PEGylated AgNP covered with cationic carbosilane dendrons to enhance antibacterial and inhibition of biofilm properties

Andrea Barrios-Gumiel,[1,2,3] Javier Sanchez-Nieves,[1,2,3] Jorge Pérez-Serrano,[4] Rafael Gómez,[1,2,3,*] F. Javier de la Mata[1,2,3,*]

[1] *Dpto. de Química Orgánica y Química Inorgánica. Universidad de Alcalá (UAH). Campus Universitario. E-28871 Alcalá de Henares (Madrid) Spain. Instituto de Investigación Química "Andrés M. del Río" (IQAR). Universidad de Alcalá (UAH). Corresponding authors: andrea.barrios@edu.uah.es; javier.delamata@uah.es*

[2] *Networking Research Center for Bioengineering. Biomaterials and Nanomedicine (CIBER-BBN). Spain*

[3] *Instituto Ramón y Cajal de Investigación Sanitaria. IRYCIS. Spain*

[4] *Dpto. de Biomedicina y Biotecnología, Universidad de Alcalá (UAH), Campus Universitario, E-28871 Alcalá de Henares (Madrid) Spain*

ABSTRACT

This work focuses on preparation of silver nanoparticles (AgNP) covered with cationic carbosilane dendrons and poly(ethylene glycol) (PEG). It is well known that AgNP and cationic carbosilane dendritic systems present antibacterial properties. On the other hand, PEG ligand provides antifouling properties and improved biocompatibility. Hence, combination of both ligands, carbosilane dendrons and PEG, on the AgNP surface can be a way to improve antibacterial capacity of AgNP. The new family of heterofunctionalized AgNP has been directly synthesized using silver precursor and cationic carbosilane dendrons and PEG ligands containing a thiol moiety. AgNP were characterized by TEM, TGA, UV, ^{1}H NMR, DLS, Z potential, XRD. The antibacterial capacity of these systems was evaluated against *E. coli* and *S. aureus*. The results confirmed the influence of both silver core and cationic carbosilane dendrons on the activity of these systems. The behaviour obtained for PEGylated systems were slightly lower than for non-PEGylated AgNP. However, hemolysis assays demonstrated that this decrease was compensated for by the greater biocompatibility. To more completely characterize the improvements of PEGylation on dendronized AgNP, one non-PEGylated and one PEGylated AgNP were tested for resistance in a planktonic state. Both AgNPs barely affected the minimum inhibitory concentration (MIC) whereas reference antibiotics generated significant resistance. In addition, relevant improvement in biofilm inhibition was achieved by dendronized AgNP after PEGylation.

Keywords: silver nanoparticle, PEG, carbosilane dendron, antibacterial, antifouling

INTRODUCTION

The emergence of resistant microorganism to multiple antibiotics constitutes one of the major worldwide health concerns (Ciorba, *et al.*, 2015; WHO, 2018). The recklessness and lack of control with antibiotic administration have caused microorganism adaptation and development of new defense mechanisms (Douafer, *et al.*, 2019; Ventola, 2015; Zaman, *et al.*, 2017). Another important factor that contributes to bacterial resistance is their ability to form biofilms. In nature, bacteria are usually arranged in complex microorganism communities, as a result of irreversible adhesion of planktonic cells to living or inert substrates (Singh, *et al.*, 2017). Due to the high resistance of bacteria in biofilms, their impact on life is of great relevance. Formation of biofilms as a survival mechanism of human pathogenic bacteria results in chronic diseases with complicated treatments and represents approximately 65% of human infectious diseases caused by bacteria (Jamal, *et al.*, 2018). Consequently, the development of new antibacterial agents with non-specific action mechanisms that are able to avoid resistance and microorganism adhesion to surfaces, has gained significant momentum (Coates, *et al.*, 2002; Qayyum and Khan, 2016).

Due to its high activity against a broad range of microorganisms, silver based compounds are currently the most widely used commercially available antimicrobial agents (Chaloupka, *et al.*, 2010; Sim, *et al.*, 2018). Unlike common drugs, silver nanoparticles (AgNP) present many action mechanisms, which make it more difficult to develop resistance. The antibacterial activity of AgNP is related with their ability to release silver ions (Ag^{+}) (Jung, *et al.*, 2008; Morones, *et al.*, 2005; Xiu, *et al.*, 2012), but also to surface oxidation and interaction with biological macromolecules. Through diffusion or endocytosis, AgNP can enter the cell, causing mitochondrial dysfunction, which generates reactive oxygen species (ROS), damaging proteins and nucleic acids, thereby inhibiting cell proliferation (Gopinath, *et al.*, 2010). AgNP can also interact directly with membrane proteins and activate a number of

metabolic pathways involved in transport and respiration, altering ATP production and membrane permeability (McShan, *et al.*, 2014; Prabhu and Poulose, 2012). AgNP aggregation is a determining factor in the toxicity and activity of these systems. This is because the activity is enhanced for dispersed AgNP when compared to aggregated AgNP. In the former case, Ag^{+} release is favorable allowing it to make contact with its target (Li and Lenhart, 2012). Aggregation of AgNP can be controlled by the presence of stabilizing agents like surfactants and polymers (Li, *et al.*, 2012).

Polycationic macromolecules represent a different type of system extensively studied as antimicrobial agents (Huang, *et al.*, 2016). Cationic multivalence is responsible for their activity since they are able to interact with negatively charged bacterial membranes (Chen, *et al.*, 2019; Gottenbos, *et al.*, 2001). These multicationic systems remove divalent cations embedded in membrane structures, inducing its destabilization and subsequent bacterial cell lysis (Clifton, *et al.*, 2015). Dendritic molecules are one type of polycationic macromolecules that have shown such high antimicrobial activity (Tülü and Ertürk, 2012). Their well-defined size, monodispersity and the multivalence of their surface offers several advantages over traditional polymers (Lee, *et al.*, 2005; Tomalia and Fréchet, 2002). Among dendritic molecules, dendrons are cone-shaped macromolecules with similar properties to dendrimers but with an extra reactive moiety at the focal point (Newkome, *et al.*, 2001). Reactivity of focal point allows for more complex structures such as dendronized nanoparticles, transferring dendritic properties to them (Peña-González, *et al.*, 2016; Peña-González, *et al.*, 2017). Cationic carbosilane dendrimers and dendrons have proven to be good antimicrobial agents due to the difference between hydrophilic and hydrophobic parts; cationic peripheral groups and carbosilane framework respectively. While cationic groups modify membrane permeability, the hydrophobic skeleton allows penetration into the phospholipid bilayer

causing membrane disintegration (Fuentes-Paniagua, *et al.*, 2016; Heredero-Bermejo, *et al.*, 2018).

