Development of di-methacrylate quaternary ammonium monomers with antibacterial activity

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ABSTRACT

Nine antibacterial di-methacrylate monomers based on bis-quaternary ammonium salts (bis-QAMs) were synthesized and structurally characterized. The biological activity of the bis-QAMs was tested in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on different bacterial strains achieving promising results and, in most cases, a complete bactericidal effect using a bis-QAM concentration lower than 1 mg/mL. Two of the structures showed comparable and superior activity against *S. mutans* than the commercial monomer 12-methacryloyloxydodecyl pyridinium bromide (MDBP). All the bis-QAMs here described were able to inhibit *S. mutans* biofilm formation at a concentration equal to the MIC value. From the analysis of the obtained data, some correlation regarding the structure and the antibacterial activity of the bis-QAMs could be drawn: a flexible alkyl C_{12} spacer between the two quaternary ammonium moieties increased the monomer antibacterial effect in comparison to the aromatic ones; the equilibrium between hydrophobic and hydrophilic moieties was directly correlated to the bactericidal range of action; the increase of the steric hindrance of the ammonium side groups might be both advantageous or disadvantageous to the antibacterial efficacy depending on the whole monomer chemical structure. Even though the possible correlation between the monomer structures and their bacteriostatic or bactericidal effect is under investigation, the monomers exhibited low cytotoxicity on human dental pulp stem cells, confirming their promising potential in the dental materials' field.

Statement of significance

The use of dental resins with antibacterial monomers might prevent the formation of secondary caries at the restoration margins. For this purpose, a series of di-methacrylate bis-quaternary ammonium monomers (QAMs) was developed. Unlike antibacterial mono-methacrylate monomers already described in the literature, the synthesized di-methacrylate monomers have the potential of acting as cross-linkers stabilizing the polymeric network and bear two quaternary ammonium groups that increase their antibacterial ability.

The QAMs exert bactericidal activity on both Gram(+) and Gram(−) bacterial strains maintaining at the same time good biocompatibility with the oral environment.

Some structural elements of the monomers were clearly related to high antibacterial properties, and this can help design new active structures and better understand their mechanism of action.

1. Introduction

A major concern in restorative dentistry is bacteria presence in the oral biofilm, which are involved in secondary caries development and demineralization of enamel and dentin at the restorations' margins [1]. Among the many aspects influencing the

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longevity of dental restorations, secondary caries' formation is considered the main reason for restoration failure [2]. Secondary caries is strongly correlated with a defective adhesive interface and all dental adhesive systems, in spite of their good performance in terms of stability and mechanical properties, are subjected to some degradation over time and do not have intrinsic antibacterial properties, which may lead to failure of the tooth-restoration bond, leading to marginal leakage and bacteria infiltration [3,4]. To overcome bacterial proliferation, several researchers have focused on developing new resin restorative materials that possess antibacterial potential. Some of the first attempts involved the development of dental resins embedding releasable antimicrobial compounds, such as chlorhexidine, fluoride, and silver particles [5–7]. One of the disadvantages of these methods was the limited time frame in which these materials could remain active [5]. Since Imazato et al. introduced the concept of "immobilized bactericide" [8], several efforts have been made for developing high-performance dental materials that would assure long-lasting antibacterial properties. The current trend involves the development of antibacterial monomers able to co-polymerize with the methacrylate monomers commonly used in dentistry, leading to the formation of a polymeric network with antimicrobial properties [9]. Thus, the synthesis of new methacrylate monomers based on quaternary ammonium salts has become a research field vividly active. These new compounds proved to be a convenient choice thanks to their activity against a broad spectrum of pathogens and the relatively low toxicity for eukaryotic cells [10,11]. In most cases, they showed strong antibacterial activity both as monomers and after their co-polymerization, without the release of antibacterial components from the polymerized matrix [10].

To the best of the authors' knowledge, up to date, various quaternary ammonium monomers (QAMs) have been proposed. However, only the mono-QAM 12-methacryloyloxydodecyl pyridinium bromide (MDPB) [8,10,12–14] has been successfully introduced in commercial dental adhesive systems, becoming a reference point for the development of new monomeric structures.

The main classification of the several QAMs proposed after MDBP is commonly based on their structural differences regarding, in particular, the number of methacrylate functionalities (*i.e.,* mono- or di-methacrylate monomers) and the number of quaternary ammonium groups (*i.e.,* QAMs with one quaternary ammonium moiety and bis-QAMs with two ammonium groups) [6]. Indeed, each structure is characterized by different intrinsic properties [9]. Despite the high antibacterial activity and biocompatibility showed by some mono-methacrylate QAMs [5,6,12], their main drawback is the impossibility of forming cross-linking bonds between polymeric chains. Thus, the incorporation of high concentrations of mono-methacrylate QAMs in di-methacrylate resins usually employed in dentistry may significantly affect the formation of the polymeric network, jeopardizing the material's final mechanical and structural properties [6]. Besides, mono-methacrylates with pendant quaternary ammonium moieties may present miscibility problems with hydrophobic di-methacrylates commonly used in dental bonding systems and composite materials [15]. A series of di-methacrylate QAMs have been developed and incorporated into experimental resins to overcome these disadvantages [5,6,16]. Differently from mono-methacrylate QAMs, which are added in 1–10% w/w to the resin mixture [13,17], di-methacrylate QAMs are usually added from a minimum concentration of 10% w/w [15,18] to a maximum concentration of 50% w/w [19] to guarantee an antimicrobial effect. Unfortunately, an increase in the di-methacrylate QAMs concentration often corresponds to a decay of the polymerized material's mechanical properties [20,21].

In order to provide an efficient antibacterial dental resin system that maintains adequate mechanical properties overcoming the disadvantages of employing very high concentrations of mono-QAMs, the use of monomeric structures bearing two quaternary ammonium moieties can be considered an improvement. Considering the direct correlation between the charge-density of cationic polymeric surfaces and their biocidal efficiency [22,23], bis-QAMs with an increased charge-density in comparison to mono-QAMs have the potential to provide a significant bactericidal effect even at very low concentrations. Moreover, di-methacrylate bis-QAMs with two active polymerizable sites could act as crosslinkers, stabilizing the polymeric network and contributing to preserving high mechanical properties. Thanks to their structural characteristics, di-methacrylate bis-QAMs should be considered advantageous candidates as dental resins components. Surprisingly, only a few examples of this kind of monomers have been reported in the literature [15,24–27]. Moreover, for none of the di-methacrylate bis-QAMs previously studied, the relationship between their chemical structure and antibacterial efficacy was investigated.

Under these considerations, in the present study, nine antibacterial di-methacrylate bis-QAMs were synthesized and characterized. Their chemical structures differ for several characteristics, such as the nature of the spacer between the two quaternary ammonium functionalities (alkyl C_{12}, xylyl, bis-phenyl), the geometry of the quaternary ammonium (sp^3, sp^2), the steric hindrance of the ammonium substituents (methyl and ethyl), the nature of the bond between the methacrylic moieties and the rest of the molecule (ester, amide) and the counterion (bromide, fluoride) of the ionic forms. The synthesized monomers' bactericidal properties were tested on different bacterial strains in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), finding a clear structure-activity correlation. Their bactericidal activity against *Streptococcus mutans* was compared with that of formerly synthesized antibacterial monomers: 12-methacryloyloxydodecyl pyridinium bromide (MDBP) [12] and 2,2'-bis[((methacryloyl)oxy)ethyl] dimethylammonium bromide-1,1'-benzyl (IDMA-2) [15]. The cytotoxicity of the most active compounds was also tested on human dental pulp stem cells to evaluate biocompatibility for their use in dental materials.

2. Materials and Methods

2.1. Synthesis of bis–quaternary ammonium di-methacrylates

The detailed synthetic conditions for preparing each new monomer were patented (Italian patent n° 102019000020949, filling date 11.12.2019). The general procedure is available in the Appendix: Supporting information.

