence of: (a) 4 ppm Ag leachate from NPs (equivalent leachate to 10 mg Ag L⁻¹ NPs exposure), (b) 4 ppm Ag from AgNO₃, (c) 8.3 ppm Ag leachate from NPs and (d) 8.3 ppm Ag from AgNO₃. All stained samples were imaged at comparable cell concentrations (scale bars = 50 μ m).

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533 Figure 4. (a) Dynamic stimulation of cellular ROS in *B. subtilis* measured by H₂DCFDA assay over its growth course (5, 30 min and 1, 3, 4.5, 6 h) upon exposure to 10 mg Ag L⁻¹ NPs (4 ppm 534 535 Ag leached into medium at equilibrium), 4 ppm Ag leachate from NPs and 4 ppm Ag from 536 AgNO₃. The detected cellular ROS was normalised to the basal ROS levels of the cell-only 537 control growth. Shown in the inset is cellular ROS detected in the presence of 8.3 ppm Ag 538 leachate from NPs and 8.3 ppm Ag from AgNO₃. (b) The corresponding dynamic cell death 539 detection probed by PI staining of *B. subtilis* throughout its growth course. Also shown is the 540 fraction of dead cells detected for the cell-only control. Statistical analysis of the data was 541 performed with one-way ANOVA followed by Dunnett's posthoc analysis in Prism (GraphPad) 542 (Figure S3). The experiments in (a) and (b) were reproduced on different days with unique bacterial inoculum and particle preparations. (c) Leaching profile of $10 \text{ mg Ag L}^{-1} \text{ NPs}$ in cell-543 21

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2 3	544	free individual components of the Luria Bertani (LB) culture medium (5 g L^{-1} NaCl, 5 g L^{-1} yeast
4 5	545	extract, 10 g L ⁻¹ tryptone dissolved in deionized water). Each data point in (a), (b), (c) is the
6 7 8	546	average of triplicate batches with the error bars representing the maximum and minimum.
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Figure 2



Figure 3

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Figure 4

Nanosilver and the Microbiological Activity of the Particulate Solids versus

the Leached Soluble Silver

Merisa B. Faiz¹, Rose Amal¹, Christopher P. Marquis², Elizabeth J. Harry³, Georgios A. Sotiriou⁴, Scott A. Rice⁵, Cindy Gunawan^{1,3*}

Supplementary Data

Quantification of the soluble and solid Ag fractions

ICP-MS analysis was performed to determine the concentration of the leached soluble Ag and the undissolved Ag particulate residue of the Ag NPs-culture medium systems (no cells).



Figure S1. Concentration of the soluble and solid Ag fractions of the Ag NPs-culture medium systems (no bacteria), samples were taken at 6 h following NPs addition.

First order kinetic prediction of growth upon exposure to Ag leachate from NPs and

AgNO₃

The presence of non- or slowly proliferating viable cells as a result of exposure of *B. subtilis* to 4 and 8.3 ppm Ag leachate as well as to the equivalent 4 and 8.3 ppm soluble Ag from AgNO₃ salt is validated by overestimation of the predicted biomass growth based on first order kinetic, *i.e.* $[A_n] / [A_{n-1}] = e^{kt}$, where $[A_{n-1}]$ is the concentration of PI-negative (viable) cells at previous time interval. The rate coefficient (k) is estimated from bacteria-only control growth.



Figure S2. First order kinetic growth prediction of *B. subtilis* upon exposure to 4 & 8.3 ppm Ag NPs leachate and to 4 & 8.3 ppm Ag from AgNO₃ as compared to the actual growth.

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Statistical analysis of silver-induced cellular reactive oxygen species generation and cell death detection

The statistical significance of cytotoxicity of Ag NPs, Ag NPs leachate and AgNO₃ relative to the bacteria-only control was analyzed by using a one-way ANOVA followed by Dunnett's posthoc analysis in Prism (GraphPad).



Figure S3. Statistical analysis of dynamic cellular ROS and cell death detection over the course of *B. subtilis* growth upon exposure to (a, b) 10 mg Ag L^{-1} NPs (4 ppm Ag leached into medium

at equilibrium) (c, d) 4 ppm Ag leachate from NPs (inset is 8.3 ppm Ag leachate from NPs) (e, f) 4 ppm Ag from AgNO₃ (inset is 8.3 ppm Ag from AgNO₃). The asterisks (*, **) correspond to p values of \leq 0.01, 0.001 respectively, and indicate statistical significance relative to the bacteria-only control (dashed lines).

