TECHNICAL SPECIFICATION

ISO/TS 16550

First edition
2014-05-15

Nanotechnologies — Determination of silver nanoparticles potency by release of muramic acid from *Staphylococcus aureus*

Nanotechnologies — Détermination de l'efficacité des nanoparticules d'argent par la libération de l'acide muramique du Staphylococcus aureus

Reference number
ISO/TS 16550:2014(E)

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Foreword

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information.

The committee responsible for this document is ISO/TC 229, *Nanotechnologies*.

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Introduction

The antimicrobial properties of silver nanoparticles (AgNPs) are being increasingly utilized in consumer products. It has been widely used for development of many biological and pharmaceutical processes, products, and applications such as coating material for medical devices, orthopaedic or dental graft materials, topical aids for wound repair, clothing, underwear and socks, textile products, and even washing machines.[1] Industries are producing wide varieties of AgNPs as an antibacterial agents with different physical properties and with little low concern to their side effects. Moreover, there is a risk of enhanced bioavailability of the nanoparticles in the different media[2][3] which might have impact on the beneficial soil flora, plants, animals, and humans. From this point of view, the potency of AgNPs should be evaluated and classified according to its final biological activities.

This Technical Specification does not describe use of this Technical Specification to evaluate what properties of silver nanomaterial are to be assessed.

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Nanotechnologies — Determination of silver nanoparticles potency by release of muramic acid from *Staphylococcus aureus*

1 Scope

This Technical Specification provides a test method for evaluating potency of silver nanoparticles to cell wall degradation of *Staphylococcus aureus* and muramic acid release as quantified by a gas chromatography-mass spectrometry (GC-MS).

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
antibacterial activity
property of compound or substance that kills (bactericidal) or slows down (bacteriostatic) the growth of bacteria

2.2
dynamic light scattering
photon correlation spectroscopy
DLS
technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution

2.3
gas chromatography–mass spectrometry
GC-MS
method that combines the features of gas-liquid chromatography and mass spectrometry to qualitatively and quantitatively analyse volatile compounds within a test sample (Annex A)

2.4
Gram positive bacteria
bacteria that are stained/resistant to bleaching by organic solvent during Gram staining

2.5
internal standard
compound of known concentration added to a sample to facilitate the qualitative identification and/or quantitative determination of the sample components (Annex B)

[SOURCE: ISO 20752, 3.2]

2.6
limit of detection
LOD
minimum amount or concentration of the analyte in a test sample which can be detected reliably, but not necessarily quantified, as demonstrated by a collaborative trial or other appropriate validation

[SOURCE: ISO 8196-1, 3.4.3]

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2.7
limit of quantification
LOQ
lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy under the experimental conditions specified in the method, as demonstrated by a collaborative trial or other validation

[SOURCE: ISO/TS 15216-1, 3.18]

2.8
linear dynamic range
LDR
regression curve of Y on X is a straight line and the regression is called LDR

[SOURCE: ISO 3534-1, 1.34]

Note 1 to entry: The coefficient of linear regression of Y on X is the coefficient of x (slope) in the equation of the regression line.

2.9
muramic acid
form of sugar amide which chemically is the ether conjugate between lactic acid and glucosamine. It occurs naturally as an N-acetyl derivative in peptidoglycan[8]

Note 1 to entry: 3-O-α-carboxyethyl-D-glucosamine.

Note 2 to entry: See Figure 1 b).