The obtained structures were characterized by nuclear magnetic resonance. ^{1}H,^{13}C, HH-COSY, HSQC, and ^{19}F NMR spectra were recorded on a Varian 400 spectrometer at operating frequencies of 400 and 101.56 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts relative to the solvent's residual signal (DMSO-d_6 ^{1}H 2.50 ppm,^{13}C 39.52 ppm; D_2O ^{1}H 4.79 ppm) were measured.

2.2. Microorganisms and media

Streptococcus mutans (*S. mutans* - ATCC 25175), *Escherichia coli* (*E. coli* - ATCC 25922), *Staphylococcus aureus* (*S. aureus* - ATCC 25923), *Streptococcus sanguinis* (*S. sanguinis* - ATCC 10556), and *Streptococcus mitis* (*S. mitis* - ATCC 49456) strains were chosen to evaluate the antibacterial activity of the compounds. *S. mutans* were grown in Brain Heart Infusion Agar/Broth at 37°C, *E. coli* and *S. aureus* were grown and tested in Muller Hinton [28] Agar/Broth at 37°C, *S. sanguinis* and *S. mitis* were grown in Brain Heart Infusion (BHI) Agar/Broth at 37°C and 5% CO_2.

2.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MICs were determined using the broth micro-dilution assay [28]. A pure single bacterium colony from the agar plate was inoculated into the bacterial culture medium and grown overnight at 37°C. Bacteria were then diluted in fresh growth-supporting broth to a concentration of 1×10^6 cfu/mL. A stock solution of 20 mg/mL of tested compounds was prepared dissolving the monomers in the bacteria culture medium.

Compound stock solutions were serially 1:1 diluted in bacterial broth into the wells of 96-well polypropylene microtiter U plates. All dilutions of the tested products were then inoculated with equal volumes of the specified microorganism. Bacteria suspension without antibacterial agent served as the negative control, culture media without bacteria suspension were used as the positive control. Positive and negative control wells were included for every microorganism to demonstrate adequate microbial growth throughout the incubation period and media sterility, respectively. The plates were incubated at 37°C and the bacterial growth in each well was detected after 24 h for *E. coli* and *S. aureus* and after 48 h for *S. mutans, S. sanguinis* and *S. mitis*. The lowest concentration of the compound at which no bacteria growth was visually evident was considered the MIC value. Each concentration was tested with N = 6 for each QAM. To determine the MBC values, the dilution representing the MIC and two of the more concentrated monomer dilutions were sub-cultured on an agar culture medium. Viable colony-forming units were enumerated after 24 h for *E. coli* and *S. aureus* and after 48 h for *S. mutans, S. sanguinis* and *S. mitis*. The MBC value was considered as the lowest concentration with ≥ 99.9% of bacterial killing.

2.4. Biofilm inhibition assay

A pure suspension of *S. mutans* in BHI (Brain Heart Infusion) added with 1% w/v sucrose was obtained after an overnight incubation at 37°C. The suspension was adjusted to a microbial concentration of approximately 3.0×10^8 cells/mL in BHI + 1% w/v of sucrose in the presence or absence of free monomers. 200 μL of bacterial suspension were then aliquoted in each well of a 96-well microtiter plate (VWR Tissue Culture Plates) and incubated at 37°C. For each monomer, the tested concentration was equal to and twice the MIC value.

After 24 h, medium and non-adherent bacteria were removed and 200 μL of fresh BHI + 1% sucrose were added. After additional incubation at 37°C for 24 h, wells were washed two times with 200 µL of PBS and then 100 µL of PBS containing 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added. The plate was incubated at 37°C for 3 h in the dark. The wells were then washed with 150 µL PBS and subsequently, formazan crystals were solubilized with 100 µL of 100 % DMSO for each well. Optical density at 550 nm was measured using a microplate spectrophotometer (Infinite M200PRO NanoQuant, Tecan). Results were displayed as optical density (OD) units. Eight replicates were tested for each monomer concentration (N = 8). The data were presented as mean OD ± standard deviation.

2.5. Human dental pulp stem cells (HDPSCs) isolation

Freshly extracted, non-carious, human third molars were collected from surgical patients (16–25 years of age). Samples were collected after obtaining patients' informed consent (Authorization by Regional Health Service- Azienda Sanitaria Universitaria Integrata di Trieste and by the Comitato Etico Unico Regionale - CEUR FVG, ID studio 2433: TERM – "Collection of biological samples for the study of biocompatibility and bioactivity on dental pulp cells of materials for restorative and regenerative dentistry"). Immediately after extraction, teeth were immersed in PBS containing antibiotics (500 U/mL penicillin, 500 mg/mL streptomycin) and promptly processed for pulp isolation. Under sterile conditions, the teeth pulp chamber was exposed using a microtome (Isomet 1000, Buehler Ltd., Lake Bluff, IL, USA). The entire pulp tissue was cut into small pieces by a scalpel blade and transferred in a Petri dish with 2 mL of proteolytic enzymes (12 mg/mL of Collagenase I from Life technology and a 16 mg/mL of Dispase II from Sigma-Aldrich in PBS) for 45 min at 37°C. Then, the digested cell suspension was transferred in a 15 mL centrifuge tube with 3 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% glutamine, 500 U/mL penicillin, 500 mg/mL streptomycin and 10% fetal bovine serum (FBS) and centrifuged for 5 min at room temperature. The cell pellet was then resuspended in complete DMEM containing L-ascorbic acid 2-phosphate 100 µM and seeded into a 25 cm^2 culture flask at 37°C and 5% pCO_2. The culture medium was changed every three days until 80% of cell confluence was achieved. The expression of the positive markers CD90, CD73, CD29 and the negative markers CD14, CD34 and CD45 was evaluated by flow cytometry analysis. On average, more than 95% of cells were positive for the markers of staminality (CD29, CD73, and CD90) and less than 5% displayed the markers of hematopoietic cells (CD14, CD34, and CD45). HDPSCs were cultured in high-glucose DMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L–glutamine and 1 mM ascorbic acid, at 37°C with 5% CO_2. Cells were always sub-cultured at a confluence of 70–80% using trypsin digestion.

2.6. Cytotoxicity assay

The cytotoxicity of the synthesized monomers was tested on HDPSCs at passages 3-5 after isolation. Cell viability was evaluated after 24 and 72 h of cell culture in the presence of the tested compounds by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) growth assay (CellTiter 96®AQueous One Solution Cell Proliferation Assay-Promega), based on the capability of viable cells to reduce MTS into a colored formazan product. Tested compounds were solubilized at different concentrations in a complete culture medium and sterilized by 0.22 µm filtration. The cells were seeded into 96-well plates at 6 × 103 cells/well. After 24 h, the cells were treated with a culture medium containing the compounds. At the established time points, the medium was replaced with a fresh one containing MTS reagent according to the manufacturer's protocol, and the cells were incubated for 4 h in a dark and humidified atmosphere of 5% CO_2 at 37°C. The medium was then collected from each well and the absorbance was measured at 490 nm using a microplate spectrophotometer (Infinite M200PRO NanoQuant, Tecan). The values obtained in the absence of cells were considered as background and subtracted from the optical density values of the samples. Cell cultured in plain medium and the presence of 0.01% v/v of Triton X-100 in DMEM were used as a negative and positive control of cell death, respectively. The percentage of vitality was calculated on the negative control. For each series of samples, eight replicates were considered (N = 8).

2.7. Statistical analysis

Statistical analysis to evaluate biofilm inhibition and cytotoxicity was conducted using the unpaired two-sample Student's t-test at a preset $\alpha = 0.01$. Statistical differences between the treated groups and the control group were evaluated considering eight replicates for each group (N = 8). The data are reported as mean value ± standard deviation.