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32	Nanosilver and the Microbiological Activity of the Particulate Solids versus
33	the Leached Soluble Silver
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35	Abstract
36	Nanosilver (Ag NPs) is currently one of the most commercialized antimicrobial nanoparticles with as yet,
37	still unresolved cytotoxicity origins. To date, research efforts have mostly described the antimicrobial
38	contribution from the leaching of soluble silver, while the undissolved solid Ag particulates are often
39	considered as being microbiologically inert, serving only as source of the cytotoxic Ag ions. Here, we
40	show the rapid stimulation of lethal cellular oxidative stress in bacteria by the presence of the undissolved
41	Ag particulates. The cytotoxicity characteristics are distinct from those arising from the leached soluble
42	Ag, the latter being locked in organic complexes. The work also highlights the unique oxidative stress-
43	independent bacterial toxicity of silver salt. Taken together, the findings advocate that future enquiries on
44	the antimicrobial potency and also importantly, the environmental and clinical impact of Ag NPs use,
45	should pay attention to the potential bacterial toxicological responses to the undissolved Ag particulates,
46	rather than just to the leaching of soluble silver. The findings also put into question the common use of
47	silver salt as model material for evaluating bacterial toxicity of Ag NPs.
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49	Keywords: silver nanoparticles; Ag solids; silver leaching; toxicity; reactive oxygen species
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58 Introduction

59 The rapid development in nanotechnology has seen inorganic nanomaterials such as nanosilver, 60 copper oxide and zinc oxide, subjected to advanced physicochemical manipulation to exhibit 61 powerful antimicrobial activity (Gunawan et al. 2009, 2011, 2013a, Hajipour et al. 2012). 62 Among these materials, nanosilver (silver nanoparticles, Ag NPs) is currently one of the most 63 commercialized due to its potent and broad-spectrum antimicrobial characteristics (Consumer 64 Products Inventory – Project on Emerging Nanotechnologies). Along with applications as core or 65 co-antimicrobial ingredients in wound dressings and internal catheters (Ge et al. 2014), Ag NPs 66 have also been incorporated in an increasing array of consumer products (Deardorff 2014), 67 ranging from personal care products, textiles and household appliances to food and beverages 68 and even children's products (Benn et al. 2010, Quadros et al. 2013). The widespread use is 69 despite the ill-defined antimicrobial mechanisms of Ag NPs, in particular the lack of knowledge 70 regarding the origins of cytotoxicity. The controversy has been at least one of the underlying reasons for regulatory bodies to still classify and regulate Ag NPs as regular bulk silver. 71 72 Therefore, the nanoparticles are subjected to the same reporting requirements, threshold levels 73 and toxicity tests as bulk silver, despite the mounting evidence indicating differences in their 74 antimicrobial potency and properties (Faunce and Watal 2010). The antimicrobial activity of Ag 75 NPs is influenced by the particles' physicochemical characteristics (e.g. size, shape, surface 76 functional groups) as well as interactions with the particles' environment. In real-world settings 77 of Ag NPs antimicrobial applications, the almost inevitable contact of the nanoparticles with 78 aqueous environments, including those in the environment and in the human body, will lead to 79 leaching of soluble silver species through oxidative dissolution of the silver metal (Trop et al. 80 2006, Benn and Westerhoff 2008, Liu et al. 2012, Sotiriou et al. 2012). Considerable research 81 efforts have described the cytotoxic activity of the leached soluble silver on bacteria, even in 82 their various forms, such as the soluble Ag(I)-chloride anionic complexes (Levard et al. 2013) 83 and organo complexes (Gunawan et al. 2009), as a result of potential interactions of the released 3

84	silver with the ubiquitous presence of halides (Cl ⁻ , Br ⁻ , I ⁻) and biomolecules in the environment
85	and in body fluids (Silver 2003, Liu et al. 2012, Eckhardt et al. 2013). Uncertainty however, still
86	lingers as to the bacterial toxicological responses to the undissolved Ag residue (Gunawan et al.
87	2009, Sotiriou and Pratsinis 2010, Xiu et al. 2012), that remains after leaching of silver. The
88	solid Ag particulates have been indicated to physically interact with cellular membranes of
89	bacteria (Sondi and Salopek-Sondi 2004, Mirzajani et al. 2011), but otherwise are often regarded
90	as being inert, indirectly contributing to the antimicrobial activity as a source of the cytotoxic Ag
91	ions. This view is inclusive of the hypothesized Trojan-horse type of Ag NPs cytotoxicity,
92	whereby leaching occurs intracellularly following uptake of particles, or, the suggested cell-
93	particle contact to cause additional leaching at the cell-particle interface and in turn, increasing
94	the uptake of Ag ions by bacteria (Lemire et al. 2013, Bondarenko et al. 2013). The elucidation
95	of the source of Ag NPs cytotoxicity will not only clarify the nanoparticles' 'true' antimicrobial
96	potency in real-world applications, but will also contribute to more accurate assessments of their
07	long term impact on the environment and human health
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98 99	Here, we investigated the origins of Ag NPs cytotoxicity through detailed investigations of
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98 99 100 101 102 103 104 105 106 107 108 109	Here, we investigated the origins of Ag NPs cytotoxicity through detailed investigations of bacterial toxicological responses to the 'overall' presence of nanosilver (<i>i.e.</i> both leached soluble Ag and Ag particulate residue are present in the systems), as compared to those of the corresponding pre-leached filtered Ag leachate samples. Nanosilver in products can be in the forms of nano-sized Ag(I) or metallic Ag ⁰ coated on or impregnated in support materials (Gunawan et al. 2017). As model material, the current work used nanosilver in the form of nano- sized Ag ₂ O deposits (d _{<i>TEM</i>} = 2 nm (Gunawan et al. 2009)) homogenously dispersed on the surface of inert TiO ₂ support (d _{<i>TEM</i>} = 30 nm (Gunawan et al. 2009)). It is noteworthy to point out that studies have observed discrepancies on the leaching behaviour as well as capability of cellular oxidative stress stimulation of Ag(I) <i>versus</i> Ag ⁰ nanoparticles (Gunawan et al. 2009, Gunawan et al. 2013b). Nonetheless, the generated knowledge of cellular responses to the two 4

