Journal of Microbiological Methods 203 (2022) 106618

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Contribution to optimization and standardization of antibacterial assays with silver nanoparticles: the culture medium and their aggregation

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ARTICLE INFO

Keywords:
Silver nanoparticles
Antibacterial effect
Culture media
Aggregation
Surfactant

ABSTRACT

The antimicrobial activity of silver nanoparticles is determined by their size and specific properties, as well as by the chemical composition of the exposure medium in which the nanoparticles are suspended. When the antibacterial tests are carried out in a culture medium, aggregation of the nanoparticles is produced, decreasing their effectiveness. This study proposes the addition of surfactants to the culture medium to prevent the aggregation of silver nanoparticles and optimizes the concentrations of these surfactants. The aggregation of silver nanoparticles was studied by dynamic light scattering (DLS) after dispersion in three liquid culture media (Mueller-Hinton (MH), Luria-Bertani (LB) and Brain Heart Infusion) in which four different surfactants (SDS, Triton X100, Tween 80 and CTAB) were added at concentrations of 0, 0.1, 0.5, 1, 1.5 and 2%. Results showed that, the optimal culture media to prevent aggregation of silver nanoparticles were MH and LB with higher concentrations of Tween 80 and Triton X100 surfactants; being MH + 2% of Tween 80 and MH + 1% Triton X100 the best combinations obtained because the results obtained were closest to the sizes of nanoparticles in ultrapure water. In addition, it has been verified that the optimal medium + surfactant combinations chosen did not affect the viability of *Escherichia coli* bacteria. Nanoparticle aggregation was not observed by single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) when nanoparticles were incubated for long incubations periods (24 h) in the optimal medium chosen.

1. Introduction

Due to the worldwide increase of multidrug resistance in microorganisms, silver nanoparticles are increasingly used for their antibacterial properties in many applications, for example, in medicine to reduce infections in burn treatments or to prevent bacteria colonization on protheses or catheters, to eliminate microorganisms on textile fabrics or for water treatment (Pal et al., 2007; Panáček et al., 2006; Hartemann et al., 2015). Silver has been known for thousands of years for its broad spectrum of bactericidal activity, mainly in the form of ionic silver, to fight infections and control spoilage, but it has been demonstrated that its substitution in the nanoparticle form may have additional advantages (Fernández et al., 2010; Marambio-Jones and Hoek, 2010; Gunawan et al., 2017).

The antimicrobial activity of colloid silver particles is influenced by size and specific properties such as shape, chemical composition, surface charge, coatings… (Rai et al., 2012). Silver nanoparticles with smaller sizes had a greater antimicrobial effect against different gram-negative

Abbreviations: AgNPs, Silver nanoparticles; Al_2O_3, Aluminum oxide; BHI, Brain heart infusion; $CaCl_2$, Calcium chloride; CFU, Colony-forming unit; CTAB, Cetyl Trimethyl Ammonium Bromide; DLS, Dynamic light scattering; *E. coli*, *Escherichia coli*; ICP-MS, Inductively coupled plasma mass spectrometry; LB, Luria-Bertani; $MgCl_2$, Magnesium chloride; MH, Mueller-Hinton; NaCl, Sodium chloride; nm, Nanometers; p, p-value; PVP, Polyvinylpyrrolidone; SD, Standard deviation; SDBS, Sodium dodecyl benzene sulfonate; SDS, Sodium dodecyl sulfate; SiO_2-NPs, Silicon dioxide nanoparticles; SP-ICP-MS, Single particle inductively coupled plasma mass spectrometry; TiO_2, Titanium dioxide; ZnO-NPs, Zinc nanoparticles; ZrO_2, Zirconium dioxide; ZrO_2-NPs, Zirconium dioxide nanoparticles; ν, Kinematic viscositiy; ρ, Densitiy.

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https://doi.org/10.1016/j.mimet.2022.106618
Received 19 September 2022; Received in revised form 4 November 2022; Accepted 4 November 2022
Available online 8 November 2022

bacteria (Morones et al., 2005; Baker et al., 2005). Moreover, the chemical composition of exposure media (pH, ionic strength, ionic composition, presence of natural organic matter) is also a relevant factor that would affect the toxicity of AgNPs (Millour et al., 2015; Vazquez-Muñoz et al., 2020; Bélteky et al., 2021).