Recently, we have reported the antibacterial and antifungi properties of silver based NPs functionalized with ammonium terminated dendrons (Peña-González, Pedziwiatr-Werbicka, Martín-Pérez, Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017). Microbiologic assays corroborated the relevance of the silver core in contrast with an innocuous gold core, since AgNP are more effective as antibacterial agents than their analogous AuNPs. The antibacterial properties of AgNP are due to the previously mentioned capacity to release silver cations in contact with air and also due to the presence of cationic dendrons.

In spite of the numerous advantages offered by AgNP, these systems could experience problems concerning toxicity. In this sense, it is well known that polyethyleneglycol (PEG) is one of the most commonly used "stealth" polymer to improve the efficiency and biocompatibility of drugs and nanosystems (Amoozgar and Yeo, 2012; Kalaycı, *et al.*, 2010). It has been demonstrated that PEGylation of NPs can reduce their hemotoxic properties and increase the circulation time. This is due to hydrophilic nature of PEG that creates a hydrated cloud, preventing interaction between neighboring NPs and/or blood components (Suk, *et al.*, 2016). On the other hand, the antifouling properties of PEG (Jeon, *et al.*, 1991) are employed to reduce protein adsorption and, consequently, bacterial adhesion and biofilm generation (Banerjee, *et al.*, 2011; Cheng, *et al.*, 2007; Lowe, *et al.*, 2015; Mukherjee, *et al.*, 2019; Xu and Siedlecki, 2017; Yu, *et al.*, 2019).

Accordingly, herein we report synthesis and characterization of heterofunctionalized AgNP covered with cationic carbosilane dendrons, with antibacterial properties, and PEG ligands, with antifouling properties. Their ability as antibacterial systems has been evaluated and resistance studies have also been carried out, comparing the results with fully

dendronized AgNP. Finally, the effect of selected nonPEGylated and PEGylated AgNP on biofilm formation has been tested.

RESULTS AND DISCUSSION

Synthesis of nanoparticles

For simplicity, the following nomenclature will be used to name dendrons described in this work: XG_nY_m, where 'X' represents the functional groups present at the focal point, 'G_n', represent the carbosilane dendron generation, 'Y' denotes nature of peripheral groups and "m" the number of these groups.

The carbosilane dendrons used for the synthesis of AgNP, $HSG_n(S\text{-}NMe_3^+)_m$ (n = 1, m = 2 (**I**); n = 2, m = 4 (**II**); n = 3, m = 8 (**III**)), were previously described (see Figure S1 for full structure) (Peña-González, *et al.*, 2017), and the PEG fragment $CH_3O(CH_2CH_2O)_nCH_2CH_2SH$ (HS-PEG, M_n = 800) was obtained from commercial sources. The synthetic procedure used for preparation of heterofunctionalized AgNP is similar to that previously described to obtain homofunctionalized AgNP (**1-3Ag**) (Peña-González, Pedziwiatr-Werbicka, Martín-Pérez, Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017).

SCHEME 1

The new AgNP were easily synthesized in water by direct reaction of $AgNO_3$ with a mixture of both thiol derivatives, dendrons **I-III** and HS-PEG, and the reducing agent $NaBH_4$ (Scheme 1). With the aim to evaluate influence of both ligands on AgNP properties, three different dendron/HS-PEG ratios were used (3/1, 1/1, 1/3). The following nomenclature was employed to name AgNP described in this work: NxAg, where number 'N' represents the

carbosilane dendron generation, and the 'x' letter represents the dendron/HS-PEG ratio (a = 3/1, b = 1/1, c = 1/3) used. Homofunctionalized AgNP follows the same nomenclature without the 'x' descriptor: NAg. Heterofunctionalized AgNP (**1a-cAg**, **2a-cAg**, **3a-cAg**) were obtained in high yield as black solids that can be dispersed in water and characterized by nuclear magnetic resonance (^{1}H-NMR), ultraviolet spectroscopy (UV-VIS), thermogravimetric analysis (TGA), transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential (ZP) (see Table 1).

Figure 1 shows TEM images and size distribution of first generation heterofunctionalized AgNP (**1a-cAg**). The corresponding images for higher generation NP are collected in the Supporting Information (Figures S2-S7). The size of the AgNP (TEM) was independent of generation or dendron/HS-PEG ratio, presenting diameters from 2 to 4 nm in all cases. These systems were stable, maintaining their size and shape for almost 12 months under inert atmosphere. According to the stabilizing capacity of PEG ligand (Díaz-Cruz, *et al.*, 2016; Li and Lenhart, 2012; Popa, *et al.*, 2007), stability of PEGylated AgNP was higher than that of non-PEGylated **1-3Ag**, whose sizes and shapes changed after 3 months (Peña-González, Pedziwiatr-Werbicka, Martín-Pérez, Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017).