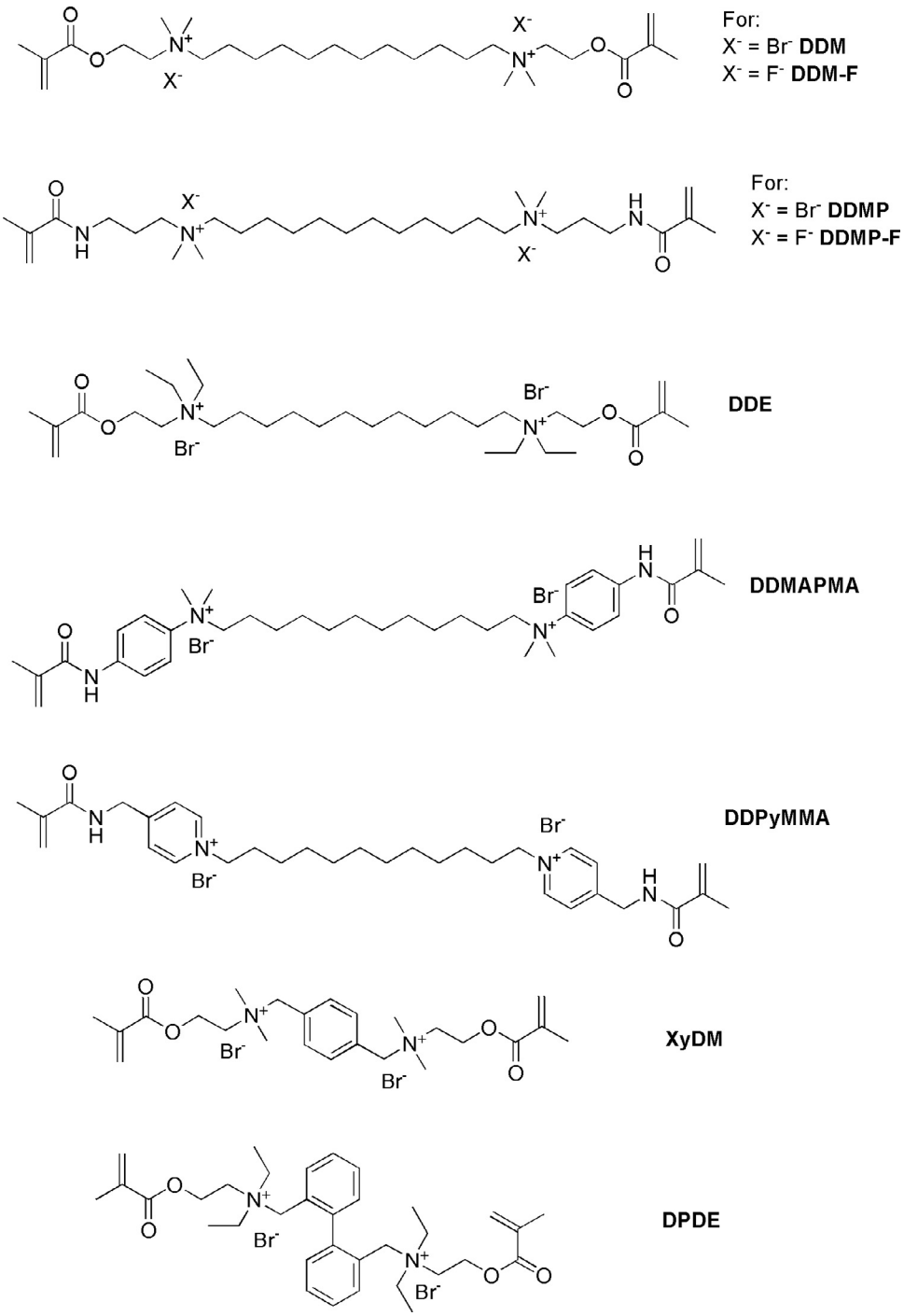

Fig. 1. Structures of newly synthesized antibacterial di-methacrylate bis-QAMs monomers. N,N′-bis[2-((methacryloyl)oxy)ethyl]-N,N,N′,N′-tetramethyl-N,N′-dodecyl diammonium bromide (DDM); N,N′-bis[2-((methacryloyl)oxy)ethyl]-N,N,N′,N′-tetramethyl-N,N′-dodecyl diammonium fluoride (DDM-F); N,N′-bis[2-((methacryloyl)amino)propyl]-N,N,N′,N′-tetramethyl-N,N′-dodecyl diammonium bromide (DDMP); N,N′-bis[2-((methacryloyl)amino)propyl]-N,N,N′,N′-tetramethyl-N,N′-dodecyl diammonium fluoride (DDMP-F); N,N′-bis[2-((methacryloyl)oxy)ethyl]-N,N,N′,N′-tetraethyl-N,N′-dodecyl diammonium bromide (DDE); N,N'-bis((4-methacryloyl)amino)phenyl-N,N,N',N'-tetramethyl-N,N'-dodecyl diammonium bromide (DDMAPMA); bis[4-(((2-methylacryloyl)amino)methyl)-1, 12-dodecyl dipyridinium bromide (DDPyMMA); N,N′-bis[2-((methacryloyl)oxy)ethyl]-N,N,N′,N′-tetramethyl-N,N′-xylyl diammonium bromide (XyDM); 2,2'-bis[((methacryloyl)oxy)ethyl]-diethylammonium bromide-1,1'-benzyl (BPDE).

3. Results

3.1. Structure validation

Fig. 1 and 2 show the structures of newly and formerly synthesized monomers used in this study and their identification acronyms. NMR characterization of the synthesized structures is available in the Appendix: Supporting information.

3.2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and antibiofilm activity

Table 1 shows the MIC and MBC values against *S. mutans* obtained for the synthesized bis-QAMs compared to the monomers already described in literature MDPB [12] and IDMA-2 [15]. Among the nine new di-methacrylate bis-QAMs, XyDM and BPDE were the less active against *S. mutans*, showing MIC and MBC values of the

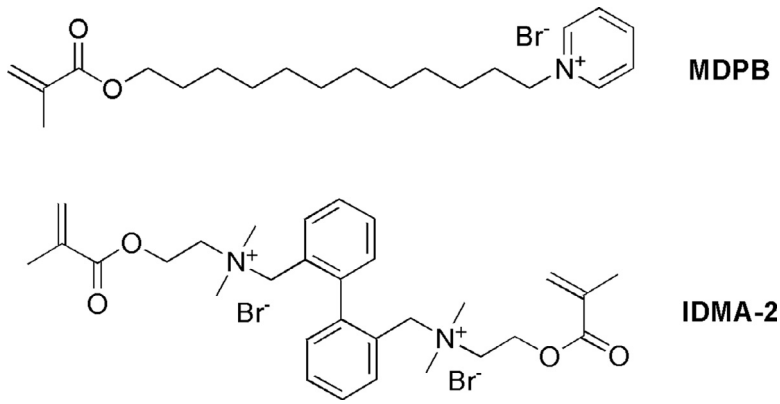

Fig. 2. Structures of the formerly synthesized antibacterial QAMs monomers. 12-methacryloyloxydodecyl pyridinium bromide (MDPB); 2,2'-bis[((methacryloyl)oxy)ethyl] dimethylammonium bromide-1,1'-benzyl (IDMA-2).

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Streptococcus mutans* of nine synthesized bis-QAMs compared with the formerly reported antibacterial monomers MDPB and IDMA-2. (N = 6).

Monomer	*S. mutans*	
	MIC (μg/mL)	MBC (μg/mL)
DDM	150	300
DDM-F	156	313
DDMP	150	310
DDMP-F	156	156
DDE	10–20	20
DDMAPMA	5	10
DDPyMMA	2.5	2.5
XyDM	2500	2500
BPDE	2500	10000
MDPB	4.0–8.0	8
IDMA-2	5000	5000

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of bis-QAMs against different bacterial strains. (N = 6).