For years, extensive research has been done on the antibacterial activity of silver nanoparticles and about how to improve their activity by controlling some properties such as the nanoparticle size (Agnihotri et al., 2014). It has been reported that the biological media (Stebounova et al., 2011) and their components could affect the size of the nanoparticles or their rate of dissolution (Pareek et al., 2018; Huynh and Chen, 2011; Li et al., 2010). Millour et al. (Millour et al., 2015) showed that the presence of salts like NaCl, $CaCl_2$ or $MgCl_2$, which are present in culture media, produces the aggregation of silver nanoparticles; as the presence of organic matter in complex media, such as tryptone or yeast extract, modifies the rate of aggregation in these culture media. In an extensive study performed by Vazquez-Muñoz et al. (Vazquez-Muñoz et al., 2020), in 2020, on the influence of the culture media on the antimicrobial activity of silver nanoparticles, they determined that the values of the minimal inhibitory concentration of silver nanoparticles against *Escherichia coli* changed up to two orders of magnitude by the influence of the composition of the culture medium. In some culture media, aggregation of silver nanoparticles is promoted so that their size increases up to 3 times.

The effect of different surfactants and polymers on the stability and aggregation of nanoparticles in aqueous media has been analyzed. For water-dispersed nanoparticles, the addition of anionic surfactants such as sodium dodecyl sulfate (SDS) (Yekeen et al., 2019) or sodium dodecyl benzene sulfonate (SDBS) (Yekeen et al., 2019; Ordóñez et al., 2020; Tran et al., 2022) decreases the sizes of nanoparticles. The presence of non-ionic surfactants such as Triton X100 (Yekeen et al., 2019; Tran et al., 2022) or PVP (Ordóñez et al., 2020) also contributes to obtain smaller sizes of nanoparticles. On the contrary, the addition of cationic surfactants such as Cetyl Trimethyl Ammonium Bromide (CTAB) (Yekeen et al., 2019; Ordóñez et al., 2020; Tran et al., 2022), does not prevent the nanoparticle aggregation. Kvítek et al. (Kvítek et al., 2008) showed that SDS, Tween 80 (non-ionic surfactant) and PVP were the most effective surfactants in preventing the nanoparticle aggregation in water. In addition, the presence of SDS and Tween 80 in water, did not modify the antibacterial properties of the silver nanoparticles, whereas the presence of PVP reduced these properties. Later, when the contact between nanoparticles and bacteria was established in a culture medium to study their antibacterial activity, the stabilizing effect of the surfactant on the nanoparticles can change. The components of the culture media matrix and the added surfactants could modify the stability of the nanoparticles and their ability to aggregate, and consequently, their interaction with the bacteria. Therefore, the standardization of methodology is very important, allowing comparison of results obtained by different authors.

In this study, the addition of surfactants to the culture medium is proposed as a solution to the problem of nanoparticle aggregation in the evaluation of the antibacterial effect of silver nanoparticles. For this purpose, four different surfactants (SDS, Triton X100, Tween 80 and CTAB) dispersed in three culture media (Mueller-Hinton, Luria-Bertani and Brain Heart Infusion) have been evaluated to prevent the aggregation of silver nanoparticles. Aggregation of silver nanoparticles was studied by dynamic light scattering analysis (DLS) in short-term experiments and by single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) for long incubation periods. *Escherichia coli* ATCC 25922 and *Escherichia coli* K12 strain J62 were used as reference bacteria to study the effect surfactants on the viability of bacteria.

2. Experimental

2.1. Culture media

Three liquid culture media were studied: brain heart infusion (BHI) (Difco, Detroit, USA), Mueller-Hinton (MH) (Scharlau, Scharlab SL, Spain) and Luria-Bertani (LB) (Labkem, Labbox.com). Four surfactants at different concentrations were added to culture media (0, 0.1, 0.5, 1, 1.5 and 2%). Surfactants tested were Tween 80, Triton X-100, SDS and CTAB (Cetyl Trimethyl Ammonium Bromide) (Sigma-Aldrich, Saint Louis, MO, USA). The control sample contained a suspension of the tested nanoparticles in ultrapure water (Milli-Q Advantage, Molsheim, France). All assays were performed in triplicate.

The mixtures of culture media + surfactants were autoclaved for 20 min at 121 °C at 1 atm. These culture media were stored at 4 °C until use.

2.2. Nanoparticle suspensions

Nanoparticles used were a suspension of monodisperse citrate-stabilized silver nanoparticles of nominal diameter 60 ± 7 nm (NanoComposix, San Diego, CA, USA). The commercially available suspension of silver nanoparticles, after 1 min sonication, was diluted in the different sterile culture media to obtain a final concentration of 2 mg L^{-1}. After dilution and before each analysis, the diluted suspensions were sonicated for 1 min and 50 W in an ultrasonic bath (Ultrasons, J.P. Selecta, Barcelona, Spain). Longer sonication times were not used to avoid excessive heating of the suspensions. The control sample consisted of a suspension of nanoparticles in ultrapure water at the same final concentration.

