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Research paper

Potential anti-adhesion activity of novel carbosilane zwitterionic dendrimers against eukaryotic and prokaryotic pathogenic microorganisms

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ABSTRACT

The development of biofilms on different surfaces continues to be a major public health problem. The antimicrobial resistance and the difficulty of finding drugs capable of combating these established biofilms generates the urgent need to find compounds that prevent cells from settling and establishing of these complex communities of microorganisms. Zwitterionic modification of nanomaterials allows the formation of a hydration layer, and this highly hydrophilic surface provides antifouling properties as well as a good biocompatibility by preventing non-specific interactions. Thus, they are appropriate candidates to prevent microbial adhesion to different surfaces and, in consequence, avoid biofilm formation. For this reason, we have incorporated zwitterionic moieties in multivalent systems, as are carbosilane dendrimers. Characterization of these systems was performed using nuclear magnetic resonance and mass spectrometry. It has been analysed if the new molecules have capacity to inhibit the biofilm formation in *Candida albicans, Staphylococcus aureus* and *Pseudomonas aeruginosa.* The results showed that they were more effective against *S. aureus,* observing a biofilm reduction of 81.5% treating with 32 mg/L of G2SiZWsf dendrimer and by 72.5% using 32 mg/L of the G3SiZWsf dendrimer. Finally, the absence of cytotoxicity was verified by haemolysis and cytotoxicity studies in human cells lines.

1. Introduction

The human body is usually colonized by normally innocuous and even beneficial microorganisms, its normal biota, and there is even a kind of symbiosis between them. However, sometimes these opportunistic microorganisms can be responsible of serious diseases that can be lethal for the host that suffers them. Among the prokaryotic microorganisms that cause serious nosocomial human infections are two bacteria, *Staphylococcus aureus* (gram positive) and *Pseudomonas aeruginosa* (gram negative) [1,2,3,4]. On the other hand, among the eukaryotic microorganisms, the pathogenic yeast, *Candida albicans*, is responsible of fungal infections that are considered a serious health problem worldwide [5]. These microorganisms are opportunistic pathogens and the increase in mortality in patients is higher when the pathogen reaches the bloodstream, causing sepsis or candidiasis. This problematic is associated to the ability of these microorganisms to form biofilms.

Biofilms are complex sessile cell structures capable of establishing themselves on biotic and abiotic surfaces [6,7]. These communities protect cells from adverse environmental conditions or the immune system and make cells more resistant against antibiotics and disinfectants. This phenomenon is produced by bacteria and fungi and causes difficulties to treat them in the medical and food fields, among others. While planktonic bacteria in suspension are more susceptible to host immune cells and antibiotics [8], bacteria in a biofilm often confers drug resistance, making it difficult to destroy biofilms once established. This fact is important in implantable medical devices, that are optimal surfaces for biofilm formation and can lead to infections. Therefore, eliminating these structures, or better yet, preventing them from being set up, would be an important strategic action to deal with the problematic associated and prevent biofilm related infections.

Dendrimers have emerged as an attractive non-antibiotic strategy against pathogenic bacteria [9]. Dendrimers are macromolecules with a

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highly branched tridimensional structure in which branches consisting of a repetitive monomer grow from the core. Each of these branching points allows the incorporation of new repetitive units, thus increasing the generation. Their multivalent nature, narrow polydispersity, unique structural geometry, and the possibility of further polymerize or modify their surface with desired functional peripheral groups bring a huge range of applications. In nanomedicine they have been used as drug transporters [10,11] as anticancer [12,13,14] or antiviral [15,16] drugs and as nucleic material carriers [17,18]. Dendrimers have also been employed in the treatment, prevention, and diagnosis of infectious diseases [9,19,20]. Their ability to prevent biofilm formation has been tested in bacteria [21,22,23] and yeast [24,25]. In most of cases it is the high density of peripheral positively charged groups that provides the antibacterial properties [20,26]. However, this positive charges often induce cytotoxicity and blood toxicity, which is undesirable for nanomaterials to be used in biomedicine to avoid biofilms. There are some different methods to reduces such toxicity: PEGylation or zwitterionation. Those methods have been widely used for developing biocompatible and non-fouling materials [27,28,29,30,31]. Both strategies allow the formation of a hydration layer which avoids unspecific binding [30,32]. Poly (ethylene glycol) (PEG) has been widely used as non-fouling material because of its commercial availability and high hydrophilicity [32]. Its hydration layer is formed by hydrogen bonding [30]. However, their antifouling properties are affected by its degradation in the presence of oxygen and transition metal ions, which results in weakened antifouling properties [27,33]. Furthermore, PEG has been reported to induce immune response and allergies [34,35]. For these reasons, the use of zwitterionic materials has increased during the last years for their outstanding biocompatibility and antiadhesive properties. Zwitterionic compounds are bio-mimetic [30] electrically neutral compounds, with an equal number of positive and negative groups, their net charge is zero. Unlike PEG, they form a highly dense hydration layer via ionic hydration [32]. These compounds have been studied as an approach for functionalizing surfaces to inhibit bacterial and non-specific protein adhesion [33,36,37].

In this work, we have synthesized for the first time in the literature four generations of a new carbosilane (CBS) dendrimer bearing zwitterionic functionalization in the periphery and a silicon atom as core. We have incorporated sulfobetaine groups by reaction of dimethylamine ending precursors with propanesultone that should provide solubility, biocompatibility, and lack of toxicity, which added to dendrimers molecular stability could be important properties for nanomaterials aimed to prevent biofilms development. To confirm this hypothesis, the ability of these newly synthesised dendrimers to inhibit biofilm formation in prokaryotic and eukaryotic pathogenic microorganisms was subsequently evaluated, and biocompatibility studies on the ability of the compounds to produce haemolysis and to be cytotoxic in different human cell lines were carried out. The results are promising for studying the use of these molecules to functionalise surfaces and prevent the appearance of biofilms on them.

2. Materials and methods

2.1. Synthesis and characterization of zwitterionic CBS dendritic systems GnSiZWsf

First, dendrimers derived from a Si atom core bearing vinyl functionalization in their periphery (G_nSiV_m) [38] were modified by thiol–ene addition of 2-dimethylaminoethanethiol hydrochloride ($HS(CH_2)_2NMe_2{\cdot}HCl$) and then neutralized with base (NaOH) to afford $G_nSi(S–NMe_2)_m$ precursor dendrimers (n = 0, m = 4 (I); n = 1, m = 8 (II); n = 2, m = 16 (III); n = 3, m = 32 (IV)) as previously reported [20]. (See synthesis of precursor G3SiV32, not previously published, detailed in Supporting Information, Scheme S1, Figure S1-S8 and Table S1).

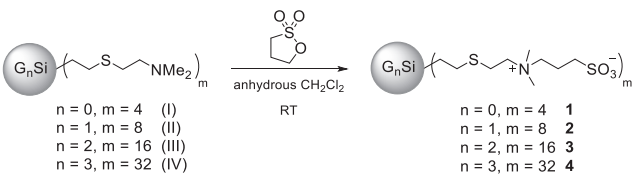
Then, neutral dendrimers $G_nSi(S–NMe_2)_m$ (I-IV) were dissolved in anhydrous dichloromethane, 1.5 equivalents per branch (1,5·m) of 1,3-propanesultone added and left to react overnight at room temperature. The formation of zwitterionic compounds GnSiZWsf can be easily observed as they precipitate. Reaction completion was confirmed by ^1H NMR in $CDCl_3$. Afterwards, reaction mixture was transferred to an extraction funnel and water added to recover zwitterionic product in the aqueous phase. Finally, water was eliminated by rotary evaporation to afford compounds **1**–**4** (Scheme 1) as white to brownish solids with quantitative yields, which were characterized by ^1H and ^{13}C NMR and MS.