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fundamental forms of nanosilver-derived microbiologically active components, that is, the leached soluble silver and the solid Ag particulates in the present study, is relevant to the countless nanosilver design with variation in the particle's properties (e.g. size, shape and oxidation states). This facile approach enables unambiguous elucidation of the source of nanoparticulate cytotoxicity without the need to employ simulation materials, such as soluble Ag salt (Gunawan et al. 2009, Sotiriou and Pratsinis 2010, Gunawan et al. 2011, Bondarenko et al. 2013, Ivask et al. 2014), which, as also shown in the current work, exhibits different cytotoxicity characteristics. We report cytotoxic activity of the solid Ag particulates on bacteria, distinct from the leached soluble silver. Methods Synthesis of Ag NPs and Preparation of Ag leachate from NPs The 5 at% Ag/TiO₂ nanoparticles as finely dispersed Ag₂O on inert TiO₂ support were synthesized using the flame spray pyrolysis (FSP) technique as earlier described (Gunawan et al. 2013b, note that at% refers to the percentage of Ag atom relative to the total number of atoms in the particle). TEM images of the particles and XPS spectra that confirm the presence of silver (I) oxide are available (Gunawan et al. 2009). The Ag-leachate was prepared by aseptically predissolving known amounts of Ag NPs (3, 6, 8, 10 mg Ag L⁻¹) in sterile Luria Bertani (LB) broth (5 g L⁻¹ yeast extract, 10 g L⁻¹ tryptone, 5 g L⁻¹ NaCl in deionized water) at 37°C, 280 rpm under dark conditions for 6 h, unsonicated. The undissolved particulates (mean aggregate size = $1.09 \pm$ 0.03 µm by dynamic light scattering (Gunawan et al. 2009)) were removed by centrifugation (5,000 rpm) followed by filtration of the leachate with 0.22 µm polyethersulfone membrane (Millipore Express). Comparable light scattering intensity of the filtered Ag leachate to that of the filtered LB medium confirmed the removal of the solid Ag residue (data not shown). The concentration of soluble silver in the filtered Ag leachate was determined by inductively coupled