The hydrodynamic diameters (d_z) of AgNP were measured by DLS, obtaining values from 16 to 40 nm. The higher diameters obtained by DLS, compared with those obtained by TEM, are due to the differences between both techniques. Whereas TEM only measures the metallic core, DLS includes dendron cover and solvent layer at the interface. The polydispersity (PDI) and the diameter obtained by DLS (d_z) can be used to calculate a theoretical diameter value ($Cd_n = d_z/(1+Q)^5$); where Q correspond with PDI) (Cho, *et al.*, 2014; Hanus and Ploehn, 1999; Peña-González, Pedziwiatr-Werbicka, Martín-Pérez, Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017). These Cd_n values were

160 closer to those measured by TEM (Table 1). UV spectra also confirmed AgNP formation
161 through the observation of the band corresponding to the surface plasmon resonance, which
162 was detected at *ca*. 440 nm for all NP.
163
164 **FIGURE 1**
165
166 The functionalization of AgNP was also followed by ^{1}H-NMR spectroscopy (Figures S8-
167 S16), in spite of extremely broad signals. The resonances corresponding to dendrons and PEG
168 ligands on **1-3,a-cAg** were observed at similar chemical shifts than the resonances of starting
169 dendrons (**I-III**) and the HS-PEG compound. The only significant difference was the
170 disappearance of the methylene groups CH_2SAg, corresponding with the focal point of the
171 dendron and PEG derivative, due to proximity to the AgNP surface. ^{1}H-NMR spectroscopy
172 also allowed calculation of final dendron/PEG molar ratio on AgNP. This was done by
173 integration of the signals of the external methoxide group of the PEG fragment and the methyl
174 groups of trimethylammonium peripheral moieties of dendrons (Table 1). The experimental
175 dendron/PEG content observed by ^{1}H-NMR was clearly dependent on the initial
176 dendron/PEG ratio and dendron generation. The molar ratio found in the case of **1a-cAg**,
177 functionalized with first generation dendrons, showed an easier entrance of PEG ligands on
178 AgNP surface than of carbosilane dendrons. In these cases, the observed dendron/PEG ratio
179 was smaller than the theoretical ratio. However, for higher generations, the entrance of HS-
180 PEG was hindered, especially in third generation AgNP (**3a-cAg**). For these AgNP, the final
181 dendron/PEG ratios were higher than those theoretically expected from initial ratios.
182 X-ray diffraction (XRD) of selected AgNPs were done (**2aAg** and **2cAg**, Figure S18). The
183 pattern obtained supports the crystalline nature of these AgNP, with peaks corresponding to
184 the 111, 200, 220, and 311 crystallographic planes of the face-centered cubic silver crystals.

185 These data matched with files 00-004-0783 (**2aAg**) and 01-087-0717 (**2cAg**) from the
186 database Joint Committee on Powder Diffraction Standards (JCPDS).
187

AgNP	Molar ratio $HSG_n(S\text{-}NMe_3^+)_m$:HS-PEG		%L[b]	d_n[c]	d_z[d]	Cd_n[e]	PDI[f]	ZP[g]
	Theoretical	Obtained[a]						
1Ag	-	-	53.6	1.7	39.3	7.20	0.404	32.3
1aAg	3:1	1:1	59.8	2.3	21.17	6.34	0.273	23.2
1bAg	1:1	1:2.4	64.3	4.1	21.72	7.17	0.248	22.5
1cAg	1:3	1:4.7	68.5	2.6	28.45	2.82	0.587	10.5
2Ag	-	-	66.7	3.0	40.01	8.26	0.371	35.3
2aAg	3:1	5:1	74.2	2.5	16.44	9.06	0.126	26.4
2bAg	1:1	1.7:1	72.6	4.0	20.97	4.5	0.360	19.9
2cAg	1:3	1:2.2	73.6	2.0	16.53	4.42	0.301	12.6
3Ag	-	-	85.8	3.9	180.0	14.3	0.659	53.0
3aAg	3:1	6.7:1	81.4	2.9	17.89	5.96	0.246	31.8
3bAg	1:1	2:1	74.3	4.9	18.04	5.05	0.290	22.5
3cAg	1:3	1:1.5	75.8	3.0	17.17	4.43	0.311	26.2
PEGAg	-	-	72.5	7.5	128.4	7.20	0.779	-1.2

Table 1. Selected data of AgNP. a) Molar ratio obtained by RMN. b) % Organic matter obtained by TGA, corresponding with dendron and PEG coating. c) Diameter (d_n, nm) obtained by TEM. d) Diameter (d_z. nm) obtained by DLS. e) Diameter calculated (Cd_n, nm): $Cd_n = d_z/(1+Q)^5$; Q corresponds with PDI, (Hanus and Ploehn, 1999). f) Polydispersity index (PDI) obtained by DLS. g) Zeta potencial (mV). **1-3Ag** were previously described (Peña-González, Pedziwiatr-Werbicka, Martín-Pérez, Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017).

Antibacterial activity

Heterofunctionalized cationic/PEG AgNP (**1a-cAg**. **2a-cAg**. **3a-cAg**) were tested as antibacterial agents. Their activity was compared with those observed for analogous AgNP homofunctionalized with cationic dendrons (**1-3Ag**) or PEG ligands (**PEGAg**). *S. aureus* and *E. coli* were chosen as models of Gram-positive and Gram-negative bacteria, respectively. Table 2 summarizes minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values.

	E. coli		***S. aureus***	
	MIC	MBC	MIC	MBC
1Ag	4	8	4	4
1aAg	8	8	8	8
1bAg	16	16	16	32
1cAg	32	32	32	32
2Ag	4	8	4	8
2aAg	8	16	4	8
2bAg	16	16	4	8
2cAg	32	16	8	16
3Ag	16	16	4	4
3aAg	16	16	4	4
3bAg	16	16	4	4
3cAg	16	16	8	16
PEGAg	256	256	256	512

Table 2. Antibacterial activity of homo and heterofunctionalized AgNP. Minimum inhibitory (MIC) and bactericidal concentrations (MBC) are in ppm ($mg \cdot L^{-1}$).

Analysis of the data for each generation showed a general trend: the antibacterial activity of AgNP diminishes with higher PEG content, with **1Ag** being the most active. This trend was not observed for third generation AgNP (**3(a-c)Ag**), probably because the PEG chain is not long enough to project beyond the cationic dendron surface. Thus, cationic groups would be exposed equally, regardless of dendron/PEG ratio.

An improvement in activity by increasing the dendritic generation was not observed. Only AgNP functionalized with first generation dendrons showed a slight decrease in activity against *S. aureus*, compared with AgNP functionalized with second and third generation dendrons. First generation AgNP proved to be good candidates as antibacterial agents, since first generation dendrons are the easiest and cheapest to synthesize.