2.3. Cell culture

For the bacteria assays, two *Escherichia coli* strains were used, *E. coli* ATCC 25922 and *E. coli* K12 strain J62 (F^-, *pro, his, trp, lac, Nal*^{r}) (Gomez-Lus et al., 1990; Hane and Wood, 1969). The strains were cultured on Mueller-Hinton agar at 37 °C for 24 h, and stored at −80 °C in sterile skimmed milk.

2.4. Procedures

2.4.1. Density and viscosity measurements of culture media

Densities (ρ) of culture media with and without surfactants were measured at 25 °C with an Anton Paar DSA 5000 vibrating tube densimeter. The standard uncertainty for temperature was 0.005 K and the uncertainty of density can be estimated in 10^{-4} g cm^{-3}.

Kinematic viscosities (ν) were determined at 25 °C using an Ubbelohde capillary viscometer and a measuring unit Schott-Geräte AVS-440. The temperature was controlled with a CT52 Schott-Geräte thermostat. The standard uncertainty for temperature was 0.01 K and the uncertainty for kinematic viscosities was 0.005 mm^2 s^{-1}. Dynamic viscosities, $\eta = \rho\nu$, were obtained from experimental kinematic viscosities and densities. The estimated uncertainty for dynamic viscosities was 0.005 mPa s.

2.4.2. Dynamic Light Scattering (DLS) measurements

Sonicated stock suspension of silver nanoparicles was added to each media of study to obtain a final concentration of 2 mg L^{-1}.

The hydrodynamic size of nanoparticles was characterized by a DLS instrument (Zetasizer Nano ZS, ZEN3600, Malvern, Worcestershire, United Kingdom) equipped with a flow cell (ZEN 0023). All the measurements were performed at 25.0 °C. Signal intensity and hydrodynamic diameter estimations were collected every 3 s.

2.4.3. Viability assays

Ten microliters of a 0.5 McFarland Turbidity Standard bacterial suspension were added to 90 μL of culture medium in a 96-well flat-

bottomed microtiter plate (Nunc, Thermo Fisher Scientific, Madrid, Spain) and incubated for 24 h at 37 °C. Afterwards, 10 μL of each combination were seeded on a Petri dish containing MacConkey agar (McK, Bio-Rad, La Coquette, France) and incubated at 37 °C for 24 h.

For those combinatios in which differences in growth were observed in relation to the culture media without surfactants, 10 μL of a 1:100 dilution of the combinations were spread plated on MacConkey agar. The plates were incubated at 37 °C for 24 h. Then the numbers of bacterial colonies (CFU) were counted. The counts were considered the surviving number of bacteria. Analyses were carried out in triplicate.

Those culture media in which no differences were observed in relation to the culture media without surfactants, for both bacterial strains, were chosen as optimal culture media, interpreting that the viability of the bacteria was not affected by surfactants. In addition, to select the optimal culture medium for subsequent SP-ICP-MS measurements, another critical criterion was the similar nanoparticle size obtained in this medium and in ultrapure water.

2.4.4. Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) measurements

Dilutions of the stock suspension of silver nanoparticles were prepared in the chosen optimal culture medium by accurately weighing (±0.1 mg) aliquots after 1 min sonication. Silver nanoparticles were incubated at 37 °C for 24 h and shaken at 130 rpm in an orbital incubator (OPAQ I10-OE, Ovan).

A Perkin-Elmer NexION 2000 ICP mass spectrometer (Toronto, Canada) was used throughout. The sample introduction system consisted of a glass concentric slurry nebulizer and a cyclonic spray chamber (Glass Expansion, Melbourne, Australia). Suspensions were measured in single particle mode using the Syngistix Nano-Application module version 2.5 (PerkinElmer Inc.). The dwell time used was 100 μs with a total acquisition time of 60s and recording 600,000 readings per time scan. The isotope monitored was ^{107}Ag.

2.4.5. Statistical analysis

All statistical analysis were performed using SPSS 26.0 (SPSS Corp., Armonk, NY USA). Variables were described with mean, standard deviation (SD) and variation coefficient. The Shapiro-Wilk test was used to verify that the distributions were normal. Analysis of variance (ANOVA) of one factor was used for the comparison between the methods of detection used for each culture media; if the distributions were not homoscedastic, Welch correction was carried out. Pair-wise comparisons were carried out with the Sidak correction if the distributions were homoscedastic (Levene test, $p > 0,05$) and Games-Howel if the distributions were not homoscedastic. Finally, t-student test was used to check if there were significant differences between the two best results obtained. The level of significance required in all the analyzes performed was <0.05.