135	plasma mass spectrometry (ICP-MS) (Nexion 300D, PerkinElmer). ICP-MS analysis was also
136	performed on the undissolved Ag residue (3-4 h digestion with 70% (v/v) HNO_3 to dissolve the
137	Ag solid). This Ag solid concentration (no cells) reflected, at least in approximation, the
138	presence of the undissolved Ag fraction in the nanoparticle-bacteria exposure systems (note the
139	comparable leaching of Ag NPs in the presence and absence of bacteria, Figure 1 and S1,
140	Supplementary Data). The ICP-MS analysis of the (digested) solid Ag residue and the
141	corresponding Ag leachate fractions (undigested) found that their concentrations added up
142	(within 10-15%) to the nominal total Ag concentrations of the nanoparticles (Figure S1). Finally,
143	the ICP-MS analysis of digested leachate samples found comparable Ag concentrations before
144	and after digestion, which further validated the removal of the solid Ag residue. Suspended
145	Ag/TiO ₂ particulates in the growth medium is expressed as mg L ⁻¹ to reflect their heterogeneous
146	presence, while the homogeneous nature of soluble Ag is referred to in ppm.
147	
148	Bacterial Growth Studies with Ag NPs, Ag leachate and AgNO3 salt
149	The growth experiments on Bacillus subtilis strain UNSW 448700 were carried out in triplicate
150	in LB culture medium at 37°C, 280 rpm under dark conditions for 6 h. To prepare the bacterial
151	inoculum, a single agar plate colony was cultured overnight at 30°C, 220 rpm in LB broth. A
152	measured volume of 1-2 mL of the overnight culture (typical OD_{600} of 6-8) was transferred into
153	50 mL fresh LB broth for a further 0.5-1 h conditioning at 37°C, 280 rpm. For the Ag NPs and
154	AgNO ₃ exposure, pre-weighed Ag NPs (1.1x of the intended dosage) and 0.5 mL (110x
155	concentrated of the intended dosage) solution of AgNO3 were aseptically added into 50 mL and
156	49.5 mL LB respectively. The experiments were initiated by the addition of 5 mL bacterial
157	inoculum into the 50 mL broth containing suspended Ag NPs or dissolved silver salt (OD_{600}
158	bacteria initial = 0.04, corresponding to $\sim 2 \times 10^7$ cfu mL ⁻¹). For the Ag leachate exposure, 5 mL
159	of the bacterial inoculum was added into 50 mL LB containing 1.1x concentrated pre-leached Ag
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NPs (particle-free). The growth profiles were determined by OD₆₀₀ measurement of the biomass 160 161 (UV/Vis spectrophotometer, Hitachi U-1100) and the growth inhibiting effects were assessed 162 relative to controls with no added silver. A cell-free silver control (particulates or soluble silver) 163 was employed as a reference to obtain the OD_{600} corresponding to the bacteria. The 164 corresponding leaching profile of Ag NPs during the bacterial exposure was measured by ICP-165 MS (Nexion 300D, PerkinElmer). For this purpose, a measured volume was sampled from the 166 NPs-exposed culture, centrifuged (5,000 rpm) then filtered with the 0.22 μ m membrane to 167 remove the bacteria and Ag solid. The resulting solution was 100x diluted in deionized water and 168 subjected to the ICP-MS analysis.

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170 Detection of Intracellular ROS and Cell Viability

171 The measurement of cellular ROS generation was performed using the cell permeable oxidative reporter dye H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Sigma-Aldrich). Following 172 173 its uptake, cellular esterases cleave the diacetate moieties of H_2DCFDA to form H_2DCF , which 174 readily transforms to the fluorescent DCF when reacts with ROS. The cell viability assay was 175 based on the fluorescent nucleic acid dye propidium iodide (Sigma-Aldrich) staining. PI enters 176 cells with damaged cytoplasmic membrane, while being excluded by healthy cells. Following removal of the culture medium by centrifugation, samples from the Ag NPs, Ag leachate and 177 AgNO₃ exposure systems (and the silver-free controls) were washed and re-suspended in sterile 178 saline (8 g L⁻¹ NaCl, 0.2 g L⁻¹ KCl) at 2.5 x 10⁸ CFU mL⁻¹. Independent cellular ROS and cell 179 180 viability assays were carried out with 10 μ M H₂DCFDA and 30 μ M PI for 1 h and 15 min 181 respectively, at room temperature under dark conditions. The stained cells were washed with 182 saline and analysed by flow cytometry (FACSCanto[™] II, BD Bioscience) at 488 nm excitation 183 with 530 nm and 670 nm emission filter settings for the detection of DCF and PI fluorescence respectively. DCF fluorescence was also measured using a microplate reader (EnsightTM) 184 185 Multimode, Perkin Elmer) at 492 nm and 520 nm excitation and emission filter settings 7

respectively. The stained cells were also visualized with a BX51WI fluorescence microscope

(Olympus) with 460–490 nm excitation filter settings. **Results and discussion** Bacterial growth inhibition: Activity of the solid Ag particulates, the leached soluble Ag and silver salt To distinguish the cytotoxicity or antimicrobial contribution of the leached soluble Ag and the undissolved Ag particulates, we exposed a model bacteria B. subtilis UNSW 448700 to 0-10mg Ag L^{-1} NPs (Ag/TiO₂) and compared the bacterial growth to that of the corresponding leachate-only systems, as a function of soluble silver detected in the exposure systems. The leachate samples were prepared by aseptically pre-dissolving Ag NPs in culture medium followed by removal of the solid Ag residue. Firstly, as shown in Figure 1a, the dose-response growth inhibiting effects of the Ag NPs correlates with the increasing concentration of soluble silver that leached from the NPs. The extent of growth of *B. subtilis* was reduced to \sim 80% upon exposure to 3 mg Ag L⁻¹ NPs (1.3 ppm silver leached into the culture medium at equilibrium) relative to silver-free control cultures after 6 h. The control cultures were characterized by a relatively short lag phase of 1 h, followed by 3 to 4 h active exponential growth phase before entering the stationary phase at 6 h (Figure 1b). Increasing the NPs dosage to 6 mg Ag L^{-1} (2.7 ppm leached Ag) saw 50% bacterial growth, while almost complete growth suppression was observed at MIC₉₅ 10 mg Ag L^{-1} NPs exposure (4 ppm leached Ag, see Figure 1b for growth profile, MIC₉₅ is minimum inhibitory concentration that cause 5% growth relative to the control). At all of the tested Ag NPs loading, leaching of Ag from NPs was rapid, with detection of ~70% soluble Ag (relative to the leached Ag concentration detected at equilibrium) within 5 min of the Ag NPs-bacterial exposure (see Figure 1c inset for leaching profile of 10 mg Ag L^{-1} NPs). Equilibrium was reached in 1 h with the soluble Ag concentration remained constant afterwards, indicating absence of the Ostwald ripening phenomenon that refers to re-deposition of the