2.2. Microorganisms and growth conditions

Eukaryotic and prokaryotic microorganisms were used in this study: *Candida albicans* from Colección Española de Cultivos Tipo (CECT 1002), *Staphylococcus aureus* CECT 240, and *Pseudomonas aeruginosa* CECT 108. The isolates were stored at − 80 ∘C with 20% glycerol (Sigma-Aldrich, Saint Louis, MO, USA). *C. albicans* was grown on Sabouraud chloramphenicol agar (Scharlab, Barcelona, Spain), while *S. aureus* and *P. aeruginosa* were grown on Plate Count Agar (PCA) overnight. To stimulate biofilm formation, few colonies were transferred into Yeast Extract–Peptone–Dextrose (YPD (1%-2%-2%), Scharlab, Barcelona, Spain) for *C. albicans*, and into Mueller Hinton broth (Scharlab, Barcelona, Spain) for *S. aureus* and *P. aeruginosa*. They were incubated at 37 °C with agitation (150 rpm) for 24 h.

2.3. Assay of biofilm inhibition formation by zwitterionic CBS dendrimers

In this study four dendrimers were tested (Fig. 1) in induced microorganisms for producing biofilm. The activity of zwitterionic CBS dendrimers on the inhibition of biofilm formation in *C. albicans* was performed as previously described [24,25]. An inoculum of *C. albicans* was adjusted to a density equivalent to 0.5 McFarland standard in RPMI 1640 medium (Sigma-Aldrich) with morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich) and glucose (RPMI + MOPS + GLU). Immediately, 50 μL of the adjusted suspension were inoculated in 96-well microtiter plates containing two-fold serial dilutions (50 μL/wells) of the dendrimers ranging from 16 to 1024 mg/L. For *P. aeruginosa,* it was performed as previously described [39,40]. An inoculum of *P. aeruginosa* was adjusted to a density equivalent to 0.5 McFarland standard in Triptizon Soy Broth (TSB) medium supplemented with glucose. Then, 50 μL of the adjusted suspension was inoculated in 96-well microtiter plates adding immediately two-fold serial dilutions of the dendrimers (50 μL/wells) ranging from 16 to 1024 mg/L. Finally, the anti-biofilm activity in *S. aureus* was performed as previously described [23]**.** An inoculum of *S. aureus* was adjusted to a density equivalent to 0.5 McFarland standard and a dilution 1:100 was elaborated in TSB medium supplemented with glucose. Then, 50 μL of the suspension was inoculated in 96-well microtiter plates adding immediately two-fold serial dilutions of the dendrimers (50 μL/wells) ranging from 16 to 1024 mg/L. Controls were included in all experiments: un-inoculated medium (negative control) and dendron free medium (positive control). Plates were sealed with Parafilm® (Bemis, Neenah, WI, USA) and incubated for 24 h at 37 °C for *S. aureus* and *P. aeruginosa,* and for 48 h at 37 °C for *C. albicans*. Experiments were performed in triplicate and repeated at least twice in independent experiments.



Scheme 1. Synthesis of zwitterionic CBS dendrimers. 'n' represents dendrimer generation and 'm' the number of peripheral groups.

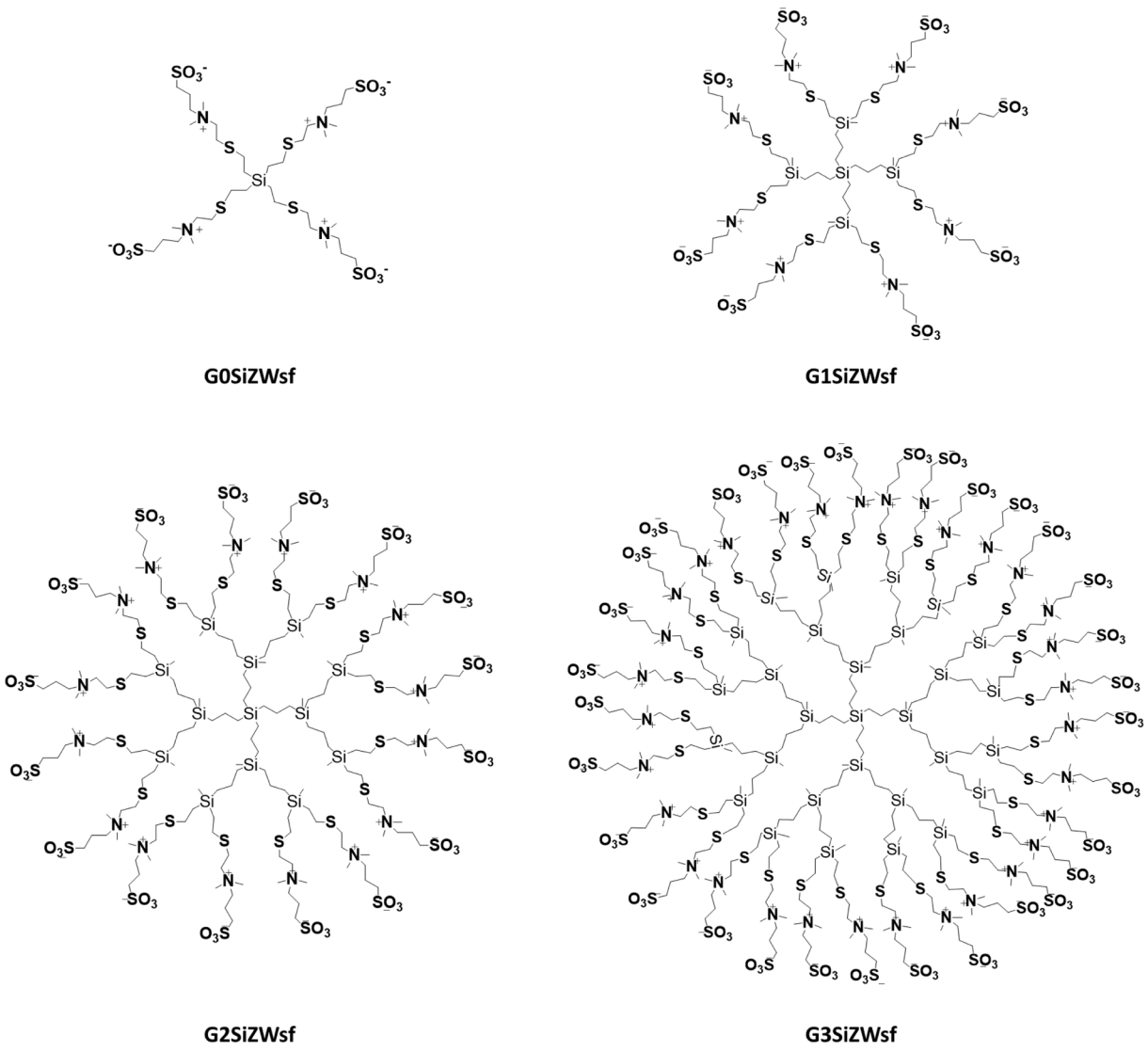


Fig. 1. Structures of zwitterionic CBS dendrimers GnSiZWsf. 'n' represents the CBS dendrimer generation, 'Si' denotes the silicon atom in the dendritic core, 'ZW' zwitterion and 'sf' represents sulfobetaine, the zwitterionic moiety chosen for dendrimers peripheral modification.