3. Results and discussion

3.1. Influence of media composition on the nanoparticle size

Nanoparticle size is a determining factor in the interaction process bewteen nanoparticles and bacteria, because the larger the nanoparticle size, the more difficult it will be for them to enter to the bacteria. Therefore, the aggregation of nanoparticles in culture media is one of the main problems in the study of their activity against microorganisms.

Due to this, hydrodynamic sizes of silver nanoparticles diluted in different culture media were measured by DLS to determine the optimal composition of culture medium that allows to keep the original size of nanoparticles.

The most frequently used culture media for the growth of enterobacteria were selected for the study, Mueller-Hinton (MH) (Acay et al., 2019; Panáček et al., 2016; Gurunathan, 2015), Luria-Bertani (LB) (El-Kheshen and El-Rab, 2012; Sadeghi et al., 2010; Abbaszadegan et al., 2015; Morones-Ramirez et al., 2013) and brain heart infusion (BHI) (Toledo et al., 2020; Ravichandran et al., 2011; Callon et al., 2016). Because nanoparticle aggregation was observed in initial experiments, each one of them was mixed with different surfactant types, which were Tween 80, Triton X110 (non-ionic surfactants), SDS (anionic surfactant) and CTAB (cationic surfactant).

Table S1 shows experimental values of density, kinematic and dinamic viscosity obtained for each one of the culture media used in the determination of the hydrodynamic sizes by DLS.

Table 1 shows experimental hydrodynamic sizes of silver nanoparticles spiked in the three culture media with different types and concentrations of surfactants. The hydrodynamic size obtained for silver nanoparticles of 60 nm diluted in ultrapure water was 68.5 ± 3.2 nm, which agrees with the size certified by the supplier that was 64 nm. Thus, nanoparticles in water were used as a control sample.

As can be seen in Table 1, nanoparticle aggregation occurs in all media without surfactants. Certain concentrations of Triton X100, Tween 80 and SDS contributed to prevent aggregation, since as can be

Table 1
Mean hydrodynamic size of silver nanoparticles spiked in several culture media with different surfactants at different concentrations (mean ± standard deviation, $n = 9$). *Welch correction.

Culture media	Surfactant concentration %	Mean hydrodynamic size nm			
		Triton X100	Tween 80	SDS	CTAB
Mueller-Hinton	0	127.7 ± 6.0	127.7 ± 6.0	127.7 ± 6.0	127.7 ± 6.0
	0.1	119.5 ± 8.1	120.8 ± 9.0	116.9 ± 10.0	149.3 ± 7.3
	0.5	107.5 ± 8.7	112.3 ± 7.3	109.8 ± 9.4	141.4 ± 7.8
	1	71.4 ± 7.8	116.0 ± 7.4	113.4 ± 6.8	139.4 ± 8.6
	1.5	101.5 ± 7.6	106.8 ± 7.2	110.7 ± 7.2	135.1 ± 4.1
	2	91.6 ± 6.7	74.9 ± 6.5	95.9 ± 8.8	114.4 ± 3.7
ANOVA (*p*-value)		<0.001	<0.001	<0.001	<0.001
Luria-Bertani	0	139.2 ± 8.3	139.2 ± 8.3	139.2 ± 8.3	139.2 ± 8.3
	0.1	118.5 ± 14.2	117.6 ± 10.4	112.0 ± 7.3	237.8 ± 24.7
	0.5	98.0 ± 8.3	109.6 ± 6.7	112.2 ± 3.9	144.6 ± 12.8
	1	87.6 ± 12.0	109.3 ± 7.6	108.9 ± 5.3	142.5 ± 0.9
	1.5	89.2 ± 11.9	94.6 ± 13.7	105.8 ± 4.4	135.4 ± 6.6
	2	81.7 ± 7.6	89.4 ± 6.8	95.9 ± 4.4	138.6 ± 7.4
ANOVA (p-value)		<0.001	<0.001	<0.001	<0.001*
BHI	0	104.2 ± 10.8	104.2 ± 10.8	104.2 ± 10.8	104.2 ± 10.8
	0.1	109.8 ± 20.2	127.4 ± 21.6	102.7 ± 10.0	225.4 ± 48.7
	0.5	82.4 ± 7.4	114.7 ± 8.7	103.9 ± 31.1	285.3 ± 58.2
	1	96.6 ± 20.9	110.6 ± 11.9	119.5 ± 21.4	181.0 ± 38.5
	1.5	88.8 ± 12.8	97.8 ± 9.1	83.8 ± 6.6	179.9 ± 66.6
	2	84.3 ± 14.4	89.9 ± 9.8	86.5 ± 10.1	146.4 ± 9.7
ANOVA (p-value)		=0.006*	<0.001	<0.001*	<0.001*

observed, the nanoparticle sizes in the presence of these surfactants were lower than in their absence, except in the case of the addition, for any concentration, of Triton X100 and Tween 80 in BHI where no significant differences in sizes were observed in relation to the absence of these surfactants. In contrast, the CTAB surfactant did not prevent aggregation, and smaller sizes were obtained in the absence of this surfactant, in almost all cases. This fact indicated that the CTAB surfactant was not suitable to prevent aggregation of the nanoparticles.