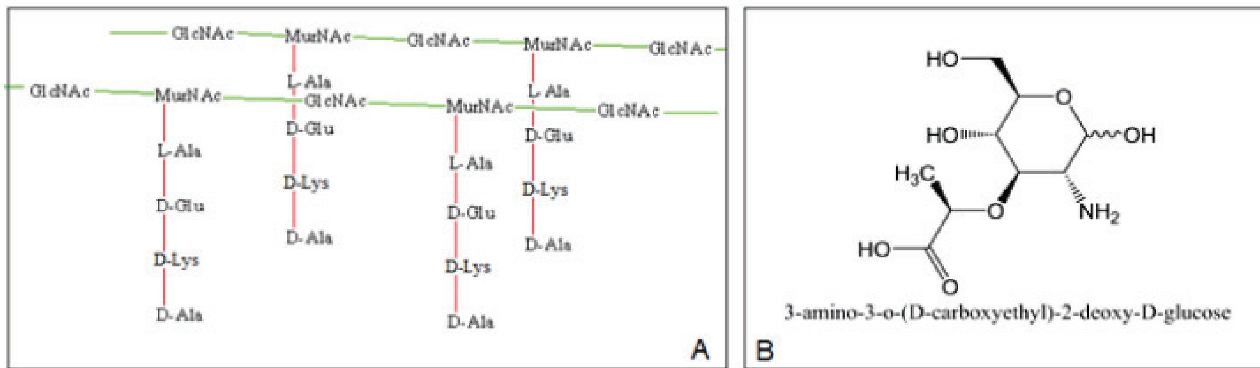

Figure 1 — Structure of a) peptidoglycan layer, b) muramic acid

2.10
peptidoglycan
(murein, PGN)
polymer consisting of sugars and amino acids that forms a mesh-like layer outside the plasma membrane of bacteria forming the cell wall

Note 1 to entry: The peptidoglycan is a specific and essential element in the cell wall of almost all bacteria. Its amount in cell walls of Gram positives is substantial, see Figure 1 a).[3]

2.11
silver nanoparticle potency
reaction of silver nanoparticles with a bacterium's cell wall as measured indirectly by muramic acid release

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2.12
Staphylococcus aureus
S. aureus
facultative anaerobic, Gram positive coccus, coagulase positive, which appears as grape-like clusters when viewed through a microscope, able to grow in a hypersaline medium with manitol as the sole carbon source producing a yellow pigment

2.13
trace
μg/ml concentration or lower

2.14
transmission electron microscopy
TEM
instrument that produces magnified images or diffraction patterns of the sample by an electron beam which passes through the sample and interacts with it

[SOURCE: ISO/TS 10797, 3.8]

2.15
ultraviolet visible spectroscopy
UV-Vis
absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region

Note 1 to entry: This means it uses light in the visible and adjacent [near-UV and near-infrared (NIR)] ranges.

2.16
x-ray power diffraction
XRD
method in which the elastically scattered intensity of X-rays is measured

[SOURCE: ISO/TS 80004-6, 3.2.4]

3 Symbols and abbreviated terms

AgNP Silver Nanoparticle

MA Muramic Acid

MHB Mueller Hinton Broth

OD Optical Density at 600 nm

SIM Selected Ion Monitoring

4 Principles

PGN is a specific and essential component in the cell wall of bacteria.[8] Its main structural features are linear glycan strands cross-linked by short peptides. The glycan strands are made up of alternating N-acetylglucosamine and N-acetylmuramic acid, MA, residues linked by β-1→4 bonds, and the peptide chain is composed of L-alanine, D-glutamine, L-lysine, and D-alanine in the case of *S. aureus* (see Figure 1 a).[8][9] It has been shown that AgNPs will damage the bacterial cell wall and production of the PGN fragments, leading to increased MA concentration in the culture media.[8][12]

AgNPs as the active entity of colloidal AgNPs could have various sizes, surface features, shapes, zeta potential, and concentrations in different trade products. The activity of AgNPs depends on the mentioned characteristics, which could have synergistic or antagonistic cross-effects.[12] Distinguishing among the effects caused by each of these parameters would require the preparation of a large number of samples

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and use of a great variety of instrumentations. Subsequently, monitoring the concentration of MA is one indicator of the effects of these characteristics on AgNPs potency.[12][13][14][15][16]

This Technical Specification utilizes a very sensitive measure of a single analyte, muramic acid as an indirect measure of a single mode of action, cell wall degradation. This method's sensitivity allows for muramic acid quantification below the minimum inhibitory concentration (MIC).[17][18] However, the effect of silver nanoparticles detected through muramic acid liberation does not indicate lethality or cessation of cell growth.