The biofilm formation was evaluated by determining the viability of the cells in the biofilm comparing to the untreated control using a resazurin colorimetric assay. For this purpose, resazurin was prepared at 0.01% in sterile water. At the end of the incubation time, 48 h for *C. albicans* and 24 h for *P. aeruginosa* and *S. aureus*, the supernatant from the wells was removed and washed with PBS. Then, 100 μL of PBS and 20 μL of resazurin were added to each well, and plates were incubated in the dark at 37°C for 20 h. The absorbances of wells were measured in a microplate reader (EpochTM, BioTek) at 570 and 600 nm. These data allowed us to determine the amount of the biofilm formed [25,41].

2.4. Viability assay of microorganisms in presence of zwitterionic dendrimer

The biocidal activity of the zwitterionic dendrimers was studied. For this purpose, only the microorganisms most sensitive to the dendrimers was evaluated (*Staphylococcus aureus*). The same process was followed as described in section 2.3. The experiment was performed on NUNC ^{TM} microtiter plates and the absorbance values were recorded every hour for 20 h using a plate reader (BioTek Instruments Inc. Model: ELX 800) at 630 nm. When the hourly absorbance was measured, a 5 μL suspension from each well was used for plating on PCA agar plates (drop plate method) and, after 24 h, visualised to determine the viability of *S. aureus* cells.

2.5. Cytotoxicity study of zwitterionic dendrimers in HeLa and MCF7

The cytotoxicity was evaluated using HeLa (ATCC® CCL-2TM) and MCF7 (ATCC –HTB22) cell lines. Experiments were performed in NUNC^{TM} plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (Sigma-Aldrich Ltd.) and 1% antibiotic mix: 10.000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL (Sigma-Aldrich Ltd.). For MCF7 cells, DMEM culture media was also supplemented with insulin (0.001 mg/ml). Cells were seeded at a density of 5×10^3 cells/well in 100 μL of fresh medium. Then, the plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere to allow the cells to form a confluent monolayer. After the incubation period, media was removed, and 10 μL of the serial concentrations of dendrimers prepared in fresh medium were added to each well to have final concentrations. Control wells (non-treated) contained only fresh medium. After 24 h incubation, cytotoxicity was evaluated using 10 μL of the Microculture Tetrazolium Assay (MTT, Sigma-Aldrich Ltd.) stock solution (5 mg/mL) added into each well. The plates were incubated for 3 to 4 h at 37 °C. Subsequently, medium was

discarded, and dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. Absorbance of the samples was recorded in a microplate absorbance reader at 570 nm (BioTek Instruments Inc. Model: ELX 800). All experiments were performed in triplicate for each concentration and repeated at least twice. Cytotoxicity values were considered as described: non-cytotoxic < 10% reduction in cell viability, low cytotoxicity 10%-25% reduction, and moderate cytotoxicity levels 25%-40% [42].

2.6. Haemolysis activity study of zwitterionic dendrimers

The haemolysis assay was adapted from the ISO 10993–4 protocol. Erythrocytes were isolated from lamb blood (RBC, Oxoid sheep erythrocytes) by centrifugation 2 mL of whole blood at 800 g for 10 min. Plasma was discarded and the pellet resuspended in 2 mL of PBS. This washing step was performed three times. Then, a 1:50 erythrocyte suspension was prepared in PBS. This erythrocyte suspension (180 μL) was incubated with each concentration to test for each dendrimer (20 μL) and incubated at 37 °C for 2 h. After incubation, each sample was centrifugated at 800 g for 15 min, 150 μL of the supernatant transferred to 96-well microplates (NUNC, polystyrene, untreated) and absorbance measured at 540 nm using BioTek Epoch 2 spectrophotometer. Final concentrations assayed for each zwitterionic dendrimer were 0.1, 1, 5, 10, 50, 65, 100 μM. Each concentration was assayed in triplicate, and a control for each concentration consisting of a well containing 20 μL of the corresponding dendrimer concentration and 180 μL of PBS was included. The negative control consisted of 20 μL PBS with 180 μL of erythrocyte suspension and the positive control consisted of 20 μL Triton X-100 20% with 180 μL of erythrocyte suspension. The latter control is considered to produce 100% haemolysis. The percentage of haemolysis (H%) was calculated as: [(Abs haemolysis [X] dendrimer – Abs negative control [X] dendrimer)] × 100 / (Abs positive control – Abs negative control).

2.7. Statistical analysis

Standard deviation calculation, one-way and two-way ANOVA and Tukey's post-hoc test, were performed. A value of $p < 0.05$ was considered statistically significant. All analyses were done using the GraphPad Prism 9.5.0 program (GraphPad Software, 2022, San Diego, CA, USA).

3. Results and discussion

3.1. Synthesis and characterization of zwitterionic CBS dendrimers

To carry out this work, we prepared four generations of zwitterionic CBS dendrimers to study the influence of dendritic core size as well as the number of zwitterionic groups in their capacity for inhibiting biofilm formation. A simple quaternization step of GnSi(S–NMe_2)$_m$ neutral dendrimers using 1,3-propanesultone afforded quantitative yield of GnSiZWsf dendrimers bearing 4, 8, 16 or 32 sulfobetaine moieties which contain a quaternary nitrogen atom and a negatively charged sulfonate group (Fig. 1). These compounds demonstrated an excellent solubility in water, a highly desired property for nanomaterials to be used for biomedical applications, considering that the CBS framework is extremely hydrophobic.

Transformation of neutral dendrimers into zwitterionic analogues was followed by ^{1}H NMR. Representative singlet signal of GnSi(S–NMe_2)$_m$ compounds at 2.25 ppm (Fig. 2B) corresponding to peripheral dimethylamino groups in ^{1}H NMR ($CDCl_3$) disappears by the time the reaction is completed. The new compounds formed are no longer