Monomer	*E. coli*		*S. aureus*		*S. sanguinis*		*S. mitis*	
	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC
DDM	625	625	156	156	310	620	310	620
DDMP	1250	1250	625	1250	620	620	620	620
DDE	78	156	20	39	20–39	39	39	39
DDMAPMA	20	20	10	10	5	5	10	10
DDPyMMA	2.5	2.5	2.5	5	10	20	20	20

same magnitude of IDMA-2 and a thousand-time factor the ones of DDMAPMA, DDPyMMA and MDPB. DDPyMMA showed excellent inhibitory and bactericidal activity at a lower concentration than MDPB, while the antibacterial efficiency of DDMAPMA was comparable to that of MDPB. DDE showed an excellent antibacterial activity with MIC and MBC only slightly higher than DDMAPMA and MDPB. DDM, DDM-F, DDMP and DDMP-F showed similar MIC and MBC values and an intermediate efficacy as bactericidal agents compared to the other monomers tested.

Table 2 shows the MIC and MBC values of DDM, DDMP, DDE, DDMAPMA and DDPyMMA against Gram-positive *S. sanguinis, S. mitis* and *S. aureus*, and Gram-negative *E. coli*. All the monomers tested showed inhibitory and bactericidal effects against the four bacterial strains comparable to that obtained for *S. mutans*. DDMAPMA showed the highest activity with MIC and MBC ranging between 2.0–10 μg/mL and the best results were found against *E. coli* and *S. sanguinis*. Similar results were obtained for DDPyMMA with MIC and MBC in the range 2.5–20 μg/mL with particularly higher efficiency against *E. coli* and *S. aureus*. DDE showed the same efficiency against *S. aureus, S. sanguinis* and *S. mitis* with MIC and MBC in the range 20–39 μg /mL, but a higher concentration was mandatory to inhibit and kill completely. *E. coli* (78–156 μg /mL). DDM and DDMP values of MIC and MBC were higher than those of the other monomers: DDM was active in concentrations between 156–625 μg/mL against all the tested bacteria, and DDMP in 625-1250 μg/mL. Both monomers showed less activity against *E. coli*.

Table 3
Effects of DDM, DDMP, DDE, DDMAPMA and DDPyMMA on *S. mutans* biofilm formation. The tested concentration was (a) equal to or (b) twofold the MIC value.

Monomer	Tested concentrations (μg/mL)			
	MIC (a)	% inhibition	2xMIC (b)	% inhibition
DDM	150	27	300	52
DDMP	150	50	300	60
DDE	15	53	30	60
DDMAPMA	5	48	10	66
DDPyMMA	2.5	68	5	70

Investigating whether the synthesized bis-QAMs could also exert an antibiofilm activity, the effects of DDM, DDMP, DDE, DDMAPMA and DDPyMMA on *S. mutans* biofilm formation was assessed using an MTT biomass staining assay, with the results shown in Table 3 and Fig. 3.

A significant ($p < 0.01$) reduction in biofilm formation was observed for all the tested samples in comparison to the "no-inhibitor" control (CNT). Comparing the results of the tested molecules with the control, it was observed that, even at a concentration equal to the MIC value, all the tested monomers significantly inhibited the formation of the bacterial biofilm and the inhibitory effect was directly correlated to the bis-QAM concentration.

3.3. Cytotoxicity

Cytotoxicity of the monomers DDM, DDE, DDMAPMA and DDPyMMA was tested using HDPSCs as a cellular model. Cytotoxicity assays were performed exposing the cells for 24 and 72 h to monomers dissolved at various concentrations in a culture medium.

Table 4 shows that toxicity was concentration-dependent, indeed increasing the monomer concentration in medium decreased

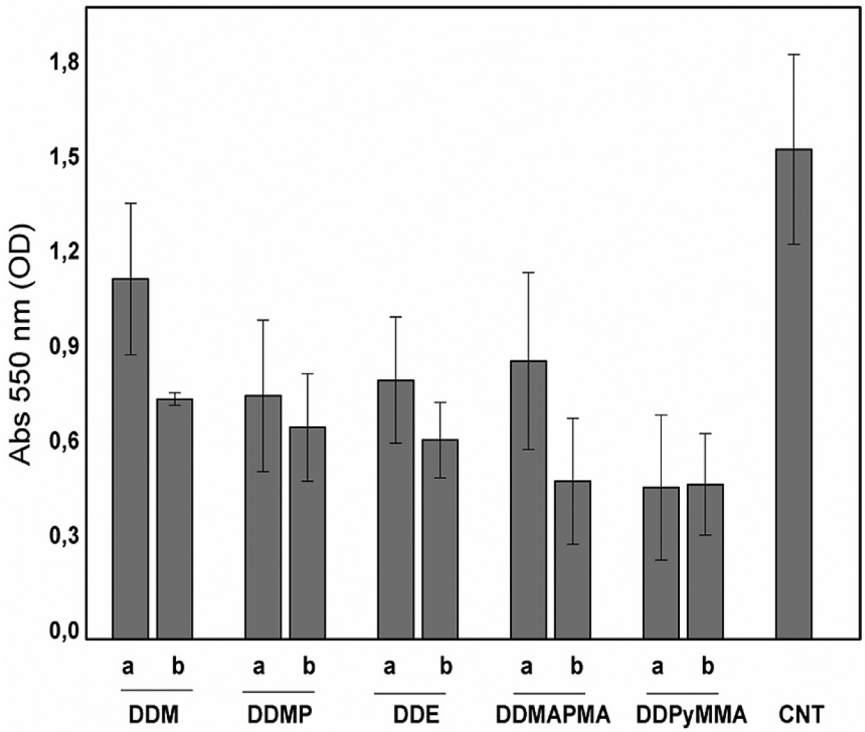

Fig. 3. Inhibition of *S. mutans* biofilm formation after 24 h in the presence of five synthesized bis-QAMs compared with bacterial biofilm grown on plate-well bottom (CNT) (mean OD ± standard deviation; N = 8). The tested concentration was (a) equal to or (b) twofold the MIC value.

Table 4
Percentage of viability of human dental pulp stem cells (mean value ± standard deviation) at 24 and 72 exposition to free monomers dissolved at various concentrations in culture medium (N = 8). n.t. = not-tested concentrations.

Monomer Conc.	DDM % Viability		DDE % Viability		DDMAPMA % Viability		DDPyMMA % Viability	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
2 mg/mL	81 ± 5	77 ± 8	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
1 mg/mL	n.t.	n.t.	(-0.4) ± (-1)	(-2) ± (-4)	3 ± 1	4 ± 2	49 ± 8	5 ± 2
500 µg/mL	87 ± 6	115 ± 12	3 ± 1	(-3) ± (-2)	2 ± 1	3 ± 4	92 ± 17	27 ± 5
100 µg/mL	103 ± 7	122 ± 7	96 ± 7	69 ± 18	11 ± 2	2 ± 1	92 ± 12	72 ± 13
50 µg/mL	106 ± 11.	111 ± 7	83 ± 5	71 ± 18	82 ± 8	72 ± 16	96 ± 11	86 ± 16
10 µg/mL	95 ± 13	110 ± 14	119 ± 7	70 ± 17	100 ± 14	98 ± 14	106 ± 12	101 ± 18
1 µg/mL	n.t.	n.t.	124 ± 6	73 ± 17	95 ± 13	103 ± 19	111 ± 16	97 ± 18

cell viability. For the monomer DDM, the highest non-toxic concentration appeared to be around 2 mg/mL, whereas for DDE, DDMAPMA and DDPyMMA it was in the range 50–100 µg/mL. All four new monomers could be deemed biocompatible at concentrations higher than their MIC and MBC values for the analyzed cells.

4. Discussion

In the present study, nine new polymerizable antibacterial di-methacrylate bis-quaternary ammonium monomers (bis-QAMs) were developed and characterized. The final goal is to provide new molecular structures that combine the advantage of bearing two methacrylate crosslinker moieties for the co-polymerization with commonly used methacrylate monomers, with an improved bactericidal effect due to the presence of two quaternary ammonium functionalities. Furthermore, the structural differences that characterize the synthesized monomers could improve the understanding of the correlation between the structure and the bactericidal efficacy of antibacterial bis-QAMs. Indeed, this topic has not yet been deeply investigated in the literature to the best of our knowledge.