Furthermore, the method relies on a single laboratory synthesized calibration sample to establish a dose response curve for later use with a widely accepted reference. Nanoparticles possess a particle-chemical duality; in that, the particle can be a reservoir for chemical release by dissolution processes. The protocol described in this Technical Specification does not offer information about broad spectrum bactericidal action of silver nanoparticles.

An effect on *Staphylococcus aureus* cannot be predictive for other Gram positive organisms and would not be expected to apply to Gram negative organisms due to the profound structural difference between the two cell wall types with respect to peptidoglycan proportion. Thus, this is not a comprehensive survey of nanoparticle effects upon bacteria.

In light of these considerations, characterization data for the laboratory-prepared silver nanoparticle calibration and system validation reference material can be found in Annex C.

5 Sample preparation for the determination of MA

5.1 Bacterial growth and AgNP treatment

5.1.1 Principle of the test method

Fresh broth cultures of *S. aureus* ATCC 25923, in the OD_{600}, approximately 0,45 to 0,5 (CFU/ml approximately 1 - 2 × 10^9 or the mid-logarithmic phase of growth) phase are treated with AgNPs and incubated for 18 h at 37 °C with shaking (180 rpm to 200 rpm). Afterwards, the culture is clarified with centrifugation and filtration prior to being dried using rotary evaporator and freeze drier instruments. In the next step, muramic acid concentration will be determined by using method described in this Technical Specificaition. It is essential that the cultures used in the experiment contain a high titre of viable bacteria. In each experiment, the titre at least should be demonstrated either from historical control data on growth curves and through the determination of OD_{600} within a time range that could ensure the high titre of viable cells (OD_{600} approximately 0,45 to 0,5).

5.1.2 Description of the method

5.1.2.1 Materials

— *S. aureus* ATCC 25923

— MHB medium[1)]

5.1.2.2 Test conditions

1) A fresh culture of bacterium should be prepared on nutrient agar (NA) plate, by striking of a single colony on the NA plate.

2) Triplicate flasks, each containing 25 ml sterile MHB medium (in 150 ml to 200 ml Erlenmeyer flasks) should be used for assay of each concentration of AgNPs.

1) MHB medium is available from Merck Co., Darmstadt, Germany. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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3) A pre-culture of bacterium should be prepared in the MHB medium about 2 h to 3 h before inoculation of Erlenmeyer flasks. A tube containing 5 ml MHB should be inoculated by a single colony of bacterium and be shacked at 37 °C to reach turbidity equal to 1 McFarland standard tube. For preparation of 20 ml of bacterial suspension 0,1 ml pre-cultured broth medium should be added to sterile 100-ml Erlenmeyer flask containing 20 ml MHB medium, and incubated at 37 °C to reach OD_{600} approximately 0,45 to 0,5 (CFU/ml approximately 1-2 × 10^9) or the mid-logarithmicphase of growth. This OD is recommended based on the experiments for drawing growth curve according to data obtained from OD_{600} reading parallel to CFU counts determination. Under the sterile condition, the prepared bacterial suspension should be used based on Table 1.

4) Cultures in the stationary phase should not be used.

5) The recommended culture temperature and rpm of shaking are 37 °C and 180 rpm to 200 rpm, respectively.

5.1.2.3 Solvent/vehicle

In the case of powdered AgNPs, the solvent/vehicle should not be suspected of chemical reaction with the test substance and bacteria. It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first (Annex D).

5.1.2.4 Exposure concentrations

At least five different treatment conditions (according to the Table 1) of the test substance should be used.

5.1.2.5 Procedure

Culture of *S. aureus* ATCC 25923 should be performed in MHB medium based on the 5.1.2.2. Before the time of AgNPs treatment, a 20 ml MHB medium should be inoculated by 0,1 ml of pre-culture of the strain and incubated at 37 °C, 180 rpm to 200 rpm to reach OD_{600} of about 0,45 to 0,5.