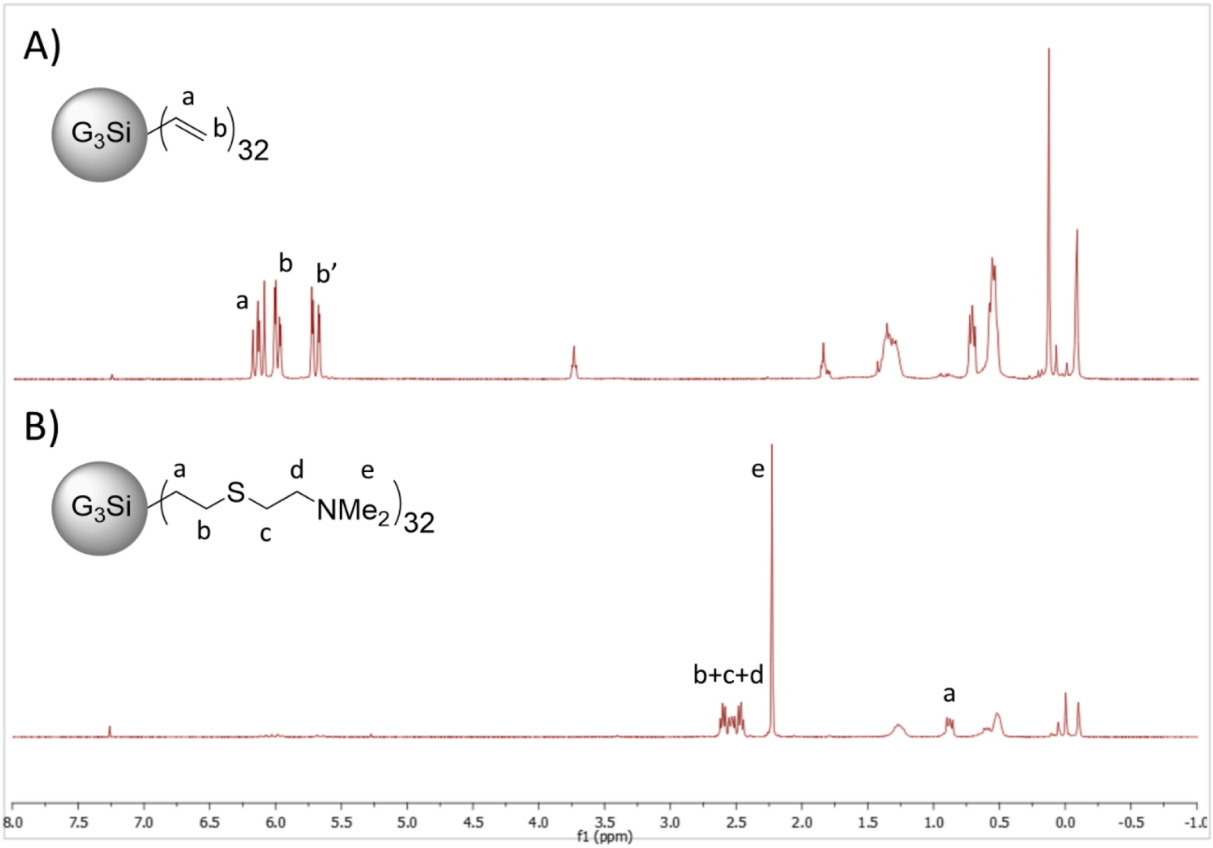


Fig. 2. Comparison of ^{1}H NMR ($CDCl_3$) spectra of G_3SiV_{32} (A) and G3Si(S–NMe_2)$_{32}$ (B). Vinyl groups disappearance is observed (A) as well as the appearance of dimethylamino groups and the two methylene groups belonging to the incorporated thiol (B).

soluble in $CDCl_3$. Furthermore, ^1H NMR in D_2O shows the methyl groups bound to the quaternary nitrogen atom as a singlet around 3.00 ppm and three new signals around 3.5 ppm, 2.2 ppm and 2.9 ppm (Fig. 3B), corresponding to the sulfobetaine chain $N(CH_2)_3S$.

3.2. Biofilm formation inhibition activity of zwitterionic CBS dendrimers

Microorganisms like *S. aureus, P. aeruginosa* and *C. albicans* are of great importance, especially in healthcare environments. Blocking the formation of biofilms create by some of these microorganisms is of great need in many fields, such as sanitary and food fields. For this reason, the antibiofilm formation behavior in yeast and bacteria of zwitterionic CBS new dendrimers with zwitterionic peripheral groups deposited in suspension was tested in this study. After the incubation time required for the biofilm establishing, the microplates were washed to remove non biofilm cells. The plates washed with PBS were incubated with resazurin and PBS for 20 h to evaluate the presence of viable biofilm.

The best anti biofilm formation results obtained were variable depending on the microorganism, the dendrimer generation and concentration (Table 1). None of the tested compounds were able to prevent biofilm formation in *C. albicans* after 48 h of incubation (0 % of biofilm formation inhibition activity). The results showed that the anti-biofilm activity in bacteria was better against *S. aureus* than against *P. aeruginosa*. This can be due to the different cell wall composition of the two bacteria as *S. aureus* is gram positive and *P. aeruginosa* is gram negative. These results are interesting since a preference for interacting with prokaryotes was observed, but not with eukaryotes. Therefore, our data indicate that the spectrum of our dendrimers is uniquely focused on prokaryotes. Probably, the new dendrimers have greater affinity to bind to the bacteria cell wall and membrane components which will prevent microorganisms from aggregating and forming biofilms.

In Fig. 4 are showed the results of anti-biofilm formation activity at the different concentrations of these zwitterionic CBS dendrimers against *S. aureus*. G0SiZWsf dendrimer was not effective against *S. aureus* biofilm-forming cells because the percentage of biofilm reduction was near 0. G1SiZWsf dendrimer showed some activity, however, it does not exceed 40% of biofilm reduction activity at any of the concentrations tested. The most effective dendrimer against *S. aureus* were G2SiZWsf and G3SiZWsf. The data showed for these later dendrimers have significant differences with the results obtained with G0SiZWsf and G1SiZWsf dendrimers ($p < 0.0001$). On the other hand, the effectiveness of G2SiZWsf dendrimer was slightly better than G3SiZWsf dendrimer at 16 and 32 mg/L. In addition, it could be observed that with a concentration of 32 mg/L G2SiZWsf the biofilm reduction is 81.5% and that higher concentrations do not significantly improve the efficacy of the compound ($p = 0.7526$), for example, using

Table 1
Maximum biofilm formation inhibition activity of G0SiZWsf, G1SiZWsf, G2SiZWsf and G3SiZWsf zwitterionic dendrimers evaluated in solution with cells induced to form biofilms of *C. albicans*, *S. aureus* and *P. aeruginosa*. The results are expressed in percentages, being 0 when biofilm formation is not inhibited. In brackets, the concentration at which it is obtained.

MICROORGANISMS	G0SiZWsf (0)	G1SiZWsf (G1)	G2SiZWsf (G2)	G3SiZWsf (G3)
C. albicans	0.0 (1024 mg/L)	0.0 (1024 mg/L)	0.0 (1024 mg/L)	0.0 (1024 mg/L)
S. aureus	2.5 (1024 mg/L)	35.6 (32 mg/L)	82.7 (512 mg/L)	81.4 (256 mg/L)
P. aeruginosa	16.0 (1024 mg/L)	18.8 (32 mg/L)	38.4 (128 mg/L)	35.8 (128 mg/L)