For the preparation of the monomers, the Menschutkin reaction [29] was used: starting from commercially available reactants, the reaction conditions were optimized avoiding extreme pressure and temperature; the purification process was simplified by precipitating the products directly from the reaction medium and obtaining the final product in high yield (≥ 70%) by filtration. Only in the case of DDPyMMA and DDMAPMA a two-steps synthetic route was followed, the first step consisting of the preparation of the intermediates under common amide-synthesis conditions. [30]. The second reaction step was an optimized Menshutkin reaction.

The monomer IDMA-2 was synthesized following a procedure slightly different from the one reported in the literature [15], using CH_3CN as a solvent and a temperature of 65°C. The monomer was collected by filtration after reaction of 2.5 equivalents (eq) of DMAEM with 1 eq of $bPhMBr_2$.

For seven of the new monomers (DDM, DDM-F, DDMP, DDMP-F, DDE, DDPyMMA and DDMAPMA), an alkyl C_{12} was chosen as a spacer for the two quaternary ammonium groups because the presence of a C_{12} alkyl spacer can provide some useful features for an antibacterial cross-linking di-methacrylate monomer. For instance, the medium length of a C_{12} chain could contribute to the balance of the structure hydrophobicity, increasing the bactericidal effect, as in the case of mono-methacrylate QAMs [6], and yet could avoid a partial shielding of the quaternary ammonium site

due to an excessive bend/curl as in case of longer (C_{16}, C_{18}) alkyl chains [31]. Also, a cross-linking bis-QAM bearing a C_{12} spacer that mimics the total length of common commercial crosslinkers, such as bisphenol A-glycidyl methacrylate (Bis-GMA) and triethylene glycol dimethacrylate (TEGDMA), could contribute to maintaining the polymeric network stability and high mechanical properties. Moreover, to investigate the effect of a more rigid spacer, the derivatives XyDM and BPDE were prepared with 1,4-xylene and bis-phenyl moieties as monomer-core.

Considering the nature of the bond between the portion derived from methacrylic acid and the rest of the molecule, among the synthesized bis-QAMs, five of them could be classified as di-methacrylamides (DDMP, DDMP-F, DDMAPMA and DDPyMMA) and four of them as di-methacrylates (DDM, DDM-F, DDE, XyDM and BPDE). Even though, in general, the ester or amide bond present in QAMs is not directly correlated to the antibacterial properties, their hydrolyzable nature could represent a drawback in terms of monomer molecular stability. In the desired and reasonable scenario in which these new bis-QAMs could be used in dental adhesive systems, their compatibility in formulation with other methacrylate monomers is an issue of primary importance. Besides primers and bonding agents in etch and rinse adhesive systems, characterized by the presence of low and high viscosity hydrophilic and hydrophobic monomers, it is mandatory to consider the extensive use of resins classified as "self-etch" and "universal." These classes of primers and bonding agents contain acidic monomers, which could promote the hydrolysis of common mono- and di-methacrylates and, indeed, QAMs [32]. Based on these premises, the well-known lower reactivity of amides compared to esters in acidic conditions [33] might allow extending the use of the new bis-QAMs as components of "self-etch" and "universal" systems avoiding hydrolysis and loss of chemical and mechanical properties of the material.

Although the detailed mechanism for the antibacterial effect of QAMs has not been determined yet, the correlation between their bactericidal effect and their contact-interaction with the bacterial membrane is generally accepted [6]. Under this consideration, three modes of action have been proposed: 1) an electric imbalance of negatively charged bacterial cells caused by contact with positively charged nitrogen that leads to increased osmotic pressure; 2) diffusion through the cell wall and binding to the cytoplasmic membrane; and 3) disruption of the cytoplasmic membrane, the release of cytoplasmic constituents and cell death [6]. In particular, the first mechanism relies on the availability of the cationic sites, implying that an increase in the material surface charge density would correspond to an increased antibacterial activity [34]. The other two hypothesized mechanisms could be correlated to the penetration of a hydrophobic chain, bonded to the ammonium, into the cell membrane, explaining the greater activity generally found for mono-methacrylate QAMs with C_{12} and C_{16} alkyl substituents in comparison to those with shorter or longer chains [31,35]. In the present study, the interest was mainly focused on the preparation of cross-linking bis-QAMs, in which the antibacterial activity would be mainly related to the increase of the charge density *per* molecule compared to common QAMs. Thus, to assure the availability of the quaternary ammonium moieties also after co-polymerization, low hindrance methyl groups were chosen as two of the nitrogen substituents for most of the prepared bis-QAMs. In the case of DDPyMMA, the intrinsic structural planarity of the aromatic ring should assure a low steric hindrance and good accessibility of the pyridinium ion. Additionally, DDE and BPDE were synthesized as DDM and IDMA-2 homologous, characterized by a slightly more hindered cationic nitrogen bonded to two ethyl groups instead of two methyl groups.

To our best knowledge, the effect of the quaternary ammonium counterion on the antibacterial activity has not been utterly elucidated and controversial results can be found in the literature [6]. For instance, Chen et al. [36] and Flemming et al. [37] described a correlation between the type of counterion and the biocide properties of quaternary ammonium-bearing dendrimers and polyurethanes, respectively, reporting a greater activity when bromide or iodide was introduced as counterion instead of chloride. However, the work carried out by Panarin et al. [38] on the synthesis of homopolymers of vinylamine and methylmethacrylate with pendant quaternary ammonium groups showed no effect for counterions on the antibacterial activities among chloride, bromide and iodide. The variability of the obtained results might be correlated to several differences among the studied structures and, consequently, to their intrinsic structure-activity relationship, suggesting that even if the effect of different types of counterion is not easily predictable, it might not be entirely negligible. Based on this idea, among the monomers developed in the present study, two fluorinated bis-QAMs (DDM-F and DDMP-F) were synthesized by anion exchange reaction from the relative bromide derivative (DDM and DDMP, respectively). Fluoride was chosen among other counterions because of its well-documented anti-cariogenic effectiveness [39] and to increase the monomers' potential against secondary caries.

The range of activity of the new bis-QAMs was tested against five different bacterial strains: four Gram-positive (*S. mutans, S. sanguinis, S. aureus* and *S. mitis*) and one Gram-negative (*E. coli*). *S. mutans, S. sanguinis* and *S. mitis* are typical inhabitants of the healthy human mouth where are found in dental plaque, while *E. coli* and *S. aureus* are two bacterial species that are not part of the normal oral microbiota.

The first screening of the antibacterial activity was against *S. mutans:* a cariogenic, aerotolerant anaerobic bacterium and the primary etiological agent of dental caries [40]. To clarify the relationship between the bis-QAMs structure and its activity against bacteria, the inhibitory and bactericidal activity in terms of MIC and MBC of the presented monomers was compared with that of one of the oldest antibacterial compounds introduced in dental materials: MDPB [8] and the bis-phenyl derivative IDMA-2 [15].