5.1.2.6 Treatment with test substance

Laboratory-prepared AgNP reference materials (see Annex C) and working AgNPs treatments should be done at different concentration levels. The treatments are explained in Table 1 based on the volumes of the AgNPs colloidal suspensions, culture media, and bacterial suspensions in the final volume of 5 ml. In the case of powder samples, please refer to Annex D.

Table 1 — AgNP treatment in concentration

No. sample	1[a,c]	2	3	4	5
AgNPs[b] (ml)	0	0,05	0,25	0,5	1,25
Bacterial suspension (ml)	2,5	2,5	2,5	2,5	2,5
Medium (gram)/ D.W. (ml)	0,052 5/2,5	0,052 5/2,45	0,052 5/2,25	0,052 5/2,0	0,052 5/1,25

a For the conditions of positive/negative controls, see Annex E.

b Colloidal/powder suspension (Annex D) of AgNPs.

c For treatment #1, the amount of MHB powder needed for the preparation of medium is the same as recommended by manufacturer for preparation of routine (1X) MHB medium; and for the others, it should be prepared in such a way ensuring the equal amounts of nutrients in the final 5 ml volumes of all treatments. For this purpose for all treatment, 0,0525 g of medium powder should be dissolved in different amounts of distilled water (D.W.), and then be autoclaved before adding to each treatment. The amount of D.W. to be used is as follow: 2,5 ml for #1, 2,45 ml for #2, 2,25 ml for #3, 2,0 ml for # 4, 1,25 ml for #5, as indicated in Table 1.

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5.1.2.7 Incubation

All flasks in a given assay should be incubated at 37 °C for 18 h with shaking (180 rpm to 200 rpm). After the incubation period, culture should be centrifuged at 10 000 g, at 4 °C for 20 min. Thereafter, the supernatant should be filtered through 3 μm [Polytetrafluoroethylene (PTFE), Hydrophilic]. The filtrate should be concentrated to about 5 ml using rotary evaporator apparatus, fully dried using freeze dry apparatus at −50 °C and should be kept at −20 °C for subsequent analysis.

5.2 Hydrolysis of cell wall

The frozen supernatant, containing 1 mg of dried culture media, should be refluxed with 30 ml of 6 M HCl under N_2 gas for 21 h at 115 °C. After cooling, the hydrolysate should be filtered and completely dried by a rotary evaporator at 40 °C in vacuum. The dried residue should be dissolved in 20 ml H_2O and its pH should be set at about 6,6 M to 6,8 M by 0,4 M KOH. It should then be evaporated again by a rotary evaporator and finally 3 ml methanol (dried using $MgSO_4$) should be added to the residue and centrifuged for 10 min at 6 000 g. After removing the salt, it should be dried under dry air or N_2.[11]

5.3 Derivatization of cell wall

In order to convert the MA into volatile state, with the dried sample, the aldononitrile derivatization method should be performed.[9] A reagent containing 32 mg/ml hydroxylamine hydrochloride and 40 mg/ml 4-(dimethylamino) pyridine in pyridine-methanol (4:1 v/v) should be prepared. To 15 ml of this reagent, a 0,05 mg *myo*-inositol should be added. From the latter, a 0,3 ml aliquot should be added to a 3 mg of dried sample. After capping and shaking the vial manually for about 30 s, it should be heated for 30 min at 75 °C to 80 °C. The vial should then be cooled to room temperature, and then 1 ml acetic anhydride should be added. After capping and shaking the vial again, it should be reheated for 20 min. Thereafter, a 2 ml dichloromethane should be added in a continuous manner to the cooled solution. This product should be cleaned thoroughly before analysing with GC-MS by washing it three times with 1 M HCl and H_2O. Finally, the organic layer should be dried at 45 °C under N_2 gas and should then be dissolved in 200 μL dichloromethane.[11]

5.4 Gas chromatography-mass spectrometry instrumentation

For evaluation of MA, a gas chromatograph equipped with a mass spectrometer is utilized. A non-polar column (30 m × 0,25 mm ID, 0,25 µm thickness) in the split ratio of 1:30 should be used. The temperature program should be set as follows.