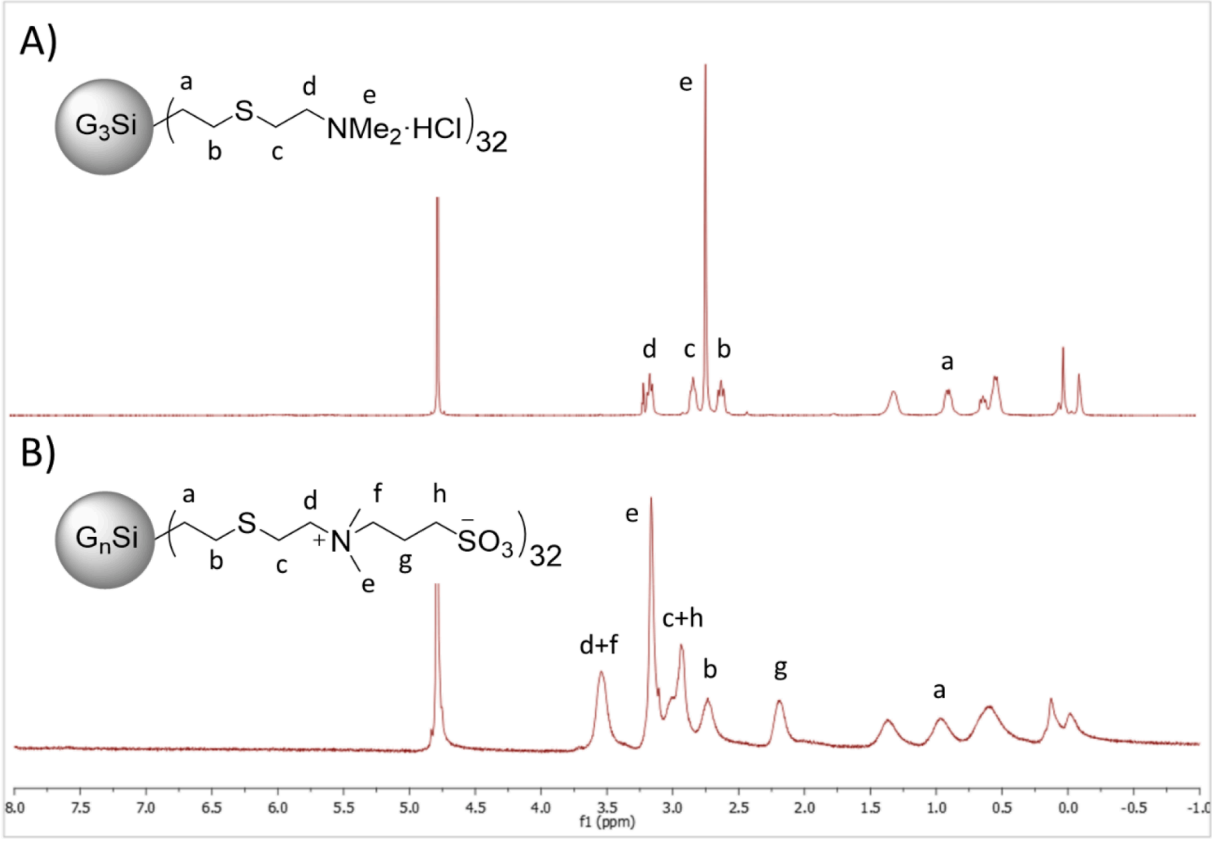


Fig. 3. Comparison of ^1H NMR (D_2O) spectra of G3Si(S–NMe_2·HCl)$_{32}$ (A) and G3SiZWsf (B). Displacement of dimethylamino groups changes from 2.71 ppm (A) to 3.08 ppm (B) and three signals belonging to the methylene groups of propanesultone moiety incorporated appear (B).

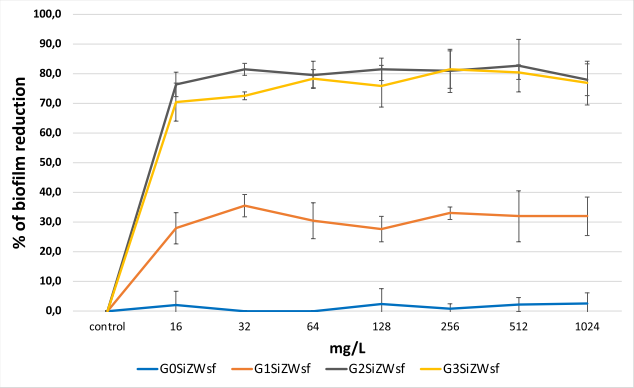


Fig. 4. Percentage of biofilm reduction activity of zwitterionic dendrimer in *S. aureus* treated: G0SiZWsf (blue), G1SiZWsf (orange), G2SiZWsf (grey) and G3SiZWsf (yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

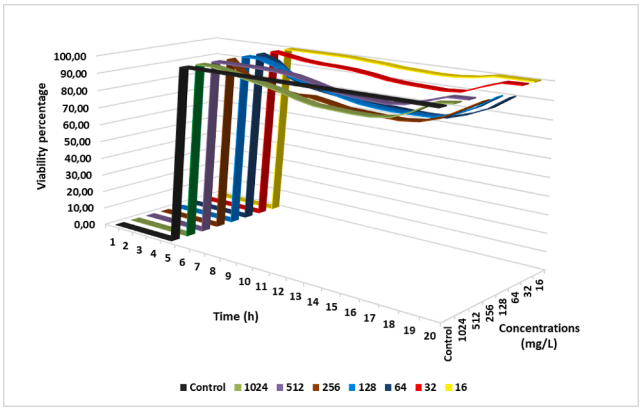


Fig. 5. Absorbance at 630 nm of *S. aureus* treated with G2SiZWsf dendrimer at different concentrations in the mix solution of the bacteria and dendrimer on the wells. Concentration in mg/L and time in hours (h).

512 mg/L G2SiZWsf on *S. aureus* results in a biofilm reduction of 82.7%. The anti-biofilm activity of G3SiZWsf at 32 mg/L was 72.5% of biofilm reduction.

Comparing the concentrations of G2SiZWsf and G3SiZWsf in μM (Table S1 in supporting information) according with the molecular weight (MW) (6 μM and 3 μM, respectively) and number of zwitterionic groups (ZW) (16 and 32, respectively) at 32 mg/mL, the same number of ZW groups were present in the mix solution of *S. aureus* and G2SiZWsf or G3SiZWsf dendrimers, however higher activity was observed for the lower generation dendrimer G2SiZWsf. This means that the size has an influence in the activity. We have observed in other types of CBS systems that not only the size and the number of functional groups determine the activity of these systems [43] but also an appropriate hydrophilic-hydrophobic balance in the structure [44] and this could be the reason of the higher activity of the second-generation dendrimer. This, together with the simpler synthesis of a lower generation dendrimer makes G2SiZWsf the best candidate for additional studies.

The activity studies of these compounds against *P. aeruginosa* indicated that they were not as that effective as against *S. aureus* ($p < 0.0001$). Once again, G2SiZWsf dendrimer was the most active, preventing *P. aeruginosa* biofilm formation. The results showed a 20.3% reduction in biofilm formation at 32 mg/L at that dendrimer. Same result was observed using G3SiZWsf dendrimer (a biofilm reduction of 20.9% at 32 mg/L). Although, a similar activity was observed for G2SiZWsf and G3SiZWsf, this did not happen with lower generation dendrimers that did not show any activity, confirming again that size has influence on activity.

3.3. Viability assay of S. Aureus when G2SiZWsf was used to avoid the formation of biofilm

A kinetic study of viability was performed to confirm the presence of alive cells in the supernatant removed prior to wash wells and run the resazurin viability assay. This study was performed with the most active compound (G2SiZWsf) and the most sensitive bacteria (*S. aureus*) using all the dendrimer concentrations during an incubation time of 20 h. The absorbance obtained at 630 nm with the different concentrations of the dendrimer could be compared with the control to calculate the viability percentage, because using the drop plate method showed the presence of viable cells at all concentrations.

In Fig. 5, after 11 h of incubation, significant differences were found for all concentrations ($p < 0.0238$), except the concentration of 16 mg/L, when compared to the control. In the hours that followed, the differences decreased gradually, and no significant differences were observed after 19 h of incubation ($p > 0.5848$). Thus, although there was a slight reduction in viability, it was stabilized over time, and the percentages of viability were equal to the control. So, the compound G2SiZWsf in solution could prevent biofilm formation on the surface but does not kill the microorganisms. Other studies have also observed a reduction in the levels of biofilm formation. For example, in Cheng [45] a reduction of adhesion can be observed in the confocal microscopy images on zwitterionic surfaces, however, no dead cells are observed.

3.4. Assays of haemolysis activity, MCF7 and HeLa cytotoxic cells study of zwitterionic dendrimers

Cytotoxicity remains a problem, especially during treatment at effective concentrations in eukaryotes. For this reason, the ability to produce haemolysis and the cytotoxicity of the most active compounds, was evaluated. The range of zwitterionic dendrimer concentrations evaluated for cytotoxicity in MCF-7 and HeLa cells was from 0.31 mg/L (0.06 μM) to 2048.24 mg/L (400 μM) for G2SiZWsf dendrimer and 0.63 mg/L (0.06 μM) to 2110.80 mg/L (200 μM) for G3SiZWsf dendrimer.