The adsorption of surfactant on the nanoparticle surface influences the electrostatic and steric interactions between the nanoparticles and surfactant molecules (Yang et al., 2015). According to DLVO theory, the total interaction energy between charged particles is a summation of the Van der Waals attraction and the Electric Double Layer repulsion (Verwey, 1947; Derjaguin and Landau, 1993). While the Van der Waals attractive force is significant at a very small separation distance, the effect of Electric Double Layer repulsive force is generally stronger and in a wider range (Adair et al., 2001). Aggregation is prevented when the electrostatic repulsion between particles is dominant over the Van der Waals attraction (Tran et al., 2022).

The addition of SDS (anionic surfactant) (Table 1, and in Fig. 1A), decreased the sizes of silver nanoparticles compared to the absence of the SDS, in all three media studied. These differences were statistically significant for all concentrations ($p < 0.005$), except for 0.1% of SDS in MH medium and for the concentrations 0.1% - 1% of SDS in BHI medium. Due to the silver nanoparticles are negatively charged (zeta potencial: −46 mV), there will be electrostatic repulsion between them and the anionic surfactant molecules, resulting in the increasing stability of the colloidal nanoparticles dispersed in SDS and the chance of

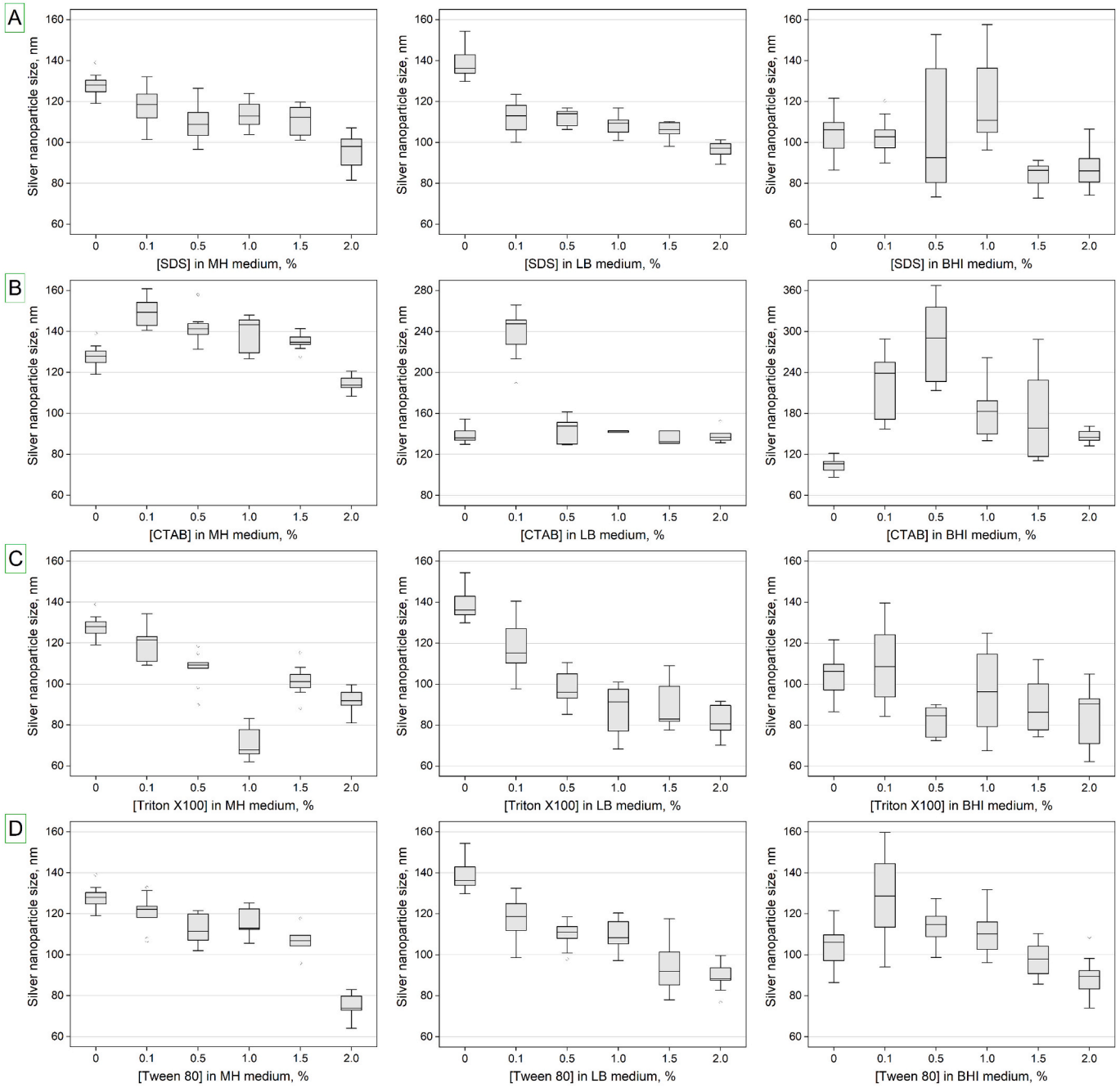

Fig. 1. Box plot representation of hydrodynamic sizes of silver nanoparticles spiked in the tested culture media with different concentrations of A: SDS (0–2%); B: CTAB (0–2%) added; C: Triton X100 (0–2%) and D: Tween 80 (0–2%).

nanoparticle aggregation is relatively low. This fact was observed in other studies with negatively charged particles (AgNPs, SiO_2-NPs, ZnO-NPs, ZrO_2-NPs) and anionic surfactants (SDS, SDBS) (Yekeen et al., 2019; Tran et al., 2022; Kvítek et al., 2008).

The addition of CTAB (cationic surfactant), increased the sizes of silver nanoparticles at almost all surfactant concentrations, in all media studied (Fig. 1B). In MH and BHI media, the nanoparticle size was significantly smaller in absence of surfactant than at any of the concentrations ($p < 0.001$), except for the concentration of 1.5% in BHI medium and for 0.1%, 0.5% and 2% in MH medium. In LB medium, no significant differences were found for any concentration of CTAB, except for the concentration of 0.1%, where the nanoparticle size was significantly higher than in the absence of CTAB. The electrostatic attraction between the cationic surfactant and the negatively charged nanoparticles can generate a multilayer structure of surfactant molecules on the nanoparticle surface and increase the hydrodynamic size (Tran et al., 2022; Fernando et al., 2019).