FIGURE 2

On the other hand, homofunctionalized AgNP with HS-PEG (**PEGAg**) were synthesized and their behavior against bacteria was assessed. **PEGAg** presented rather low activity against both *E. coli* and *S. aureus* (MIC = 256 ppm). Hence, this datum confirms that biocidal capacity is due to the cooperative effect of the silver core and cationic dendrons. Nanoparticles of low active metals such as gold covered with the same cationic dendrons were also barely active (Peña-González, Pedziwiatr-Werbicka, Martín-Pérez, Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017).

It is important to note that the bactericidal properties of AgNP is related to their ability to release silver cations in aerobic conditions (Xiu, Zhang, Puppala, Colvin and Alvarez, 2012). This process could be the reason for higher activity of AgNP covered with smaller dendrons, which enables silver cation release (Peña-González, Pedziwiatr-Werbicka, Martín-Pérez,

Szewczyk, Copa-Patiño, Soliveri, Pérez-Serrano, Gómez, Bryszewska, Sánchez-Nieves and de la Mata, 2017). For the purpose of checking Ag^+ release from AgNP, **1bAg** was selected as model to study. On the first day after purification of these systems, a stock AgNP solution (360 ppm) was prepared and stored under inert atmosphere. 10 mL of this solution was collected, bubbled with an oxygen flow, to favor Ag^+ release, and ultrafiltrated (MWCO = 1000 Da). Then, the silver content was analyzed by ICP. This process was repeated after 2, 3, 6 and 12 weeks. Results revealed minimum Ag^+ ions released for all of the samples. Moreover, the antibacterial capacity of solutions treated as above were analyzed and were not affected during this time. These results reveal the high stability for these systems if they are stored under inert atmosphere (Figures S18-S19).

Biocompatibility

In regard to the use of these nanoparticles as antibacterial agents, preliminary biocompatibility studies were carried out. Hemolysis was determined by measuring the free hemoglobin content after erythrocytes incubation with AgNP for 2 h. in different concentrations. Table 3 summarizes the concentration of AgNP when 50% hemolysis is observed (H50) (Figure 3) and the percentage of the hemolysis at the MBC for the two different types of bacteria evaluated (*S. aureus*, Figure 4a; *E. coli*, Figure 4b).

	H50[a] (ppm)	***S. aureus***		***E. coli***	
		MBC	%H(MBC)[b]	MBC	%H(MBC)[b]
1Ag	16	8	2	8	2.0
1aAg	29.7	8	3.6	8	3.6
1bAg	511	32	0.9	16	0.7
1cAg	428	64	6.7	32	0.5
2Ag	7	4	31.3	8	71.2

2aAg	12	4	21.0	16	61.5
2bAg	15	8	30.5	16	50.3
2cAg	21	16	35.3	16	35.3
3Ag	6	8	56.8	16	91.2
3aAg	10	4	30.4	16	72.8
3bAg	9	4	27.8	16	65.7
3cAg	9	4	33.5	16	74.8
PEGAg	>1024	256	20.6	256	20.6

Table 3. Hemolytic activity of AgNP. a) Concentration of AgNP when 50% hemolysis is reached. b) Percentage of hemolysis at the MBC.

The data showed lower values of H50 for non-PEGylated **1-3Ag** with respect to PEGylated (**1-3,a-cAg**). This result demonstrates that biocompatibility of these systems is favored by PEGylation. The PEGylation effect was considerably more remarkable for first generation nanoparticles, with **1bAg** and **1cAg** being significantly less toxic than **1aAg** and non-PEGylated **1Ag**, with H50 values of 512, 428, 29, and 16 ppm, respectivley. The biocompatibility of the second generation was slightly enhanced when PEG proportion was higher, whereas the PEGylation of third generation AgNP did not present major improvements. These results are in agreement with the assessment done for the effect of PEGylation on antibacterial activity, the higher size of third generation dendron cover PEG chains avoiding their exposure.

FIGURE 3

FIGURE 4

Hemolysis percentage at MBC was calculated in order to know the selectivity of these systems and the possibility to be used as biocides (Table 3, Figure 4). Striking results were found for all AgNP functionalized with first generation dendrons, with hemolysis being lower than 10% at this concentration. This value was even below 1% for PEGylated **1bAg**. However, for AgNP decorated with second generation dendrons, the hemolysis at the MBC was clearly higher. In general, the activity of AgNP against *S. aureus* was higher than against *E. coli*. Thus, the percentages of hemolysis reached at the MBC in *S. aureus* were below 50%, except for non-PEGylated **3Ag**, whereas for *E. coli* were above 50%.

Hence, taking into account antibacterial acitivity, toxicity, and synthetic cost, non-PEGylated **1Ag** and PEGylated **1bAg** were selected as the best candidate for further antibacterial studies.

Bacterial resistance studies

Antibiotic resistant bacteria are a main worldwide concern (Alanis, 2005). Therefore, we assessed the ability of **1Ag** and **1bAg** to induce bacterial resistance. Commercial antibiotics such as erythromycin or penicillin (PenVK), active against *S. aureus*, and gentamycine or tetracycline, active against *E. coli*, were also included in this study with the aim of establishing a comparison with our systems. These assays were carried out through the daily assessment of the MIC during 15 cycles, using a subculture arising from the MIC determined in the previous cycle. Comparisons between MIC obtained on the last day (MIC_{15}) and that obtained on the first day (MIC_1) allowed for the determination of the evolution of the inhibitory capacity of the tested compounds (Figure 5).

FIGURE 5

While commercial antibiotics employed here induced high resistance on *S. aureus* ($MIC_{15}/MIC_1 > 1000$), AgNP (**1bAg, 1Ag**) did not generate bacterial resistance ($MIC_{15}/MIC_1 = 2$). Regarding *E. coli*, commercial drugs had a lesser tendency to generate bacterial resistance than in *S. aureus* ($MIC_{15}/MIC_1 = 16$). However, this value was still higher than that obtained for heterofunctionalized **1bAg** ($MIC_{15}/MIC_1 = 4$) and homofunctionalized **1Ag** ($MIC_{15}/MIC_1 = 1$). These results are in accordance with a non-specific action mechanism of AgNP covered with cationic dendrons.