Data obtained from the cytotoxicity studies performed with HeLa and MCF7 cell lines confirmed the absence of cytotoxicity, including for the main active compounds, G2SiZWsf and G3SiZWsf dendrimers (Fig. 6A and Fig. 6B). For example, our results showed a low toxicity at

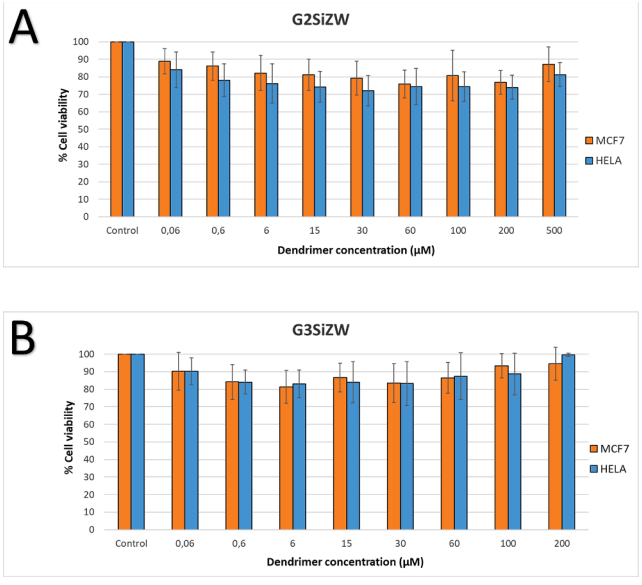


Fig. 6. Cytotoxicity of zwitterionic dendrimers in two human cell lines. (A) G2SiZWsf dendrimer and (B) G3SiZWsf dendrimer.

concentrations of G2SiZWsf dendrimer that managed to eliminate 81.5% (32 mg/L) of the biofilm-forming cells of *S. aureus* (Fig. 6A). The same result was observed for the G3SiZWsf compound at 32 mg/L (biofilm reduction of 72.5%) (Fig. 6B). Data obtained for G0SiZWsf and G1SiZWsf are included in supporting information (Figure S9). There were no significant differences between the values obtained in the viability studies using HeLa and MCF7 cell lines ($p > 0.05$).

Data obtained from the haemolysis studies confirm the absence of cytotoxicity of the compounds with the best activity, G2SiZWsf and G3SiZWsf dendrimers (0% haemolysis at the concentrations tested in the anti-biofilm activity studies.

4. Conclusion

In this work, four generations of a zwitterionic CBS dendrimer have been straightforward synthesized for the first time by reaction of GnSi (S–NMe_2)m with propanesultone. These dendrimers were characterized by NMR and MS and their cytotoxicity, blood compatibility and anti-biofilm properties evaluated.

In contrast to other studies in which the molecules are previously anchored on the surface [31,33,36], in our study they have been used in suspension together with the microorganisms as a first step to verify if these new dendritic synthesis molecules have anti-biofilm activity.

Our data concluded that the dendrimer used in this study had a higher ability to prevent biofilm formation in prokaryotes than in eukaryotes. We also found differences when it comes to gram positives (*S. aureus*) and gram negatives (*P. aeruginosa*) bacteria, that can be due to the difference in the cell wall of these bacteria. This fact corroborates the importance of the components and charge of the microorganism's membrane, which determines the affinity of the dendrimer with the membrane. The size of the dendrimer is also important. G2SiZWsf (32 mg/L) and G3SiZWsf (32 mg/L) dendrimers were the best generations that inhibit biofilm establishment for both bacteria, *S. aureus* and *P. aeruginosa*, but the anti-biofilm activity was higher against the former genus. Therefore, G2SiZWsf will be the best candidate for further studies against *S. aureus* due to the easier synthesis. Our results suggest that zwitterionic dendrimers of second and third generation, G2SiZWsf and G3SiZWsf, present an absence of toxicity and are effective molecules capable of inhibiting the development of biofilms on surfaces. The reduction of biofilm formation in the surface could be due to the zwitterionic nature of the chains with negative charges in the outer sphere of the dendrimers.

These results present these molecules as promising compounds in cleaning solutions to prevent the formation of biofilms and as possible compounds for the functionalization of surfaces and materials to prevent the formation of prokaryotic biofilms. Therefore, the aim for future studies will be to anchor these dendrimers, G2SiZWsf and G3SiZWsf, to different biomaterial surfaces and evaluate whether the zwitterionic surface of the dendrimers would provide anti-adhesive properties and prevent the formation of biofilms on the surfaces. This would be a promising alternative to prevent prokaryotic infections, e.g. in healthcare environments.

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CRediT authorship contribution statement

Natalia Gómez-Casanova: Resources, Writing – review & editing, Writing – original draft, Data curation, Formal analysis, Investigation, Conceptualization, Methodology. **Ángela Martín-Serrano Ortiz:** Resources, Writing – review & editing, Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. **Irene Heredero-Bermejo:** Supervision, Resources, Funding acquisition, Writing – review & editing, Writing – original draft, Data curation, Conceptualization, Methodology, Formal analysis, Investigation. **Javier Sánchez-Nieves:** Resources, Funding acquisition, Writing – review & editing. **José Luis Copa-Patiño:** Supervision, Resources, Funding acquisition, Writing – review & editing, Conceptualization, Formal analysis, Investigation. **F. Javier de la Mata:** Supervision, Resources, Writing – review & editing, 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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.07.021.

References

[1] X. Xiao, Y. Li, L. Li, Y. Xiong, Identification of Methicillin-Resistant Staphylococcus aureus (MRSA) Genetic Factors Involved in Human Endothelial Cells Damage, an Important Phenotype Correlated with Persistent Endovascular Infection, Antibiotics (Basel). 11 (3) (2022) 316, https://doi.org/10.3390/antibiotics11030316.

[2] U. Tasneem, K. Mehmood, M. Majid, S.R. Ullah, S. Andleeb, Methicillin resistant Staphylococcus aureus: A brief review of virulence and resistance, J. Pak. Med. Assoc. 72 (3) (2022) 509–515, https://doi.org/10.47391/JPMA.0504.

[3] A. Cottalorda, S. Dahyot, A. Soares, K. Alexandre, I. Zorgniotti, M. Etienne, E. Jumas-Bilak, M. Pestel-Caron, Phenotypic and genotypic within-host diversity of Pseudomonas aeruginosa urinary isolates, Sci. Rep. 12 (1) (2022) 5421, https://doi.org/10.1038/s41598-022-09234-5.

[4] K.D. Roberts, Y. Zhu, M.A.K. Azad, M.L. Han, J. Wang, L. Wang, H.H. Yu, A. S. Horne, J.A. Pinson, D. Rudd, N.H. Voelcker, N.A. Patil, J. Zhao, X. Jiang, J. Lu, K. Chen, O. Lomovskaya, S.J. Hecker, P.E. Thompson, R.L. Nation, M.N. Dudley, D. C. Griffith, T. Velkov, J. Li, A synthetic lipopeptide targeting top-priority multidrug-resistant Gram-negative pathogens, Nat. Commun. 13 (1) (2022) 1625, https://doi.org/10.1038/s41467-022-29234-3.

[5] K.R. Iyer, N. Robbins, L.E. Cowen, The role of Candida albicans stress response pathways in antifungal tolerance and resistance, iScience. 25 (3) (2022), 103953, https://doi.org/10.1016/j.isci.2022.103953.

[6] M. Jamal, W. Ahmad, S. Andleeb, F. Jalil, M. Imran, M.A. Nawaz, T. Hussain, M. Ali, M. Rafiq, M.A. Kamil, Bacterial biofilm and associated infections, J. Chin. Med. Assoc. 81 (1) (2018) 7–11, https://doi.org/10.1016/j.jcma.2017.07.012.

[7] R. Pereira, R.O. Dos Santos Fontenelle, E.H.S. de Brito, S.M. de Morais, Biofilm of Candida albicans: formation, regulation and resistance, J. Appl. Microbiol. 131 (1) (2021) 11–22, https://doi.org/10.1111/jam.14949.