Fig. 1C and D show that in presence of Triton X100 and Tween 80 (non-ionic surfactants), a statistically significant decrease in nanoparticle sizes ($p < 0.005$) was observed in LB and MH media, except for the concentration of 0.1% of both surfactants in MH medium. In BHI medium, although the addition of both surfactants decreased the sizes of the nanoparticles for almost all concentrations, statistically significant differences were not observed in relation to their absence. The stability of the nanoparticle suspension with non-ionic surfactants was high. Probably, the balance between the repulsive and the attractive forces does not produce significant interaction between the non-ionic surfactant and the charged surfaces of the nanoparticles (Yekeen et al., 2019; Kvítek et al., 2008).

In agreement with the previously published results (Yekeen et al., 2019; Kvítek et al., 2008), the non-ionic surfactants Triton X100 and Tween 80 showed the highest ability to stabilize and prevent aggregation of silver nanoparticles, compared to anionic and, especially, cationic surfactants.

Generally, increasing the surfactant concentration, the nanoparticle size decreased ($p < 0.05$). In the case of the lowest concentrations of SDS (0.1%, 0.5% and 1%) in BHI medium and for 0.1% of SDS, Triton X100 and Tween 80 in MH medium, the nanoparticle sizes did not differ significantly regarding the absence of surfactants ($p > 0.05$). Therefore, higher concentrations of surfactants were chosen to prevent aggregation of the nanoparticles. In contrast, some authors describe that in water, increasing surfactant concentration, nanoparticle sizes increased (Tran et al., 2022), showing that the composition of the culture media could affect the behavior of the surfactants and nanoparticles.

As mentioned above, the addition of Triton X100 and Tween 80 in BHI medium did not produce significant differences in relation to the absence of these surfactants, while the addition of them in MH and LB media allowed to obtain significant smaller sizes. In addition, BHI medium showed the higher coefficients of variation than in other media, mainly with the addition of CTAB and with higher surfactant concentrations. Therefore, BHI medium was considered the least stable, and therefore the least suitable to prevent nanoparticle aggregation, compared to LB and MH media.