— Hold the initial column temperature of 90 °C for a minute, then increase to 250 °C at the rate of 10 °C/min and hold at this temperature for 3 min.

— Put off the ion source ionization mode for 10 min, followed by the SIM method (for trace samples) for 10 min at the detection range of 50 m/z to 1 000 m/z.

— Adjust the ion source temperature at 200 °C.

To improve the precision of quantitative analysis, the internal standard (for example, *myo*-inositol) should be used. Figure 2 represents the separation of MA and *myo*-inositol.

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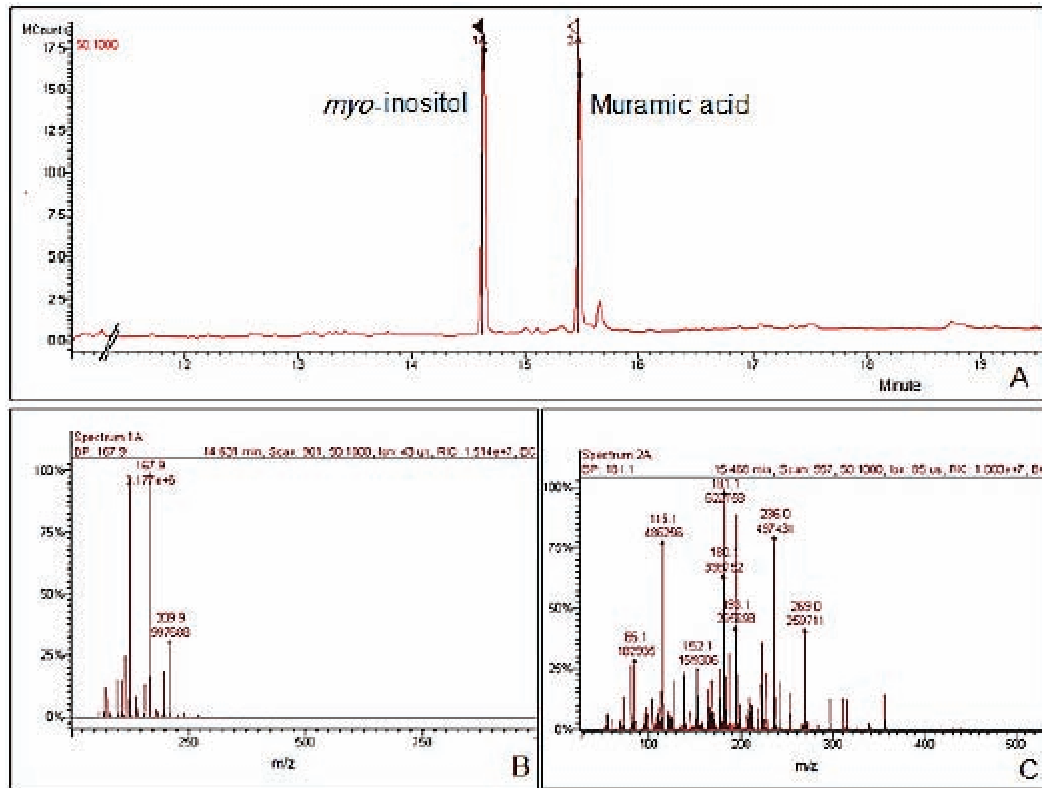

Figure 2 — GC-MS analysis of standard solutions a) chromatogram, b) spectrum of *myo*-inositol and c) muramic acid

6 Data analysis and results interpretations

A stock solution of MA (1 000 µg/ml) should be prepared as outlined earlier. The stock solution should be diluted to the final concentrations in the range of 1 µg/ml to 1 000 µg/ml of MA, including the *myo*-inositol, as an internal standard. The peak area of the *myo*-inositol (internal standard is used to modify the muramic acid peak area and omit the variation between injections. Each concentration should be analysed in three replicates. The modified MA peak areas are calculated using Formula 1.

$$modified\ response = \frac{average\ values\ of\ MA\ areas}{average\ values\ of\ myo-inositol\ areas} \quad (1)$$

The modified responses are plotted against the corresponding concentrations. The results are shown in Table 2. The limit of detection (LOD) and limit of quantification (LOQ) should then be determined as modified responses giving rise to signal-to-noise ratios of 3 and 10, respectively. In order to estimate the LOD and LOQ of method, sample solution should be spotted three times, followed by the proposed method and the signal-to-noise ratio determined. The standard deviation of three replication of samples were ± 0,0128.