[8] S. Tian, L. Su, Y. Liu, J. Cao, G. Yang, Y. Ren, F. Huang, J. Liu, Y. An, H.C. van der Mei, H.J. Busscher, L. Shi, Self-targeting, zwitterionic micellar dispersants enhance antibiotic killing of infectious biofilms-An intravital imaging study in mice, Sci. Adv. 6 (33) (2020) eabb1112, https://doi.org/10.1126/sciadv.abb1112.

[9] M.Á. Ortega, A. Guzmán Merino, O. Fraile-Martínez, J. Recio-Ruiz, L. Pekarek, G. L. Guijarro, N. García-Honduvilla, M. Álvarez-Mon, J. Buján, S. García-Gallego, Dendrimers and Dendritic Materials: From Laboratory to Medical Practice in

Infectious Diseases, Pharmaceutics. 12 (9) (2020) 874, https://doi.org/10.3390/pharmaceutics12090874.
[10] J. Pan, S.A. Attia, N. Filipczak, V.P. Torchilin, 10 - Dendrimers for drug delivery purposes, in: Woodhead Publishing Series in Biomaterials. Nanoengineered Biomaterials for Advanced Drug Delivery, Elsevier, 2020, pp. 201–242, https://doi.org/10.1016/B978-0-08-102985-5.00010-3.
[11] N. Rabiee, S. Ahmadvand, S. Ahmadi, Y. Fatahi, R. Dinarvand, M. Bagherzadeh, M. Rabiee, M. Tahriri, L. Tayebi, M.R. Hamblin, Carbosilane dendrimers: Drug and gene delivery applications, J. Drug Delivery Sci. Technol. 59 (2020), 101879, https://doi.org/10.1016/j.jddst.2020.101879.
[12] B. Todaro, S. Achilli, B. Liet, E. Laigre, C. Tiertant, D. Goyard, N. Berthet, O. Renaudet, Structural influence of antibody recruiting glycodendrimers (ARGs) on antitumoral cytotoxicity, Biomater. Sci. 9 (11) (2021 Jun 4) 4076–4085, https://doi.org/10.1039/d1bm00485a. PMID: 33913968.
[13] M. Sánchez-Milla, L. Muñoz-Moreno, J. Sánchez-Nieves, M. Malý, R. Gómez, M. J. Carmena, F.J. de la Mata, Anticancer Activity of Dendriplexes against Advanced Prostate Cancer from Protumoral Peptides and Cationic Carbosilane Dendrimers, Biomacromolecules 20 (3) (2019) 1224–1234, https://doi.org/10.1021/acs.biomac.8b01632.
[14] N. Sanz Del Olmo, R. Carloni, A.M. Bajo, P. Ortega, A. Fattori, R. Gómez, M. F. Ottaviani, S. García-Gallego, M. Cangiotti, F.J. de la Mata, Insight into the antitumor activity of carbosilane Cu(ii)-metallodendrimers through their interaction with biological membrane models, Nanoscale 11 (28) (2019) 13330–13342, https://doi.org/10.1039/c9nr03313k.
[15] E. Arnáiz, E. Vacas-Córdoba, M. Galán, M. Pion, M.A. Rafael Gómez, F. Ángeles Muñoz-Fernández, J. de la Mata, Synthesis of anionic carbosilane dendrimers via "click chemistry" and their antiviral properties against HIV, J. Polym. Sci. A Polym. Chem. 52 (8) (2014) 1099–1112, https://doi.org/10.1002/pola.27090.
[16] D. Sepúlveda-Crespo, R. Lorente, M. Leal, R. Gómez, F.J. De la Mata, J.L. Jiménez, M.Á. Muñoz-Fernández, Synergistic activity profile of carbosilane dendrimer G2-STE16 in combination with other dendrimers and antiretrovirals as topical anti-HIV-1 microbicide, Nanomedicine 10 (3) (2014) 609–618, https://doi.org/10.1016/j.nano.2013.10.002.
[17] C.E. Gutierrez-Ulloa, Y.u. Marina, E.K. Buyanova, A.G. Apartsin, F. Venyaminova, J. de la Mata, R. Gómez, Carbon Nanotubes Decorated with Cationic Carbosilane Dendrons and Their Hybrids with Nucleic Acids, ChemNanoMat 4 (2) (2018) 220–230, https://doi.org/10.1002/cnma.201700351.
[18] K. Białkowska, K. Miłowska, S. Michlewska, P. Sokołowska, P. Komorowski, T. Lozano-Cruz, R. Gomez-Ramirez, F.J. de la Mata, M. Bryszewska, Interaction of Cationic Carbosilane Dendrimers and Their siRNA Complexes with MCF-7 Cells, Int. J. Mol. Sci. 22 (13) (2021) 7097, https://doi.org/10.3390/ijms22137097.
[19] E. Fuentes-Paniagua, J.M. Hernández-Ros, M. Sánchez-Milla, M.A. Camero, M. Maly, J. Pérez-Serrano, J.L. Copa-Patiño, J. Sánchez-Nieves, J. Soliveri, R. Gómez, F.J. de la Mata, Carbosilane Cationic Dendrimers Synthesized by Thiol-Ene Click Chemistry and Their Use as Antibacterial Agents, RSC Adv. 4 (3) (2014) 1256–1265, https://doi.org/10.1039/C3RA45408H.
[20] E. Fuentes-Paniagua, J. Sánchez-Nieves, J.M. Hernández-Ros, A. Fernández-Ezequiel, J. Soliveri, J.L. Copa-Patiño, R. Gómez, F.J. de la Mata, Structure–activity relationship study of cationic carbosilane dendritic systems as antibacterial agents, RSC Adv. 6 (2016) 7022–7033, https://doi.org/10.1039/C5RA25901K.
[21] F. Ben Jeddou, L. Falconnet, A. Luscher, T. Siriwardena, J.L. Reymond, C. van Delden, T. Köhler, Adaptive and Mutational Responses to Peptide Dendrimer Antimicrobials in Pseudomonas aeruginosa, Antimicrob. Agents Chemother. 64 (4) (2020) e02040–e02119, https://doi.org/10.1128/AAC.02040-19.
[22] C. Llamazares, N. Sanz Del Olmo, P. Ortega, R. Gómez, J. Soliveri, F.J. de la Mata, S. García-Gallego, J.L. Copa-Patiño, Antibacterial Effect of Carbosilane Metallodendrimers in Planktonic Cells of Gram-Positive and Gram-Negative Bacteria and *Staphylococcus aureus* Biofilm, Biomolecules 9 (9) (2019) 405, https://doi.org/10.3390/biom9090405.
[23] J. Fernandez, Á. Martin-Serrano, N. Gómez-Casanova, A. Falanga, S. Galdiero, F. Javier de la Mata, I. Heredero-Bermejo, P. Ortega, Effect of the Combination of Levofloxacin with Cationic Carbosilane Dendron and Peptide in the Prevention and Treatment of Staphylococcus aureus Biofilms, Polymers 13 (13) (2021) 2127, https://doi.org/10.3390/polym13132127.
[24] I. Heredero-Bermejo, N. Gómez-Casanova, S. Quintana, J. Soliveri, F.J. de la Mata, J. Pérez-Serrano, J. Sánchez-Nieves, J.L. Copa-Patiño, In Vitro Activity of Carbosilane Cationic Dendritic Molecules on Prevention and Treatment of Candida Albicans Biofilms, Pharmaceutics. 12 (10) (2020) 918, https://doi.org/10.3390/pharmaceutics12100918.