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212	leached Ag on larger particulates (Sotiriou et al. 2012). Increasing the Ag NPs loading saw
213	detection of elevated soluble Ag concentration at equilibrium, with the extent of leaching
214	essentially comparable at 38 – 40% relative to the total added Ag (Figure 1c). This is consistent
215	to earlier studies under comparable conditions (Gunawan et al. 2009, Sotiriou and Pratsinis
216	2010) with the relatively high degree of leaching was due to, at least in part, the presence of
217	organics in the culture medium as shown later in this study. Note that at all of the tested Ag NPs
218	loadings, similar extent of leaching were observed in the absence of bacteria, therefore excluding
219	the possibility of microbial-induced leaching of Ag (Figure S1).
220	

Despite the correlation between Ag NPs growth inhibiting effects and Ag leaching, a 221 222 comparison with bacterial growth in the corresponding leachate-only systems yields an 223 interesting observation. Exposure of *B. subtilis* to the pre-leached soluble Ag in fact resulted in 224 much less growth inhibition when compared to those of the corresponding Ag NPs samples 225 (Figure 1a). The presence of \sim 1.3 ppm Ag leachate for example, was benign to the cultures as 226 they grew to a similar extent as the silver-free control cultures after 6 h. This was in contrast to the ~20% growth reduction of the bacteria when exposed to the corresponding 3 mg Ag L^{-1} NPs 227 228 with comparable leached soluble Ag content. At higher exposure, the bacterial growth in 4 ppm 229 Ag leachate system was ~85% relative to the control cultures (refer to Figure 1b for growth 230 profile), in contrast to the near complete growth suppression observed in the corresponding 10 mg Ag L⁻¹ NPs system. Even doubling the concentration of Ag leachate to 8.3 ppm only slightly 231 232 reduced the bacterial growth to \sim 75%. The findings suggest predominant cytotoxicity 233 contribution from the undissolved Ag particulates, rather than that arising from the leached 234 soluble Ag. Further antimicrobial simulation with an equivalent concentration of soluble Ag 235 from AgNO₃ salt as shown in Figure 1a, saw more severe growth inhibiting activity of the salt. In 236 the presence of 4 ppm soluble Ag from AgNO₃ for example, ~25% B. subtilis growth was