On the other hand, it was observed that for the lowest concentration (0.1%) of Triton X100, Tween 80 and SDS in LB medium, significantly smaller sizes were obtained compared to the absence of them, whereas for the same concentrations of these surfactants in MH medium, no significant differences were observed. This indicated that LB was more suitable for low concentrations of surfactants than MH medium.

It has been reported that the presence of salts such as NaCl allows the aggregation of nanoparticles because the thickness of the electrical double layer around the silver nanoparticles decreases as the ionic strength increases, and it enhances the close encounter of nanoparticles. Furthermore, anions such as Cl^-, can precipitate with silver ions around the silver nanoparticles and create a new coating that increases the size of the nanoparticles (Millour et al., 2015; Pareek et al., 2018). NaCl was included in LB and BHI composition, whereas it was not in MH. This could be an important factor for choosing the optimal medium, but in this study, no differences were observed between NaCl-containing and NaCl-free media. The presence of organic matter in culture media like tryptone and yeast extract, could affect the aggregation of silver nanoparticles by increasing the coagulation critical concentration, and so, the concentration of NaCl in the media might be lower than the coagulation critical concentration required to produce nanoparticle aggregation (Millour et al., 2015). Vazquez-Muñoz et al. observed that culture media with different compositions, such as MH and LB, had a similar effect on the hydrodynamic size of silver nanoparticles (Vazquez-Muñoz et al., 2020), which was consistent with those obtained in this study. LB, MH and BHI media contain proteins from different sources, such as beef (MH and BHI) or yeast (LB), and different concentrations of inorganic salts (high concentration in LB and BHI, and no salts in MH). Therefore, different mechanisms and interactions may occur between nanoparticles and components of the culture medium and affect the aggregation of silver nanoparticles (Vazquez-Muñoz et al., 2020).

Considering the results obtained, MH and LB with higher surfactant concentrations were chosen as a culture media for future studies. The optimal combinations of the culture media that prevented the aggregation of nanoparticles, and with which the results closest to the sizes of nanoparticles in ultrapure water were obtained, were: LB medium plus 1–2% of Triton X100, MH medium plus 1% of Triton X100, MH and LB media plus 2% of Tween 80 and SDS, and BHI medium plus 1.5% of SDS; being MH + 2% of Tween 80 and MH + 1% Triton X100, the best combinations of all.

3.2. Effect of the composition of the media on the bacterial viability

It must be taken into account that high concentrations of surfactant can affect the viability of the bacteria, negatively affecting the results of the bactericidal effectiveness of the nanoparticles. The follow culture media and surfactant concentrations were selected for the study of bacterial viability: MH + 2% of Triton X100; MH + 1% of Triton X100; LB + 2% of Triton X100; LB + 1% of Triton X100; MH + 2% of Tween 80; LB + 2% of Tween 80 and BHI + 1.5% of SDS, as well as, controls of the media MH, LB and BHI without surfactants.

A significant bacterial growth was observed on control media without surfactants for both *E. coli* strains. For *E. coli* ATCC, significant differences in growth were not observed in any of the media studied; except for BHI + 1.5% of SDS, where there was a considerable reduction in bacterial growth. In the case of *E. coli* J62, significant differences were observed only for medium of LB + 2% of Triton X100, where a growth reduction of some orders of magnitude was obtained.

For *E. coli* ATCC, the bacterial growth on LB control medium was of the same order as on the medium of LB + 2% of Triton X100, having a growth of $5 \times 10^5 \pm 6 \times 10^4$ CFU mL^{-1} and $3 \times 10^5 \pm 4 \times 10^4$ CFU mL^{-1}, respectively. On the contrary, $4 \times 10^5 \pm 5 \times 10^4$ CFU mL^{-1} grew on BHI medium, whereas there was no growth on the medium of BHI + 1.5% of SDS, which confirmed that SDS at this concentration affected the viability of the bacteria.

The massive growth of *E. coli* J62 in the Petri dishes did not allow colony counting. When 2% of Triton X100 was added to LB medium, countless bacteria also grew, but a qualitative reduction in growth and smaller colony size were observed, suggesting that the surfactant affected their development.

As a results of these viability studies, LB + 2% of Triton X100 and BHI + 1.5% of SDS were rejected as media for antibacterial experiments. Finally, because the viability of bacteria was not affected by surfactant, and the size of nanoparticles on these media was similar to that of nanoparticles in water, it was determined that the two most suitable media which prevent the aggregation of the silver nanoparticles studied were MH + 2% of Tween 80 and MH + 1% of Triton X100. Between both chosen media, no statistically significant differences were observed, and therefore, either of the two media could be chosen as the optimal

medium, although MH + 1% of Triton X100 medium had a higher standard deviation.