Table 2 — Validation and quantification of results

	Amount
LOD (µg/ml)	1
LOQ (µg/ml)	3,3
LDR (µg/ml)	3,3 to 1 000
Coefficient of regression (r^2)	0,9950

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7 The test method for silver nanoparticle sample

The solutions of silver nanoparticles test sample suspension and working/reference silver nanoparticle (Annex C) should be prepared in different treatment levels (Table 1). The difference of muramic acid released by test sample of silver nanoparticle with respect to muramic acids released by reference silver nanoparticle shows potency of test sample. The acceptable level of potency is determined based on the agreement between the manufacturer and user. The negative control should contain the solvent/vehicle of the silver nanoparticle test sample.

Tandem mass spectrometry and selected ion monitoring methods can be used for increased sensitivity when muramic acid concentration is very low and sample medium is complex.

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Annex A
(informative)

Gas chromatography-mass spectrometry

— **Gas chromatography (GC)**: separation of mixtures of compounds in their gaseous form; can be achieved by passing them in an inert gas, called the carrier gas (such as helium) through a column containing a stationary phase for which select for various components will have different affinities. The column is held in an oven at a carefully controlled constant/programmable temperature.

— **Mass spectrometry (MS)**: an instrument which measures the ratio of mass to the number of charges of ions produced from elements and compounds. MS identifies substances by accelerating ions through a mass analyser, breaking the molecules into charged fragments, and detecting the difference between them.

— **Selected ion monitoring (SIM)**: the selected ions are a characteristic of the target analyte. In a SIM, only a selected m/z value (or more) is/are detected. This has the potential to increase the overall sensitivity of the mass spectrometer response by avoiding the time spent, in scanned spectra, on areas of the mass range where no ions of interest will be found. SIM experiments can be performed using mass spectrometry (MS) or tandem mass spectrometry (MS/MS) instruments.

— **Tandem mass spectrometry**: it is also known as MS/MS or MS^2, involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring in between the stages. Multiple stages of mass analysis separation can be accomplished with individual mass spectrometer elements separated in space or using a single mass spectrometer with the MS steps separated in time. This technique is used to improve the detection limit and to overcome the identification of trace analytes in complex mixtures.

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Annex B
(informative)

Internal standard

The method of internal standards is used to improve the precision of quantitative analysis. Internal standards are especially useful for analyses in which the quantity of sample is analysed, or for the instrument response which might vary slightly from run-to-run, for reasons that are difficult to control. Internal standards can then be used for calibration by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. This calibration is done to correct for the loss of analyte during sample preparation or sample inlet. The internal standard is a compound that is very similar, but not identical to the chemical species of interest in the samples, as the effects of sample preparation should, relative to the amount of each species, be the same for the signal from the internal standard as for the signal(s) from the species of interest in the ideal case.

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Annex C
(informative)