237	observed relative to the control cultures after 6 h (growth profile is shown in Figure 1b), in
238	contrast to the ~85% growth in the leachate system with comparable Ag concentration. Such
239	differences in cytotoxicity may arise from unique cellular physiological responses to the
240	different silver species; the leached soluble Ag and the undissolved Ag particulates from Ag
241	NPs, and the soluble silver from silver salt, as investigated in the following.
242	
243	Dynamic stimulation of cellular oxidative stress and cell death
244	We carried out dynamic tracking of intracellular reactive oxygen species (ROS) generation
245	(measured by H ₂ DCFDA assay) and cell viability (measured by propidium iodide assay,
246	whereby PI enters cells with damaged cytoplasmic membrane, which is indicative of cell death)
247	over the 6 h growth course of <i>B. subtilis</i> in the presence of the various forms of silver; the Ag
248	NPs (MIC ₉₅ 10 mg Ag L ⁻¹ as reference point, contained 4 ppm leached Ag), its corresponding
249	Ag leachate system (4 ppm Ag) and the equivalent AgNO ₃ system (4 ppm Ag).
250	
251	The solid Ag particulates and the leached soluble Ag
252	At 5 min exposure to 10 mg Ag L^{-1} NPs, a 3-fold higher cellular ROS level was detected in <i>B</i> .
253	subtilis relative to the basal ROS levels of the silver-free control cultures, which are by-products
254	of aerobic metabolism in bacteria (Choi and Hu 2008, Gunawan et al. 2011, Eckhardt et al.
255	2013) (Figure 2a, 2b, 4a). Within 30 min of Ag NPs exposure, the cellular ROS level doubled to
256	~6-fold of the control. A secondary oxidative stress response, the cellular ROS stimulation has
257	been increasingly realized as one of the major cellular toxicological responses to Ag NPs in
258	bacteria (Choi and Hu 2008, Hwang et al. 2008, Lemire et al. 2013, Gunawan et al. 2013b). The
259	ROS generation is thought to result from destruction of the iron-sulfur [4Fe-4S] clusters of
260	proteins by Ag metal (Xu and Imlay 2012, Lemire et al. 2013) and in turn, releasing the Fenton-
261	active free Fe into the cytoplasm for subsequent reaction with cellular H_2O_2 to produce hydroxyl
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262	radicals (OH [•]) (Imlay et al. 1988). Alternatively, indirect destruction of the iron-sulfur clusters
263	could result from inhibition of respiratory enzymes by Ag NPs in bacteria (Li et al. 2010, 2011).
264	The resulting premature leakage of electrons to oxygen will generate superoxide radicals (O_2^{\bullet})
265	(Imlay 2003) that in turn again, induces the release of free Fe from iron-sulfur clusters in
266	proteins (Kohanski et al. 2007). Indeed, there have been reports on the cytoplasmic presence of
267	the solid Ag particulates upon bacterial exposure to Ag NPs, as well as the presence of the solids
268	within the bacterial membrane layers (Morones et al. 2005, Grigor'eva et al. 2013, Pal et al.
269	2007). Here, 75-90% PI-positive non-viable bacteria had been detected within 5 to 30 min
270	exposure to Ag NPs, then close to 100% bactericidal or cell death toxicity at as early as 1 h
271	exposure (Figure 2a, 2b, 4b), which indicates cytoplasmic membrane as one of the target
272	destruction sites of the Ag NPs-stimulated cellular ROS (1-8% non-viable cells were detected in
273	the control cultures over the 6 h growth course) (D'Autreaux et al. 2007, Lemire et al. 2013). As
274	expected, the levels of cellular ROS drastically dropped following the rapid high level
275	stimulation, with the majority if not all of the bacterial population were already killed (Sintubin
276	et al. 2011, Gunawan et al. 2013b). Up to this stage, the data suggest that the generation of high
277	levels of cellular ROS and associated bacteria killing was likely to be responsible for the near
278	complete suppression of <i>B. subtilis</i> growth (Figure 1a, 1b).
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Interestingly, such cellular ROS stimulation was absent in the bacteria when studied in the corresponding 4 ppm Ag leachate system. Over the 6 h growth course, only basal ROS levels, comparable to those of the silver-free control cultures were detected (Figure 2a, 3a, 4a) and not surprisingly, the little to no changes in the fraction of non-viable cells relative to the control (Figure 2a, 3a, 4b). The stimulation of lethal levels of cellular oxidative stress by the presence of solid Ag particulates therefore suggests their substantial contribution to the cytotoxicity effects observed in the growth studies. Recalling the observed ~15% growth inhibition of the bacteria in

the presence of 4 ppm Ag leachate (Figure 1a, 1b), it would be reasonable to deduce that the
exposure only resulted in sub-lethal cytotoxicity, causing a minor fraction of the viable cells
uncultivable or slowly proliferating, as further indicated by our growth prediction based on the
fraction of viable cells (Figure S2). Indeed, doubling the Ag leachate concentration to 8.3 ppm
still saw typical cellular ROS (Figure 3c, 4a inset) and dead cells (Figure 3c, 4b inset) detection
as those of the control cultures, despite the slightly higher growth suppression, at ~25% (Figure 293 1a).

295 The leached soluble Ag and silver salt

The minimal cellular ROS stimulation was also seen upon exposure of *B. subtilis* to the equivalent 4 ppm soluble Ag from AgNO₃. Similar to the 4 ppm Ag leachate system, no elevated level of cellular ROS was observed over the 6 h growth course relative to the control cultures (Figure 2a, 3b, 4a). Unlike the leachate samples however, up to $\sim 40\%$ non-viable cells were detected in the salt system (Figure 3b, 4b), indicating attacks on cytoplasmic membrane (Eckhardt et al. 2013). Considering the comparable Ag content, such discrepancies in cytotoxicity are most likely to result from differences in the chemical speciation of the soluble silver, as herein described. Our Ag NPs leaching study (at the MIC₉₅ 10 mg Ag L⁻¹ NPs) in the individual culture medium components revealed a characteristic trend of complexation-assisted dissolution of nanoparticles (Gunawan et al. 2011), with higher extent of Ag leaching in the peptide-rich components, in particular tryptone (90% leaching relative to the total added Ag), compared to those in the deionized water (60% leaching) or NaCl (10% leaching) (Figure 4c). A soft Lewis acid, Ag(I) forms silver-peptide complexes upon its release from NPs (Bolea et al. 2014), which is most likely to result from its strong affinity to the NH_x donor groups of histidine (NH^+) , arginine $(-NH_2^+)$ and lysine $(-NH_3^+)$ amino acids and also to the thiol $(-S^-)$ donor groups of cysteine and methionine amino acids (Eckhardt et al. 2013). Silver-peptide complexes also form with $AgNO_3$ (Bolea et al. 2014), with a fraction of silver is thought to remain as free ions in