Different types of Tween (80 and 20) have been used in studies with silver nanoparticles (Kvítek et al., 2008; Fernando et al., 2019), where it has been observed that Tween 80 provided a considerable enhancement of the silver nanoparticle stability; whereas Triton X100 has been used in studies with silica, ZrO_2, Al_2O_3 or TiO_2 nanoparticles (Yekeen et al., 2019; Tran et al., 2022). Due to this, the medium chosen as optimal for researching interactions between bacteria and nanoparticles was MH + 2% of Tween 80.

3.3. Study of stability over time on size nanoparticles by SP-ICP-MS

A study of stability on size nanoparticles incubated 24 h in the chosen culture medium (MH + 2% of Tween 80) was performed to determine if there is a change in the original size of silver nanoparticles during long incubation periods.

Nanoparticle sizes were measured by ICP-MS after 24 h of incubation in MH + 2% of Tween 80 medium and were compared with nanoparticles diluted in ultrapure water. Results showed that nominal size obtained after the incubation time in the culture medium was 58.1 ± 0.1 nm, whereas the size for silver nanoparticles of 60 nm diluted in ultrapure water was 59.7 ± 0.4 nm. These values are in agreement with each other and with the nominal size certified by the supplier (60 ± 7 nm). Thus, this showed that nanoparticles are stable and do not suffer size transformations during incubation.

Fig. 2 shows the size distributions of 60 nm silver nanoparticles diluted in ultrapure water and incubated in MH + 2% of Tween 80 medium. Both distributions are similar, with mean sizes around 60 nm. No larger nanoparticles were observed in the size distribution, indicating that nanoparticles did not aggregate. In addition, nanoparticle recovery was 91.4 ± 2.5% in the case of the incubation on culture medium, confirming that nanoparticles were not aggregated.

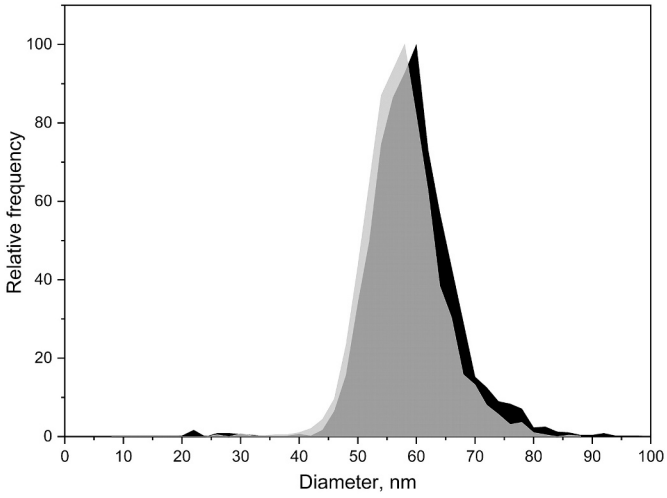

Fig. 2. Size distributions of 60 nm silver nanoparticles in water (black area) and incubated in MH + 2% of Tween 80 medium (gray area).

4. Conclusions

Based on the results of this study, it was determined that non-ionic surfactants (Triton X100 and Tween 80) have a greater ability to stabilize and prevent the aggregation of silver nanoparticles, compared to anionic (SDS) and cationic (CTAB) surfactants. In addition, higher concentrations of surfactants prevent aggregation of silver nanoparticles. In relation to the culture media studied, it was determined that MH and LB were the most stable growth media. Therefore, the optimal culture media were MH and LB with higher concentrations of Tween 80 and Triton X100 surfactants, and as it was demonstrated, these optimal culture media did not affect the viability of bacteria. Aggregation did not occur when nanoparticles were incubated in the medium for long incubations periods, so, the size of nanoparticles was stable over time.

CRediT authorship contribution statement

Ana C. Gimenez-Ingalaturre: Conceptualization, Data curation, Validation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Encarnación Rubio:** Methodology, Data curation, Formal analysis, Writing – review & editing, Visualization, Funding acquisition. **Patricia Chueca:** Data curation, Validation, Resources, Writing – review & editing, Visualization, Funding acquisition. **Francisco Laborda:** Conceptualization, Validation, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Pilar Goñi:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the Spanish Ministry of Science, Innovation and Universities and the European Regional Development Fund, project RTI2018-096111-B-I00 (MICINN/FEDER) and the Government of Aragon (E29_20R and B43_20R).

A.C.G. thanks the Government of Aragón (DGA) for a predoctoral contract.

The authors would like to acknowledge Carlos Lafuente and Héctor Artigas (Group Platon of University of Zaragoza) for the use of their equipment and facilities for density and viscosity measurements; and the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106618.

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