From the data obtained in the present study, some hypotheses could be drawn on the correlation between the antibacterial efficiency of di-methacrylate bis-QAMs and the type of spacer separating the two quaternary ammonium moieties. The use of a xylyl or a bis-phenyl-type spacer corresponded to an evident decrease of the monomer inhibitory activity in comparison to structures with alkyl C_{12} spacer and comparable methacrylate portion, ammonium substituents and counterion ($MBC_{DDM} << MBC_{XyDM} < MBC_{IDMA\text{-}2}$; $MBC_{DDE} << MBC_{BPDE}$). XyDM, BPDE and IDMA-2 showed MIC and MBC values ten or even a thousand times higher than their homologous, suggesting that the spacer's aromatic nature might be correlated to this effect. The chemical behavior of cationic gemini surfactants in an aqueous solution might be helpful to explain this behavior. Cationic gemini surfactants consist of two symmetric quaternary ammonium groups bonded with an alkyl or aromatic spacer [41] and several studies showed a correlation between the chemical characteristics of the spacer and the molecule antibacterial activity [41]. Thus, it is possible to speculate about the results obtained in the present study hypothesizing a similar behavior for non-immobilized bis-QAMs: in fact, in amphiphilic molecules, such as surfactants and QAMs, the balance between the hydrophilic and the hydrophobic portion is a crucial parameter for achieving good antibacterial properties and, to this end, the spacer nature plays a paramount role [42]. Laatiris et al. [43] and, more recently, Zhu et al. [44] reported an interesting inverse correlation between the antibacterial activity of cationic gemini surfactants and their aggregation in an aqueous solution, meaning that to a high surface activity corresponded a low antibacterial activity. The chemical properties of the spacer can be directly associated with the

aggregation behavior of amphiphilic gemini surfactants, in particular to the critical micellar concentration (CMC) and to the surface activity, which was found to decrease and increase respectively, with the spacer hydrophobicity and π-π stacking interactions [45]. The ability of gemini surfactants with xylyl spacer to tightly pack on the water-air interface is well known [46], as well as the general negative effect of molecular aggregation on the antibacterial activity [43]. In the same way, di-methacrylate bis-QAMs characterized by aromatic spacers, such as xylyl and bis-phenyl, have the potential to pack more tightly than the ones with alkyl spacers, forming aggregates in aqueous solutions thanks to π-π stacking interactions. The higher the stability of the formed aggregates, the lower their solubility [47], therefore decreasing the single molecule's ability to transfer into the bacterial membrane and subsequently decreasing their inhibitory and bactericidal effect.

As expected, the methacrylate and the methacrylamide homologous DDM and DDMP showed no distinct differences in antibacterial efficacy against *S. mutans*, neither did the counterion exchange from bromide to fluoride in their structures. Thus DDM, DDMP, DDM-F and DDMP-F could be considered all components of the same bactericidal family, in which the few structural differences did not affect the antibacterial activity.

Interestingly, the bis-QAMs derivatives bearing two ethyl lateral groups as ammonium substituents, DDE and BPDE, showed a surprising activity variation compared to their respective homologous DDM and IDMA-2. DDE showed a significant decrease in both MIC and MBC values with a bactericidal activity in concentration tenfold lower than the one used for DDM; this result was opposite to what expected, as the increase of the steric hindrance around the ammonium would partially shield the cationic charge, affecting its interaction with the bacterial membrane. Speculating about this result, it is possible to consider that free bis-QAMs in solution have a potential bactericidal action through all the three mechanisms reported before, following the structure-activity general rules determined for mono-QAMs. Under this point of view, the principle of "the more hydrophobic N-substituent, the higher antibacterial activity" [31,35] might be considered and it is possible that even a small increase of hydrophobicity due to the presence of ethyl substituents instead of methyl, might provide more effective interaction between the monomer and the bacterial membrane. Differently, contrasting results were obtained comparing MIC and MBC values of BPDE and IDMA-2, as BPDE showed inhibitory activity in lower concentration, but the bactericidal effect in higher concentration than IDMA-2. In this case, the effects of the ethyl substituents were not completely clear and complex to explain: the antibacterial activity of BPDE and IDMA-2 was probably correlated to the equilibrium among rigidity and aggregation-ability due to the aromatic spacer, to the increase of hydrophobicity due to the ethyl substituents and the availability of the quaternary ammonium moieties.

DDMAPMA and DDPyMMA showed superior bactericidal activity against *S. mutans* than DDM, DDE and DDMP and comparable to MDPB. This result might be correlated to the increased lipophilicity of DDMAPMA and DDPyMMA provided by the introduced aromatic moieties compared to the C_2 and C_3 alkyl chain of DDM, DDE and DDMP, and the consequent improvement of the bacterial membrane affinity. A comparable effect for mono-QAMs [48] and quaternary ammonium pyridinium salts [49] was reported in the literature, confirming the increase of antibacterial effectiveness in the case of molecules bearing aromatic groups.

The bactericidal activity showed by DDM, DDMP, DDE, DDMAPMA and DDPyMMA against Gram-positive bacteria such as *S. sanguinis, S. aureus* and *S. mitis* was indeed promising and most of the same magnitude as the one observed against *S. mutans*. DDMP was the sole to require a concentration higher than 1 mg/mL to achieve complete killing of *S. aureus.* Furthermore, DDE and, most of all, DDMAPMA and DDPyMMA showed to be also highly effective against *E. coli* and to be able to successfully interact with the double bilayer cell membrane that characterizes Gram-negative bacteria, probably thanks to increased lipophilicity correlated to their structures.

The response of eukaryotic cells in direct contact with an antimicrobial material is a piece of important information. The cytotoxicity of the four bis-QAMs showing the best bactericidal activity and the broader range of action was tested on HDPSCs. All the tested monomers displayed a certain degree of dose-dependent cellular toxicity. However, this was predictable since in the last years several studies have investigated and identified the cytotoxicity and genotoxicity of non-antibacterial methacrylate monomers commonly used in dentistry such as TEGDMA and HEMA [50].

From the obtained results, DDM was shown to be the less cytotoxic monomer and, theoretically, the use of this monomer in concentration up to 2 mg/mL in dental resins would lead to optimal biocompatible antibacterial materials. This result was not surprising as, in general, cytotoxicity was directly related to the bactericidal activity of the analyzed compound, in particular: higher the MIC and MBC values, higher the monomer concentration that can be used without relevant cellular toxicity. This can be probably associated with the fact that a certain extent of the molecular mechanisms driving the toxicity against bacterial cells can be correlated to the ones that affect eukaryotic cell viability, such as binding and disruption of the cytoplasmic membrane [6]. However, it is noteworthy that monomers with different antibacterial activity, in terms of MIC and MBC, and different structures such as DDE and DDPyMMA, showed similar cytotoxicity. On the other hand, DDMAPMA and DDPyMMA showed a significant difference in the value of minimum concentration affecting cell viability, despite having some comparable structural features. On the basis of the obtained results, the best performing monomer was DDPyMMA that combined the lowest values of MIC and MBC with the lowest cytotoxicity.

Moreover, it is noteworthy that the toxicity of all the new monomers tested was lower than those of other monomers described in the literature, such as: MDBP, which has been reported to have a non-cytotoxic effect at a concentration up to 40 µg/mL on HDPSCs [12]; methacrylate and di-methacrylate QAMs [11]; and commonly used not-antibacterial Bis-GMA (2 µg/mL) [51] tested on human gingival fibroblasts.

The different behavior of bis-QAMs towards procaryotic and eukaryotic cells could be correlated to different characteristics of the cell membranes [52,53]: a different affinity between the membrane lipids and QAMs seems reasonable, even though not fully interpreted yet could affect the antibacterial activity of the monomers. Basing on the obtained results, it is possible to speculate that probably, thanks to their negatively charged membranes that could interact with the positively charged ammonium salts, prokaryotic cells are more sensitive to the QAMs antibacterial mechanism of action than eukaryotic ones.

One of the major issues toward the development of antibacterial materials for medical use is the possible correlation between the employment of quaternary ammonium compounds (QACs) and the development of antimicrobial resistance. Some evidence reported in the literature shows that mono-QAM dimethylaminododecyl methacrylate (DMADDM) and chlorhexidine (CHX) caused drug resistance in *S. gordonii* [54] and associate the development of *S. mutans* persistence with the use of DMADDM in concentration 10- and 100-fold the MIC value [55]. In the field of restorative dentistry, the development of bactericidal adhesives containing QAMs aims to avoid bacterial proliferation at the restoration margins, requiring a close contact between the QAM and the bacterial membrane to be effective. This means that not all the bacteria present in the oral environment will be killed, but only those

adhering to the tooth surface near the restoration margins. In this scenario, if oral bacteria will develop some resistance to the QAMs, the result would only be the loss of the adhesive's antibacterial properties, which would become inactive against secondary caries development. Moreover, considering that the adhesive interface between the tooth and the restoration has a thickness limited to few microns and that the concentration of QAMs in the resin will not exceed 5% w/w, the potential risk of dysbiosis in the whole oral environment is very low.