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313	the organic-rich medium (Percival et al. 2005). Thermodynamically feasible, the co-existence of
314	free metal ions and organo metal complexes has been reported for the chemical speciation of
315	soluble copper salts, also a soft Lewis acid metal, in similar culture medium as that used here
316	(Gunawan et al. 2011) (note that the current technology for elemental analysis does not
317	differentiate free Ag ions to those locked in organo complexes (Eckhardt et al. 2013)). When
318	compared to free Ag ions, the hindered transport of the bulkier silver-peptide complexes into
319	bacteria (Solioz and Odermatt 1995) is thought to be at least in part, responsible for the
320	passivated, in this case, sub-lethal cytotoxicity of the Ag leachate. Unlike free Ag ions, research
321	indicates that soluble organo Ag complexes are not recognized by the P-type ATPase transporter
322	present in bacteria (Luoma 2008). As also observed in the current study with the AgNO ₃ systems,
323	exposure of bacteria to Ag ions has been reported to suppress their proliferation, which was
324	indicated to result from a ROS-independent inhibition of metabolic enzymes (dehydratases) (Xu
325	and Imlay 2012), the lack of cellular ROS stimulation also apparent in this work. Further,
326	complete suppression of <i>B. subtilis</i> growth was seen at 8.3 ppm Ag from AgNO ₃ (Figure 1a),
327	despite there being no change in the fraction of non-viable cells when compared to the 4 ppm Ag
328	exposure (Figure 3d, 4b inset). Our growth prediction based on the fraction of viable cells
329	indicates major presence of non- or slowly proliferating viable cells with the AgNO ₃ exposure
330	(Figure S2). This loss in replication could also result from the known interactions of Ag ions
331	with DNA in bacteria (most likely with the phosphorus moieties) causing DNA condensation
332	(Feng et al. 2000). The seemingly higher cytotoxic effects of Ag ions as compared to the organo
333	Ag complexes are in agreement with other bacterial studies, whereby extracellular presence of
334	thiol-containing reduced gluthathione (GSH) as silver complexing agent lowered the
335	antimicrobial activity of Ag ions on the Gram-positive Staphylococcus aureus and the Gram-
336	negative Escherichia coli and Pseudomonas aeruginosa (Mulley et al. 2014). Finally, the
337	detection of only basal cellular ROS levels in the AgNO3 exposure systems, even at the double
338	8.3 ppm Ag (Figure 3d, 4a inset), rules out the oxidative stress stimulation as the main 13

mechanisms of AgNO₃ cytotoxicity. Indeed, studies have found no differences in the

antimicrobial activity of Ag ions under aerobic and anaerobic conditions on bacteria (Sintubin et al. 2011). Conclusions Here, we report multiple cytotoxicity origins of Ag NPs towards bacteria. Presence of undissolved Ag particulates in a biological environment is not inert. In their presence, rapid generation of lethal cellular ROS levels were detected in bacteria, while the corresponding leached soluble Ag, being locked in organo complexes, only imparts sub-lethal cytotoxicity. The observed differences in bacterial toxicological responses to the solid versus soluble Ag corroborate earlier reports on the distinct extent of growth inhibiting activity of the Ag NPs' soluble and solid components (Gunawan et al. 2009, Sotiriou and Pratsinis 2010). With regard to the widespread use of Ag NPs, the resolved unique toxicological responses are expected to result in better recognition of the antimicrobial potency of the nanoparticles in real-world settings and importantly, the long-term impact. Research inquiries have shown elevated and persistent presence of silver in wounds, bladder and even in sewage and estuaries, being associated with the intended or in some cases, accidental release from nanosilver applications; the use of wound dressings, pesticides and washing machines are among the examples (Chen et al. 2004, Trop et al. 2006, Reidy et al. 2013, Donner et al. 2015, Beddow et al. 2017). The current findings imply bacterial toxicological responses to not only the leached soluble Ag, but also the Ag particulates in the microbial habitats. Indeed, studies have observed disruptions in the dynamic and balance of microbial communities from natural aquatic waters upon exposure to nanosilver (Das et al. 2012, Beddow et al. 2017), with the work also detecting presence of soluble Ag and aggregates of Ag from nanosilver in these environmental samples (Beddow et al. 2017). The resolved toxicological responses is key to the elucidation of the recently discovered bacterial potential for adaptation to Ag NPs cytotoxicity (Das et al. 2012, Gunawan et al. 2013b). Finally, the work