Inhibition of *S. aureus* biofilm formation assays

One of the main goal of heterofunctionalization of cationic AgNP with PEG ligands was to endow these systems with antifouling capacity (Michel, *et al.*, 2005). With this in mind, the ability to prevent or minimize biofilm formation of *S. aureus* of selected PEGylated and not-PEGylated AgNP (**1bAg**, **1Ag**) was carried out. Results showed that both AgNP totally inhibited the growth of biofilms at concentrations above MIC values, although below these concentrations biofilm formation was not inhibited. Nevertheless, unlike homofunctionalized cationic AgNP (**1Ag**) that did not present any effect below MIC, PEGylated AgNP (**1bAg**) reduced this formation at all tested concentrations below MIC up to 40% (Figure 6).

FIGURE 6

CONCLUSIONS

Direct reaction of different thiol derivatives with a silver salt is a suitable procedure for synthesis of heterofunctinalized AgNP. With this approach a new family of AgNP functionalized with cationic carbosilane dendrons, with antibacterial properties, and PEG chains, demonstrating biocompatibility and with antiadherent properties, was obtained. A dependence on dendron/PEG ratio and dendritic generation on the functionalization degree

was clearly observed. The PEG entrance on AgNP surface was easier when first generation dendrons were used. Although the antibacterial activity of heterofunctionalized AgNP, with cationic dendrons and PEG, is slightly below that of homofunctionalized AgNP with cationic dendrons, this reduction is compensated for by the greater biocompatibility of these systems. In the particular case of **1bAg**, hemolysis was reduced to only 1% at the MBC. Due to the successful results achieved for **1bAg**, this AgNP and its analogous non-PEGylated **1Ag** were selected to study bacterial resistance and biofilm formation. None of these AgNP generated resistance against *S. aureus* and only **1bAg** produced a slight resistance against *E.coli*, due to the nonspecific mechanism of systems. In addition, the capacity to avoid biofilm formation was evaluated with of **1bAg** and **1Ag**, concluding that PEGylation improves inhibition of biofilm formation with respect to homofunctionalized systems, in accordance with the anti-adhesive capacity of PEG. Therefore, heterofunctionalization of AgNP with cationic dendrons and PEG ligands can be considered an attractive tool in the search for new treatments against bacterial infections.

MATERIALS AND METHODS

General considerations

All reactions were carried out under inert atmosphere and solvents were purified from appropriate drying agents when necessary. Unless otherwise stated, reagents were obtained from commercial sources and used as received. Compounds $HSG_n(S\text{-}NMe_3Cl)_m$ (where n = 1, m = 2; n = 2, m = 4; n = 3, m = 8) were synthesized as published (Peña-González, Pedziwiatr-Werbicka, Shcharbin, Guerrero-Beltrán, Abashkin, Loznikova, Jiménez, Muñoz-Fernández, Bryszewska, Gómez, Sánchez-Nieves and de la Mata, 2017). Thiol-ene reactions were carried out employing a HPK 125W Mercury Lamp from Heraeus Noblelight with maximum energy at 365 nm, in normal glassware under inert atmosphere. NMR spectra were recorded on a

Varian Unity VXR-300 (300.13 (^{1}H). Chemical shifts (δ) are given in ppm. ^{1}H resonances were measured relative to solvent peaks considering TMS = 0 ppm. UV-vis absorption was measured with a Perkin-Elmer Lambda 18 spectrophotometer. The spectra were recorded by measuring dilute samples in a quartz cell with a path length of 1 cm. The silver content of filtered solutions were determined by ICP (Inductive Coupling Plasma) using an ICP Optical Emission Spectrometer Varian 720-ES at 328.068 nm. The detection limit is below 10 ppb. The X-ray diffraction data collection (XRD) was done using a Malvern Panalalytical Empyrean, using Cu-Kα radiation source from 5º to 90º (2θ).

Synthesis of compounds

A description of the synthesis of first generation AgNP with equal HSG_1(S-NMe_3Cl)/HS-PEG ratio (**1bAg**) is as follows. The procedures and data of all compounds can be found in the Supporting Information.

*AgNP@(SG_1(S-$NMe_3Cl)_2$@PEG (1/1) (**1bAg**).* An aqueous solution (24 mL, 12.5 mM) of a mixture containing compounds HSG_1(S-$NMe_3Cl)_2$ (0.15 mmol. 74.6 mg) and HS-PEG Mn = 1000 (0.15 mmol, 120 mg) was added dropwise to an aqueous solution of $AgNO_3$ (10 mL, 30 mM, 0.3 mmol, 50.9 mg). Afterwards, a solution of $NaBH_4$ in water (7.5 mL, 200 mM, 1.5 mmol, 56.7 mg) was added dropwise and the mixture was stirred 4 h at room temperature. Then, nanoparticles formed in this reaction were purified by dialysis (MWCO 10 kDa, yielding **1bAg** (74.7 mg).

Data for **1bAg**: NMR (D_2O): ^{1}H NMR: δ 0.06 (SiCH_3), 0.60 ($SCH_2CH_2CH_2CH_2Si$), 0.90 ($SiCH_2CH_2S$), 1.40 ($SCH_2CH_2CH_2CH_2Si$), 1.78 ($SCH_2CH_2CH_2CH_2Si$), 2.74 ($SiCH_2CH_2S$), 2.97 (SCH_2CH_2N), 3.10 (NCH_3), 3.50 (SCH_2CH_2N). Reagents molar ratio HSG_1(S-$NMe_3Cl)_2$/HS-PEG = 1/1. Calc. molar ratio (NMR) HSG_1(S-$NMe_3Cl)_2$/HS-PEG = 1/1. TGA (%): Ag, 35.7; L, 64.3. SPR (UV-vis): 447.3 nm. Zeta Potential (mV): 22.5. DLS (nm): 21.7. Mean diameter of silver core (TEM) = 4.1 nm.