5. Conclusions

From the obtained results, among the nine newly synthesized bis-QAMs, DDM, DDE, DDMAPMA and DDPyMMA could be recognized as very promising bactericidal agents to be used in the dental field.

The analysis of the proposed structures and their comparison with previously published antibacterial QAMs allowed to define some general structure-activity correlations which could be assessed for free di-methacrylate bis-QAMs:

- The presence of a flexible alkyl C_{12} spacer between the two quaternary ammonium moieties (such as for DDM, DDM-F, DDMP, DDMP-F, DDE, DDPyMMA and DDMAPMA) increased the monomers antibacterial effect in comparison to the aromatic ones, such as XyDM, IDMA-2 and BPDE;
- The equilibrium between hydrophobic and hydrophilic moieties in the structure was paramount for the design of efficient monomeric biocides, as it was directly correlated to the bactericidal ability and the range of action;
- The increase of steric hindrance of the ammonium side groups might be both advantageous or disadvantageous depending on the global structure of the monomer;
- Formulating a cytotoxicity prediction of a structure based on its antibacterial activity in terms of MIC and MBC is not a trivial task, although the correlation between toxicity and QAM nature is not clear yet.

Declaration of Competing Interest

The authors declare to be also the inventors of the patent presenting the structures and activity of the newly synthesized monomers.

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2021.05.012.

References

[1] M. Hanning, Transmission electron microscopy of early plaque formation on materials in vivo, Eur. J. Oral. Sci. 107 (1) (1999) 55–64, doi:10.1046/j.0909-8836.1999.eos107109.x.

[2] F. Demarco, K. Collares, M. Correa, M. Cenci, R. Moraes, N. Opdam, Should my composite restorations last forever? why are they failing? Braz. Oral. Res. 28 (31) (2017) e56 (suppl 1), doi:10.1590/1807-3107BOR-2017.

[3] A. Frassetto, L. Breschi, G. Turco, G. Marchesi, R. Di Lenarda, F. Tay, D. Pashley, M. Cadenaro, Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability - a literature review, Dent. Mater. 32 (2016) e41–e53, doi:10.1016/j.dental.2015.11.007.

[4] L. Breschi, T. Maravic, S. Cunha, A. Comba, M. Cadenaro, L. Tjäderhane, D. Pashley, F. Tay, A. Mazzoni, Dentin bonding systems: from dentin collagen structure to bond preservation and clinical applications, Dent. Mater. 34 (2018) 76–96, doi:10.1016/j.dental.2017.11.005.

[5] A. Cocco, W. Rosa, A. Silva, R. Lund, E. Piva, A systematic review about antibacterial monomers used in dental adhesive systems: current status and further prospects, Dent. Mater. 31 (11) (2015) 1345–1362, doi:10.1016/j.dental.2015.08.155.

[6] P. Makvandi, R. Jamaledin, M. Jabbari, N. Nikfarjam, A. Borzacchiello, Antibacterial quaternary ammonium compounds in dental materials: a systematic review, Dent. Mater. 34 (6) (2018) 851–867, doi:10.1016/j.dental.2018.03.014.

[7] L. Chen, B.I. Suh, J. Yang, Antibacterial dental restorative materials: a review, Am. J. Dent. 31 (Sp Is B) (2018) 6B–12B.

[8] S. Imazato, R. Russell, J. McCabe, Antibacterial activity of MDPB polymer incorporated in dental resin, J. Dent. 23 (3) (1995) 177–181, doi:10.1016/0300-5712(95)93576-n.

[9] e.-R. Kenawy, S. Worley, R. Broughton, The chemistry and applications of antimicrobial polymers: a state-of-the-art review, Biomacromolecules 8 (5) (2007) 1359–1384, doi:10.1021/bm061150q.

[10] S. Imazato, J. Chen, S. Ma, N. Izutani, F. Li, Antibacterial resin monomers based on quaternary ammonium and their benefits in restorative dentistry, Jpn. Dent. Sci. Rev. 48 (2) (2012) 115–125, doi:10.1016/j.jdsr.2012.02.003.

[11] F. Li, M. Weir, A. Fouad, H. Xu, Time-kill behaviour against eight bacterial species and cytotoxicity of antibacterial monomers, J. Dent. 41 (2013) 881–891, doi:10.1016/j.jdent.2013.07.006.

[12] S. Imazato, N. Ebi, H. Tarumi, R. Russel, T. Kaneko, S. Ebisu, Bactericidal activity and cytotoxicity of antibacterial monomer MDPB, Biomaterials 20 (9) (1999) 899–903, doi:10.1016/s0142-9612(98)00247-6.

[13] S. Imazato, Y. Kinomoto, H. Tarumi, S. Ebisu, F. Tay, Antibacterial activity and bonding characteristics of an adhesive resin containing antibacterial monomer MDPB, Dent. Mater. 19 (4) (2003) 313–319, doi:10.1016/s0109-5641(02)00060-x.

[14] S. Imazato, D. Horikawa, M. Nishida, S. Ebisu, Effect of monomers eluted from dental resin restoratives on osteoblast-like cells, J. Biomed. Mater. Res. B: Appl. Biomater. 88 (2009) 378–386.

[15] J. Antonucci, D. Zeiger, K. Tang, S. Lin-Gibson, B. Fowler, N. Lin, Synthesis and characterization of dimethacrylates containing quaternary ammonium functionalities for dental applications, Dent Mater 28 (2) (2012) 219–228, doi:10.1016/j.dental.2011.10.004.

[16] Y. Ge, S. Wang, X. Zhou, H. Wang, H. Xu, L. Cheng, The use of quaternary ammonium to combat dental caries, Materials (Basel) 8 (6) (2015) 3532–3549, doi:10.3390/ma8063532.

[17] Y. Xiao, S. Ma, J. Chen, Z. Chai, F. Li, Y. Wang, Antibacterial activity and bonding ability of an adhesive incorporating an antibacterial monomer DMAE-CB, J. Biomed. Mater. Res. B. Appl. Biomater. 90 (2) (2009) 813–817, doi:10.1002/jbm.b.31350.

[18] L. Huang, X. Sun, Y. Xiao, Y. Dong, Z. Tong, X. Xing, F. Li, Z. Chai, J. Chen, Antibacterial effect of a resin incorporating a novel polymerizable quaternary ammonium salt MAE-DB against Streptococcus mutans, J. Biomed. Mater. Res. B. Appl. Biomater. 100 (5) (2012) 1353–1358, doi:10.1002/jbm.b.32703.

[19] X. Liang, Q. Huang, F. Liu, J. He, Z. Lin, Synthesis of novel antibacterial monomers (UDMQA) and their potential application in dental resin, J. Appl. Polym. Sci. 129 (6) (2013) 3373–3381, doi:10.1002/app.39113.

[20] L. Cheng, K. Zhang, M. Melo, M. Weir, X. Zhou, H. Xu, Anti-biofilm dentin primer with quaternary ammonium and silver nanoparticles, J. Dent. Res. 91 (6) (2012) 598–604, doi:10.1177/0022034512444128.

[21] L. Cheng, M. Weir, P. Limkangwalmongkol, G. Hack, H. Xu, Q. Chen, X. Zhou, Tetracalcium phosphate composite containing quaternary ammonium dimethacrylate with antibacterial properties, J. Biomed. Mater. Res. B. Appl. Biomater. 100B (3) (2012) 726–734, doi:10.1002/jbm.b.32505.