[25] N. Gómez-Casanova, T. Lozano-Cruz, J. Soliveri, R. Gomez, P. Ortega, J.L. Copa-Patiño, I. Heredero-Bermejo, Eradication of Candida albicans Biofilm Viability. In Vitro Combination Therapy of Cationic Carbosilane Dendrons Derived from 4-Phenylbutyric Acid with AgNO3 and EDTA, J Fungi (Basel) 7 (7) (2021) 574, https://doi.org/10.3390/jof7070574.
[26] I. Heredero-Bermejo, J. Sánchez-Nieves, J. Soliveri, R. Gómez, F.J. de la Mata, J. L. Copa-Patiño, J. Pérez-Serrano, In vitro anti-Acanthamoeba synergistic effect of chlorhexidine and cationic carbosilane dendrimers against both trophozoite and cyst forms, Int. J. Pharm. 509 (1–2) (2016) 1–7, https://doi.org/10.1016/j.ijpharm.2016.04.075.
[27] Z. Xiong, M. Shen, X. Shi, Zwitterionic Modification of Nanomaterials for Improved Diagnosis of Cancer Cells, Bioconjug. Chem. 30 (10) (2019) 2519–2527, https://doi.org/10.1021/acs.bioconjchem.9b00543.
[28] E. Royo-Rubio, I. Rodríguez-Izquierdo, M. Moreno-Domene, T. Lozano-Cruz, F. J. de la Mata, R. Gómez, M.A. Muñoz-Fernández, J.L. Jiménez, Promising PEGylated cationic dendrimers for delivery of miRNAs as a possible therapy against HIV-1 infection, J Nanobiotechnology. 19 (1) (2021) 158, https://doi.org/10.1186/s12951-021-00899-0.
[29] L. Wang, Z. Wang, G. Ma, W. Lin, S. Chen, Reducing the Cytotoxity of Poly (amidoamine) Dendrimers by Modification of a Single Layer of Carboxybetaine, Langmuir 29 (28) (2013) 8914–8921, https://doi.org/10.1021/la400623s.
[30] S. Chen, L. Li, C. Zhao, J. Zheng, Surface hydration: Principles and applications toward low-fouling/nonfouling biomaterials, Polymer 51 (2010) 5283–5293, https://doi.org/10.1016/j.polymer.2010.08.022.
[31] J.B. Schlenoff, Zwitteration: coating surfaces with zwitterionic functionality to reduce nonspecific adsorption, Langmuir 30 (32) (2014) 9625–9636, https://doi.org/10.1021/la500057j.
[32] H. Wang, D.E. Christiansen, S. Mehraeen, G. Cheng, Winning the fight against biofilms: the first six-month study showing no biofilm formation on zwitterionic polyurethanes, Chem. Sci. 11 (18) (2020) 4709–4721, https://doi.org/10.1039/c9sc06155j.
[33] B.R. Knowles, D. Yang, P. Wagner, S. Maclaughlin, M.J. Higgins, P.J. Molino, Zwitterion Functionalized Silica Nanoparticle Coatings: The Effect of Particle Size on Protein, Bacteria, and Fungal Spore Adhesion, Langmuir 35 (5) (2019) 1335–1345, https://doi.org/10.1021/acs.langmuir.8b01550.
[34] F. Cox, K. Khalib, N. Conlon, PEG That Reaction: A Case Series of Allergy to Polyethylene Glycol, J. Clin. Pharmacol. 61 (6) (2021) 832–835, https://doi.org/10.1002/jcph.1824.
[35] P. Sellaturay, S. Nasser, P. Ewan, Polyethylene Glycol-Induced Systemic Allergic Reactions (Anaphylaxis), J. Allergy Clin. Immunol. Pract. 9 (2) (2021) 670–675, https://doi.org/10.1016/j.jaip.2020.09.029.
[36] W. Ma, T. Chen, S. Nanni, L. Yang, Z. Ye, M.S. Rahaman, Zwitterion-Functionalized Graphene Oxide Incorporated Polyamide Membranes with Improved Antifouling Properties, Langmuir 35 (5) (2019) 1513–1525, https://doi.org/10.1021/acs.langmuir.8b02044.
[37] M. Colilla, I. Izquierdo-Barba, M. Vallet-Regí, The Role of Zwitterionic Materials in the Fight against Proteins and Bacteria, Medicines (Basel). 5 (4) (2018) 125, https://doi.org/10.3390/medicines5040125.
[38] T. Strašák, J. Čermák, J. Sýkora, J. Horský, Z. Walterová, F. Jaroschik, D. Harakat, Carbosilane Metallodendrimers with Titanocene Dichloride End Groups, Organometallics 31 (19) (2012) 6779–6786, https://doi.org/10.1021/om300559y.
[39] S. Peppoloni, E. Pericolini, B. Colombari, D. Pinetti, C. Cermelli, F. Fini, F. Prati, E. Caselli, E. Blasi, The β-Lactamase Inhibitor Boronic Acid Derivative SM23 as a New Anti-Pseudomonas aeruginosa Biofilm, Front. Microbiol. 11 (2020) 35, https://doi.org/10.3389/fmicb.2020.00035.
[40] S. Quintana-Sanchez, N. Gómez-Casanova, J. Sánchez-Nieves, R. Gómez, J. Rachuna, S. Wąsik, J. Semaniak, B. Maciejewska, Z. Drulis-Kawa, K. Ciepluch, F. J. Mata, M. Arabski, The Antibacterial Effect of PEGylated Carbosilane Dendrimers on P. aeruginosa Alone and in Combination with Phage-Derived Endolysin, Int. J. Mol. Sci. 23 (3) (2022) 1873, https://doi.org/10.3390/ijms23031873.
[41] N. Gómez-Casanova, J.L. Copa-Patiño, I. Heredero-Bermejo. Novel Treatment Approach against Candida spp.: Evaluation of Antifungal and Antibiofilm In Vitro Activity of Dendritic Molecules. 2022; In (Ed.), Candida and Candidiasis [Working Title]. IntechOpen. https://doi.org/10.5772/intechopen.106892.
[42] J. Lorenzo-Morales, C.M. Martín-Navarro, A. López-Arencibia, M.A. Santana-Morales, R.N. Afonso-Lehmann, S.K. Maciver, B. Valladares, E. Martínez-Carretero, Therapeutic potential of a combination of two gene-specific small interfering RNAs against clinical strains of Acanthamoeba, Antimicrob. Agents Chemother. 54 (12) (2010) 5151–5155, https://doi.org/10.1128/AAC.00329-10.
[43] K. Winnicka, M. Wroblewska, P. Wieczorek, P.T. Sacha, E.A. Tryniszewska, The effect of PAMAM dendrimers on the antibacterial activity of antibiotics with different water solubility, Molecules 18 (7) (2013) 8607–8617, https://doi.org/10.3390/molecules18078607.
[44] S. Quintana-Sanchez, N. Gómez-Casanova, J. Sánchez-Nieves, R. Gómez, J. Rachuna, S. Wąsik, J. Semaniak, B. Maciejewska, Z. Drulis-Kawa, K. Ciepluch, F. J. Mata, The Antibacterial Effect of PEGylated Carbosilane Dendrimers on P. aeruginosa Alone and in Combination with Phage-Derived Endolysin, Int. J. Mol. Sci. 23 (3) (2022) 1873, https://doi.org/10.3390/ijms23031873.
[45] G. Cheng, Z. Zhang, S. Chen, J.D. Bryers, S. Jiang, Inhibition of bacterial adhesion and biofilm formation on zwitterionic surfaces, Biomaterials 28 (29) (2007) 4192–4199, https://doi.org/10.1016/j.biomaterials.2007.05.041.