Hemolytic studies

2 mL of defibrinated sheep blood (RBC) were centrifuged at 800 g for 10 min. The supernatant is eliminated and the pellet (RBC) is washed three times with phosphate buffer saline (PBS. pH 7.4; NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM and KH_2PO_4 1.8 mM) and resuspended in 2 mL of PBS. A 1:50 dilution of this RBC solution in PBS was done and incubated for 15 minutes at 37ºC. AgNP solutions were prepared in the range of 2.5 to 5120 ppm. 20 µL of each solutions and 180 µL of RBC incubated solution were added to Eppendorf tubes, obtaining solutions ten times less concentrated (0.25 to 1024 ppm). These solutions were incubated for 2 hours. Subsequently, tubes were centrifuged (15 min at 800 g) and the supernant was transferred to 96 well tray microplates. Hemolysis (H_x) was monitored by measuring the hemoglobin release by the absorbance at 540 nm. Controls of all concentrations were done by adding 20 µL of nanoparticles and 180 µL of PBS (H_{x0}). 100% hemolysis (H_{100}) was achieved by adding of 20 µL of Triton X100 at 20% solution to 180 µL of RBC. As 0% hemolysis (H_0) control value was used the absorbance of the supernant of 20 µL of PBS and 180 µL RBC solution. The percentage of hemolysis was calculated as [$(H_x - H_{xo})/(H_{100} - H_0)$]. The AgNP concentrations required to cause 50% hemolysis (H_{50}) was calculated by interpolation between the closest points. H_{MBC} corresponds to the percentage of hemolysis at MBC concentration.

Antibacterial activity

Bacterial strains. *Escherichia Coli* (CECT 515, Gram-negative), *Staphylococcus aureus* (CECT 240, Gram-positive) were obtained from the Spanish Type Culture Collection (CECT). The rest of the resistant strains were generated in the resistance induction assays carried out in this work.

MIC and MBC. The minimal inhibitory concentration (MIC) of the products was measured in 96-well tray microplates by microdilution tray preparations following the

international standard methods ISO 20776-1. Solutions of the products were prepared in the range of 0.25 to 1024 ppm adding 100 μL of one of these solutions to each well: 100 μL of double concentration Mueller Hinton (Scharlau. ref. 02-136) and 5 μL of a bacteria suspension of 2 x 10^7 CFU ml^{-1}. Microplates were incubated at 37 ºC for 20 h using an ultra-microplate reader ELX808iu (Bio Tek Instrument), considering the MIC the minimal concentration for which no turbidity was observed. The minimal bactericidal concentration (MBC) was calculated by inoculating Petri dishes containing Muller-Hinton agar with 5 μL of the sample used for MIC assessment. Samples were tested as droplets on the plates. Microbial growth on plates was monitored after 24 h of incubation at 37ºC. The MBC was determined as the minimal concentration at which no growth was detected.

Induction of resistances. Initial MIC values (MIC_1) were determined for *E. coli* CECT 515 and *S. aureus* CECT 240 as described above. MIC values were monitored daily for 15 days as follows. The first day MIC was calculated as described above (MIC_n). Then, for next day, 100 μL were taken from the well treated at the concentration below MIC. This suspension was incubated and adjusted to 2 x 10^7 CFU mL^{-1}. Then, the protocol to calculate MIC was done again (MIC_{n+1}). This procedure was repeated for 15 days (MIC_{15}). The relative MIC value (MIC_{15}/MIC_1) was determined by calculating the ratio of the MIC obtained for the 15^{th} subculture (MIC_{15}) to MIC obtained for the first culture (MIC_1).

Biofilm formation and quantification assay. The biofilm formation of *S. aureus* CECT 240 was established in microtiter plates (96 wells, NUNC). From Petri dish where *S. aureus* was growing, 10 CFU (colony forming unit) was used to inoculate 4 mL of commercial trypticase soy broth (TSB) supplemented with 0.4% glucose and 0.3% yeast extract. Then, it was kept for 3 h at 37 ^{0}C in a bath with shaking (110 rpm) for obtaining a pre-inoculum with a value of absorbance at 625 nm of 0.6–0.9 units. Culture was then diluted 1:100 in fresh TSB medium supplemented, and 200 mL was dispensed into each well of the NUNC microtiter

plates. After 20 h of incubation at 37 ^{0}C in static, the absorbance at 630 nm was measured in an ultra-microplate reader (Biotek, ELx 808). Moreover, in the microtiter plates where the solution of the wells were retired, the biofilm formed was stained with 200 mL of 10% crystal violet, rinsed three times with 200 mL of commercial phosphate buffered saline (PBS) and air dried. The crystal violet-stained biofilm formation was quantified by solubilizing the crystal violet with 200 mL 33% acetic acid solution. This solution was added to wells in a new microtiter plate and the absorbance at 630 nm was measured in the microplate reader.

Pre-biofilm treatment. The ability of **1bAg** and **1Ag** compounds to inhibit the biofilm formation of *S. aureus* was analyzed. Solutions of the products were prepared in the range of 0.0625 to 1024 ppm adding to each well 100 mL of one of these solutions, 100 mL of *S. aureus* inoculum done in double concentration commercial TSB. Microplates were incubated at 37ºC for 20 h in static. The planktonic and sessile cells, and the amount of biofilm formed were measured as explained above.

SUPPLEMENTARY DATA

Full experimental data, dendrons structures, TEM images and size distribution, ^{1}H-NMR spectra of all AgNP and Ag^{+} release graphic can be found in Supplementary Information.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Alanis, A. J.; 2005. Resistance to antibiotics: Are we in the post-antibiotic era? Arch Med Res, 36, 697-705.

Amoozgar, Z. and Yeo, Y.; 2012. Recent advances in stealth coating of nanoparticle drug delivery systems. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 4, 219-233.

Banerjee, I., Pangule, R. C. and Kane, R. S.; 2011. Antifouling coatings: Recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms. Adv. Mater., 23, 690-718.