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2 3	365	highlights the unsuitability of soluble silver salt as model material for Ag NPs cytotoxicity in
4 5 6	366	biological environments, noting a distinct ROS-independent antimicrobial characteristic of
7 8	367	soluble Ag when supplied as AgNO ₃ salt.
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17 18 19	372	under the Chancellor's Postdoctoral Research Fellowship Program.
20 21	373	
22 23	374	Declaration of interest
24 25	375	The authors declare no conflict of interest.
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503	Supplementary material is available: Supplementary Figure S1, S2 and S3.
504	
505	Figure captions
506	Figure 1. Bacterial growth in the presence of Ag NPs, Ag NPs leachate, silver salt and leaching
507	of Ag NPs. (a) Growth of <i>B. subtilis</i> (6 h) relative to cell-only control upon exposure to Ag NPs
508	(3, 6, 8, 10 mg Ag L^{-1}), Ag leachate from NPs and AgNO ₃ as a function of soluble silver
509	detected in the bacterial exposure systems (the growth studies were performed in LB medium).
510	(b) Growth profiles of the bacteria in the presence of 10 mg Ag L ⁻¹ NPs (4 ppm Ag leached into
511	medium at equilibrium), 4 ppm Ag leachate from NPs and 4 ppm Ag from AgNO ₃ . Also shown
512	is the cell-only control growth profile (dashed line). The growth in the presence of Ag was
513	normalised to the extent of growth of the control (in colony forming units, cfu). (c) The
514	corresponding equilibrium leaching of Ag NPs in the bacterial exposure systems, shown in the
515	inset is the leaching profile for 10 mg Ag L ⁻¹ NPs. Each data point in (a), (b), (c) is the average
516	of triplicate experiments with error bars representing the maximum and minimum values
517	detected. The growth studies were performed under dark conditions to render the TiO_2 support 20
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photocatalytically inactive and the benign effect of the TiO₂ support on *B. subtilis* growth had

been confirmed (Gunawan et al. 2013b). The growth studies were reproduced on different days

Figure 2. Detection of cellular reactive oxygen species (ROS, H₂DCFDA staining, green cells)

and cell death (PI staining, red cells) of B. subtilis over its growth course: (a) cell-only control

and (b) in the presence of 10 mg Ag L^{-1} NPs. All stained samples were imaged at comparable

Figure 3. Detection of cellular reactive oxygen species (ROS, H₂DCFDA staining, green cells)

and cell death (PI staining, red cells) of B. subtilis over its growth course, in the presence of: (a)

4 ppm Ag leachate from NPs (equivalent leachate to 10 mg Ag L^{-1} NPs exposure), (b) 4 ppm Ag

from AgNO₃, (c) 8.3 ppm Ag leachate from NPs and (d) 8.3 ppm Ag from AgNO₃. All stained

Figure 4. (a) Dynamic stimulation of cellular ROS in *B. subtilis* measured by H₂DCFDA assay

over its growth course (5, 30 min and 1, 3, 4.5, 6 h) upon exposure to 10 mg Ag L⁻¹ NPs (4 ppm

Ag leached into medium at equilibrium), 4 ppm Ag leachate from NPs and 4 ppm Ag from

AgNO₃. The detected cellular ROS was normalised to the basal ROS levels of the cell-only

control growth. Shown in the inset is cellular ROS detected in the presence of 8.3 ppm Ag

leachate from NPs and 8.3 ppm Ag from AgNO₃. (b) The corresponding dynamic cell death

detection probed by PI staining of *B. subtilis* throughout its growth course. Also shown is the

performed with one-way ANOVA followed by Dunnett's posthoc analysis in Prism (GraphPad)

fraction of dead cells detected for the cell-only control. Statistical analysis of the data was

(Figure S3). The experiments in (a) and (b) were reproduced on different days with unique

samples were imaged at comparable cell concentrations (scale bars = $50 \mu m$).

with unique bacterial inoculum and particle preparations.

cell concentrations (scale bars = $50 \mu m$).

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bacterial inoculum and particle preparations. (c) Leaching profile of $10 \text{ mg Ag L}^{-1} \text{ NPs}$ in cell-

- 544 free individual components of the Luria Bertani (LB) culture medium (5 g L^{-1} NaCl, 5 g L^{-1} yeast
 - 545 extract, 10 g L⁻¹ tryptone dissolved in deionized water). Each data point in (a), (b), (c) is the
 - 546 average of triplicate batches with the error bars representing the maximum and minimum.

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