Preparation of laboratory-prepared silver nanoparticles reference materials

To prepare AgNP colloid, $AgNO_3$ ($1,0 \times 10^{-3}$ M) aqueous solution containing 0,1 % polyvinyl alcohol (PVA) as a surfactant should be reduced using a dropwise addition of 3-sodium citrate solution at near the boiling point (97 °C ± 0,5 °C). After 20 min, a yellowish gray suspension could be obtained. The optical properties of AgNPs should be evaluated using a UV-Vis spectrophotometer.[12] In order to study their shape and morphology, TEM should be employed. Size of the prepared AgNPs should be studied using a DLS equipped with 632,8 nm HeNe-laser. The size measurement should be accomplished with respect to the UV absorption coefficient (ε) of the suspension at 632,8 nm and its refractive index (RI). The resulting nanoparticles should be purified by centrifugation at 16 000 g × 4 and washing with deionized water. Based on XRD study, the powder microcrystalline sample could be loaded into an aluminium sample holder that is rotated during data collection to improve particle statistics and to minimize preferred orientation effects. Diffraction data should be collected in the range of 1 to 80 (2Θ), on powder X-ray diffractometer equipped with a scintillation detector, secondary monochromator, and Cu Ka1 radiation (λ = 1,5406 A°). The particle size is in the range of 16 nm to 19 nm.[12] Further information of physico-chemical characterization of sample and standard can be obtained according on ISO Technical Reports.[19][20] DLS and UV/Vis physical measurements of the reference material stock solution should be performed before and after the time the AgNP reference material is used as a calibrant in the test method (e.g. >18 h). Statistical comparison of these size measurements establishes that the reference stock sample remained stable over that time of the treatment. These values should be recorded and used to set validated operating specifications. Ensuring that nanoparticle reference materials meet these specification during every experiment provides confidence in the comparability of day-to-day and laboratory-to-laboratory reference material preparations. The laboratory-prepared AgNP reference materials should be discarded after 24 h of preparation.

NOTE 1 This laboratory-prepared reference material is a colloidal, spherical nanoparticle. It can be used until well-defined widely accepted reference AgNP reference materials are available.

NOTE 2 Reference nanoparticle: The reference silver nanoparticle will be introduced by ISO.

NOTE 3 Working nanoparticle: The sample should be analysed.

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Annex D
(informative)

Silver nanoparticle suspension preparation

The silver nanoparticle powder should be suspended in aqueous (double deionized water) base media (Table 3) in order to perform the interaction of the silver nanoparticles with the bacteria; for homogenization of the suspension, an ultrasonic cleaner (350 W) should be used. After that, the prepared solutions should be added to 25 ml bacterial suspension as well as synthesized AgNPs (5.1.2.6).

In the whole processes, silver nanomaterial only should be used in the 24 h of suspension preparation.

Table D.1 — Treatment conditions, each in triplicate

No. sample	1	2	3	4	5
AgNPs (mg)	0	0,005	0,025	0,05	0,125
Water for suspension (ml)	1,0	1,0	1,0	1,0	1,0
Culture media (1X, ml)	1,5	1,5	1,5	1,5	1,5
NOTE The role of dissolution in silver nanoparticle toxicity is significant. For specific formulations, such as the citrate-capped material, there might be a time-dependency for dissolution that can influence the acceptable product shelf-life.					

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Annex E
(informative)

Treatment control conditions; positive, negative, and bacterial suitability controls

There is no need to carry out the positive/negative controls in the routine analysis. If needed, use the following control substances:

— **Cefixime (as a positive control)**: 3 ml of bacterial suspension and 2 ml culture media containing 16 µg/ml cefixime.

— **Negative control**: 3 ml of bacterial suspension and 2 ml culture media.

— **Supernatant control**: The supernatant control is needed in the case of each experiment and related to the sample vehicle. In the case of introduced homemade nanoparticles, it contains: $NaNO_3$ ($1,0 \times 10^{-3}$ M) aqueous solution containing 0,1 % polyvinyl alcohol (PVA) and 3-sodium citrate (10 %). The treatment is with the same conditions shown in Table 1, including 18 h with shaking.

— **Bacterial suitability control**: A control experiment with fully dissolved silver ion salt (e.g. 0,003 uM $AgNO_3$, 0,01 uM $AgNO_3$, 0,03 uM $AgNO_3$, 0,1 uM $AgNO_3$, and 3 uM $AgNO_3$ in culture media) should be performed each time new bacterial cultures are started to ensure that the dose-dependent muramic acid release response to Ag ions is consistent between new bacterial culture preparations. This control establishes that large changes in the bacterial culture and the sensitivity to silver ions have not occurred during expansion of the culture. The silver concentration at which a 50 % relative release in muramic acid occurs should be recorded and used to generate operating specifications for the culture. This EC50 value is nominally approximately 0,9 uM.

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