Ciorba, V., Odone, A., Veronesi, L., Pasquarella, C. and Signorelli, C.; 2015. Antibiotic resistance as a major public health concern: Epidemiology and economic impact. Ann Ig, 27, 562-579.

Clifton, L. A., Skoda, M. W., Le Brun, A. P., Ciesielski, F., Kuzmenko, I., Holt, S. A. and Lakey, J. H.; 2015. Effect of divalent cation removal on the structure of gram-negative bacterial outer membrane models. Langmuir, 31, 404-412.

Coates, A., Hu, Y., Bax, R. and Page, C.; 2002. The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov, 1, 895-910.

Chaloupka, K., Malam, Y. and Seifalian, A. M.; 2010. Nanosilver as a new generation of nanoproduct in biomedical applications. Trends Biotechnol, 28, 580-588.

Chen, Y., Yu, L., Zhang, B., Feng, W., Xu, M., Gao, L., Liu, N., Wang, Q., Huang, X., Li, P. and Huang, W.; 2019. Design and synthesis of biocompatible, hemocompatible and highly

selective antimicrobial cationic peptidopolysaccharides via click chemistry. Biomacromolecules, doi: 10.1021/acs.biomac.1029b00179.

Cheng, G., Zhang, Z., Chen, S., Bryers, J. D. and Jiang, S.; 2007. Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces. Biomaterials, 28, 4192-4199.

Cho, T. J., MacCuspie, R. I., Gigault, J., Gorham, J. M., Elliott, J. T. and Hackley, V. A.; 2014. Highly stable positively charged dendron-encapsulated gold nanoparticles. Langmuir, 30, 3883-3893.

Díaz-Cruz, C., Alonso Nuñez, G., Espinoza-Gómez, H. and Flores-López, L. Z.; 2016. Effect of molecular weight of peg or pva as reducing-stabilizing agent in the green synthesis of silver-nanoparticles. Eur Polym J, 83, 265-277.

Douafer, H., Andrieu, V., Phanstiel, O. and Brunel, J. M.; 2019. Antibiotic adjuvants: Make antibiotics great again! J. Med. Chem, DOI: 10.1021/acs.jmedchem.1028b01781.

Fuentes-Paniagua, E., Sánchez-Nieves, J., Hernández-Ros, J. M., Fernández-Ezequiel, A., Soliveri, J., Copa-Patiño, J. L., Gómez, R. and Javier de la Mata, F.; 2016. Structure–activity relationship study of cationic carbosilane dendritic systems as antibacterial agents. RSC Adv, 6, 7022-7033.

Gopinath, P., Gogoi, S. K., Sanpui, P., Paul, A., Chattopadhyay, A. and Ghosh, S. S.; 2010. Signaling gene cascade in silver nanoparticle induced apoptosis. Colloids Surf B, 77, 240-245.

Gottenbos, B., Grijpma, D. W., van der Mei, H. C., Feijen, J. and Busscher, H. J.; 2001. Antimicrobial effects of positively charged surfaces on adhering gram-positive and gram-negative bacteria. J Antimicrob Chemother, 48, 7-13.

Hanus, L. H. and Ploehn, H. J.; 1999. Conversion of intensity-averaged photon correlation spectroscopy measurements to number-averaged particle size distributions. 1. Theoretical development. Langmuir, 15, 3091-3100.

Heredero-Bermejo, I., Hernández-Ros, J. M., Sánchez-García, L., Maly, M., Verdú-Expósito, C., Soliveri, J., de la Mata, F. J., Copa-Patiño, J. L., Pérez-Serrano, J., Sánchez-Nieves, J. and Gómez, R.; 2018. Ammonium and guanidine carbosilane dendrimers and dendrons as microbicides. Eur Polym J, 101, 159-168.

Huang, K.-S., Yang, C.-H., Huang, S.-L., Chen, C.-Y., Lu, Y.-Y. and Lin, Y.-S.; 2016. Recent advances in antimicrobial polymers: A mini-review. Int J Mol Sci, 17, E1578.

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M. and Kamil, M. A.; 2018. Bacterial biofilm and associated infections. J Chin Med Assoc, 81, 7-11.

Jeon, S. I., Lee, J. H., Andrade, J. D. and Degennes, P. G.; 1991. Protein—surface interactions in the presence of polyethylene oxide: I. Simplified theory. J. Colloid Interface Sci., 142, 149.

Jung, W. K., Koo, H. C., Kim, K. W., Shin, S., Kim, S. H. and Park, Y. H.; 2008. Antibacterial activity and mechanism of action of the silver ion in staphylococcus aureus and escherichia coli. Appl Environ Microbiol, 74, 2171-2178.

Kalaycı, Ö. A., Cömert, F. B., Hazer, B., Atalay, T., Cavicchi, K. A. and Cakmak, M.; 2010. Synthesis, characterization, and antibacterial activity of metal nanoparticles embedded into amphiphilic comb-type graft copolymers. Polym Bull, 65, 215–226.

Lee, C. C., MacKay, J. A., Fréchet, J. M. and Szoka, F. C.; 2005. Designing dendrimers for biological applications. Nat Biotechnol, 23, 1517-1526.

Li, X. and Lenhart, J. J.; 2012. Aggregation and dissolution of silver nanoparticles in natural surface water. Environ Sci Technol, 46, 5378-5386.

Li, X., Lenhart, J. J. and Walker, H. W.; 2012. Aggregation kinetics and dissolution of coated silver nanoparticles. Langmuir, 28, 1095-1104.

Lowe, S., O'Brien-Simpson, N. M. and Connal, L. A.; 2015. Antibiofouling polymer interfaces: Poly(ethylene glycol) and other promising candidates. Polym. Chem., 6, 198-212.

McShan, D., Ray, P. C. and Yu, H.; 2014. Molecular toxicity mechanism of nanosilver. J Food Drug Anal, 22, 116-127.

Michel, R., Pasche, S., Textor, M. and Castner, D. G.; 2005. Influence of peg architecture on protein adsorption and conformation. Langmuir, 21, 12327-12332.

Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramirez, J. T. and Yacaman, M. J.; 2005. The bactericidal effect of silver nanoparticles. Nanotechnology, 16, 2346-2353.

Mukherjee, I., Ghosh, A., Bhadury, P. and De, P.; 2019. Matrix assisted antibacterial activity of polymer conjugates with pendant antibiotics, and bioactive and biopassive moieties. J. Mater. Chem. B., 7, 3007-3018.

Newkome, G. R., Moorefield, C. N. and Vögtle, F.; 2001. Dendrimers and dendrons: Concepts, syntheses, applications. Wiley-VCH Verlag GmbH,

Peña-González, C. E., García-Broncano, P., Ottaviani, M. F., Cangiotti, M., Fattori, A., Hierro-Oliva, M., González-Martín, M. L., Pérez-Serrano, J., Gómez, R., Muñoz-Fernández, M. A., Sánchez-Nieves, J. and de la Mata, F. J.; 2016. Dendronized anionic gold nanoparticles: Synthesis, characterization, and antiviral activity. Chem. Eur. J., 22, 2987-2999.

Peña-González, C. E., Pedziwiatr-Werbicka, E., Martín-Pérez, T., Szewczyk, E. M., Copa-Patiño, J. L., Soliveri, J., Pérez-Serrano, J., Gómez, R., Bryszewska, M., Sánchez-Nieves, J. and de la Mata, F. J.; 2017. Antibacterial and antifungal properties of dendronized silver and gold nanoparticles with cationic carbosilane dendrons. Int J Pharm, 528, 55-61.

Peña-González, C. E., Pedziwiatr-Werbicka, E., Shcharbin, D., Guerrero-Beltrán, C., Abashkin, V., Loznikova, S., Jiménez, J. L., Muñoz-Fernández, M. Á., Bryszewska, M.,

Gómez, R., Sánchez-Nieves, J. and de la Mata, F. J.; 2017. Gold nanoparticles stabilized by cationic carbosilane dendrons: Synthesis and biological properties. Dalton Trans, 46, 8736-8745.

Popa, M., Pradell, T., Crespo, D. and Calderón-Moreno, J. M.; 2007. Stable silver colloidal dispersions using short chain polyethylene glycol. Colloids Surf. A, 303, 184-190.

Prabhu, S. and Poulose, E. K.; 2012. Silver nanoparticles: Mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. Int Nano Lett, 2, 32.

Qayyum, S. and Khan, A. U.; 2016. Nanoparticles vs. Biofilms: A battle against another paradigm of antibiotic resistance. Med. Chem. Commun., 7, 1479-1498.

Sim, W., Barnard, R. T., Blaskovich, M. A. T. and Ziora, Z. M.; 2018. Antimicrobial silver in medicinal and consumer applications: A patent review of the past decade (2007-2017). Antibiotics (Basel), 7, E93.

Singh, S., Singh, S. K., Chowdhury, I. and Singh, R.; 2017. Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. Open Microbiol J, 11, 53-62.

Suk, J. S., Xu, Q., Kim, N., Hanes, J. and Ensign, L. M.; 2016. Pegylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev, 99, 28-51.

Tomalia, D. A. and Fréchet, J. M.; 2002. Discovery of dendrimers and dendritic polymers: A brief historical perspective. J Polym Sci Part A: Polym Chem, 40, 2719-2728.

Tülü, M. and Ertürk, A. S.; 2012. Dendrimers as antibacterial agents. A search for antibacterial agents, IntechOpen.

Ventola, C. L.; 2015. The antibiotic resistance crisis: Part 1: Causes and threats. Pharm Ther, 40, 277-283.

WHO; 2018. Global action plan on antimicrobial resistance. Antimicrobial Resistance,

Xiu, Z. M., Zhang, Q. B., Puppala, H. L., Colvin, V. L. and Alvarez, P. J.; 2012. Negligible particle-specific antibacterial activity of silver nanoparticles. Nano Lett, 12, 4271-4275.

Xu, L. C. and Siedlecki, C. A.; 2017. Protein adsorption, platelet adhesion, and bacterial adhesion to polyethylene-glycol-textured polyurethane biomaterial surfaces. J Biomed Mater Res B Appl Biomater, 105, 668-678.

Yu, M., Ding, X., Zhu, Y., Wu, S., Ding, X., Li, Y., Yu, B. and Xu, F.-J.; 2019. Facile surface multi-functionalization of biomedical catheters with dual-microcrystalline broad-spectrum antibacterial drugs and antifouling poly(ethylene glycol) for effective inhibition of bacterial infections. ACS Applied Bio Materials, 2, 1348-1356.

Zaman, S. B., Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T. and Hossain, N.; 2017. A review on antibiotic resistance: Alarm bells are ringing. Cureus, 9, e1403.

Scheme 1. Synthesis of heterofunctionalized AgNP (n=1, m=2 (**1a-cAg**); n=2, m=4 (**2a-cAg**); n=3, m=8 (**3a-cAg**)). The full structure of dendrons can be seen in Figure S1.

Figure 1. TEM image and size distribution histogram associated to a) **1aAg**, b) **1bAg** and c) **1cAg**.

Figure 2. MBC plots of synthesized AgNP with different dendritic generation and $HSG_n(S\text{-}NMe_3^+)_m$/HS-PEG ratios against *S. aureus* (A) and *E. coli* (B).

Figure 3. Concentration of 50% hemolysis (H50) for homofunctionalized and heterofunctionalized AgNP.

Figure 4. Hemolysis percentage at MBC. a) *Staphylococcus aureus*. b) *Escherichia coli.*

Figure 5. Induction resistance tests of **1Ag** and **1bAg** in *S. aureus* (a) and *E. coli* (b) after 15 survival cycles in the presence of antibacterial agents. MIC_{15}/MIC_1 is the ratio obtained from the 15^{th} subculture (MIC_{15}) and the 1^{st} subculture (MIC_1).

Figure 6. Inhibition of *S. aureus* biofilm formation with **1bAg** (blue) and **1Ag** (red) calculated from pre-biofilm treatment assay.