[22] R. Kuegler, O. Bouloussa, F. Rondelez, Evidence of charge-density threshold for optimum efficiency of biocidal cationic surfaces, Microbiology 151 (Pt5) (2005) 1341–1348, doi:10.1099/mic.0.27526-0.

[23] H. Murata, R. Koepsel, K. Matyjaszewski, A. Russel, Permanent, non-leaching antibacterial surfaces-2: how high density cationic surfaces kill bacterial cells, Biomaterials 28 (32) (2007) 4870–4879, doi:10.1016/j.biomaterials.2007.06.012.

[24] S. Punyani, H. Singh, Preparation of iodine containing quaternary amine methacrylate copolymers and their contact killing antimicrobial properties, J. Appl. Polym. Sci. 102 (2006) 1038–1044, doi:10.1002/app.24181.

[25] Y. Weng, X. Guo, V. Chong, L. Howard, R. Gregory, D. Xie, Synthesis and evaluation of a novel antibacterial dental resin composite with quaternary ammonium salts, J. Biomed. Sci. Eng. 4 (2011) 147–157, doi:10.4236/jbise.2011.43021.

[26] J. He, E. Soederling, L. Lassila, P. Vallittu, Preparation of antibacterial and radio-opaque dental resin with new polymerizable quaternary ammonium monomer, Dent. Mater. 31 (3) (2015) 575–582, doi:10.1016/j.dental.2015.02.007.

[27] P. Makvandi, M. Ghaemy, M. Mohseni, Synthesis and characterization of photocurable bis-quaternary ammonium dimethacrylate with antimicrobial activity

for dental restoration material, Eur. Polym. J. 74 (2016) 81–90, doi:10.1016/j.eurpolymj.2015.11.011.
[28] R. Kantor, C. Norden, In vitro activity of netilmicin, gentamicin, and amikacin, Antimicrob. Agents Chemother. 11 (1) (1977) 126–131, doi:10.1128/aac.11.1.126.
[29] W. Brasen, C. Hauser, o-Methyethylbenzyl Alcohol, Org Synth 34 (1954) 58, doi:10.15227/orgsyn.034.0058.
[30] C. Montalbetti, V. Falque, Amide bond formation and peptide coupling, Tetrahedron 61 (2005) 10827–10852, doi:10.1016/j.tet.2005.08.031.
[31] F. Li, M. Weir, H. Xu, Effects of quaternary ammonium chain length on antibacterial bonding agents, J. Dent. Res. 92 (10) (2013) 932–938, doi:10.1177/0022034513502053.
[32] U. Salz, J. Zimmermann, F. Zeuner, N. Moszner, Hydrolytic stability of self-etching adhesive systems, J. Adhes. Dent. 7 (2005) 107–116.
[33] N. Nishiyama, K. Suzuki, H. Yoshida, H. Teshima, K. Nomoto, Hydrolytic stability of methacrylamide in acidic aqueous solution, Biomaterials 25 (2004) 965–969, doi:10.1016/s0142-9612(03)00616-1.
[34] F. Li, M. Weir, J. Chen, H. Xu, Effect of charge density of bonding agent containing a new quaternary ammonium methacrylate on antibacterial and bonding properties, Dent. Mater. 30 (4) (2014) 433–441, doi:10.1016/j.dental.2014.01.002.
[35] G. Lu, D. Wu, R. Fu, Studies on the synthesis and antibacterial activities of polymeric quaternary ammonium salts from dimethylaminoethyl methacrylate, React. Funct.Polym. 67 (4) (2007) 355–366, doi:10.1016/j.reactfunctpolym.2007.01.008.
[36] C. Chen, N. Beck-Tan, P. Dhurjati, T. van Dyk, R. LaRossa, S. Cooper, Quaternary ammonium functionalized poly(propylene imine) dendrimers as effective antimicrobials: Structure-activity studies, Biomacromolecules 1 (3) (2000) 473–480, doi:10.1021/bm0055495.
[37] R. Flemming, C. Capelli, S. Cooper, R. Proctor, Bacterial colonization of functionalized polyurethanes, Biomaterials 21 (3) (2000) 273–281, doi:10.1016/s0142-9612(99)00176-3.
[38] E. Panarin, M. Solovskii, N. Zaikina, G. Afinigenov, Biological activity of cationic polyelectrolytes, Macromol. Chem. Phys. 9 (1985) 25–33.
[39] J. Arends, G. Dijkman, A. Dijkman, Review of fluoride release and secondary caries reduction by fluoridating composites, Adv. Dent. Res. 9 (4) (1995) 367–376, doi:10.1177/08959374950090040501.
[40] S. Forssten, M. Bjorklund, A. Ouwehand, Streptococcus mutans, caries and simulation models, Nutrients 2 (3) (2010) 290–298, doi:10.3390/nu2030290.
[41] D. Shukla, V. Tyagi, Cationic gemini surfactants: a review, J. Oleo. Sci. 55 (8) (2006) 381–390, doi:10.5650/jos.55.381.
[42] K. Taleb, M. Mohamed-Benkada, N. Benhamed, S. Saidi-Besbes, Y. Grohens, A. Derdour, Benzene ring containing gemini surfactants: synthesis, surface properties and antibacterial activity, J. Mol. Liq. 241 (2017) 81–90, doi:10.1016/j.molliq.2017.06.008.
[43] A. Laatiris, M. Achouri, M. Infante, Y. Bensouda, Antibacterial activity, structure and CMC relationship of alkanediyl-a,w-bis(dimethylammonium bromide) surfactants, Microbiol. Res. 163 (6) (2008) 645–650, doi:10.1016/j.micres.2006.09.006.
[44] H. Zhu, Z. Hu, X. Ma, J. Wang, D. Cao, Synthesis, surface and antimicrobial activities of cationic gemini surfactants with semi-rigid spacers, J. Surfact. Deterg. 19 (2016) 265–274, doi:10.1007/s11743-015-1777-4.
[45] Z. Zhang, P. Zheng, Y. Guo, Y. Yang, Z. Chen, X. Wang, X. An, W. Shen, The effect of the spacer rigidity on the aggregation behavior of two ester-containing gemini surfactants, J. Colloid Interface Sci 379 (1) (2012) 64–71, doi:10.1016/j.jcis.2012.04.052.
[46] L. Mivehi, R. Bordes, K. Holmberg, Adsorption of cationic gemini surfactants at solid surfaces studied by QCM-D and SPR: effect of the rigidity of the spacer, Langmuir 27 (2011) 7549–7557, doi:10.1021/la200539a.
[47] M. Zhou, H. Liu, H. Yang, X. Liu, Z. Zhang, Y. Hu, Spontaneous crystallization at the air-water interface: an unusual feature of gemini surfactant with a rigid spacer, Langmuir 22 (2006) 10877–10879, doi:10.1021/la061772x.
[48] X. Xu, Y. Wang, S. Liao, Z. Wen, Y. Fan, Synthesis and characterization of antibacterial dental monomers and composites, J. Biomed. Mater. Res. B Appl. Biomater. 100 (4) (2012) 1151–1162, doi:10.1002/jbm.b.32683.
[49] T. Zhao, G. Sun, Hydrophobicity and antimicrobial activities of quaternary pyridinium salts, J. Appl. Microbiol. 104 (3) (2008) 824–830, doi:10.1111/j.1365-2672.2007.03616.x.
[50] M. Kostic, A. Pejcic, M. Igic, N. Gligorijevic, Adverse reactions to denture resin materials, Eur. Rev. Med. Pharmacol. Sci. 21 (23) (2017) 5298–5305, doi:10.26355/eurrev_201712_13909.
[51] L. Huang, Y. Xiao, X. Xing, F. Li, S. Ma, L. Qi, J. Chen, Antibacterial activity and cytotoxicity of two novel cross-linking antibacterial monomers on oral pathogens, Arch. Oral. Biol. 56 (4) (2011) 367–373, doi:10.1016/j.archoralbio.2010.10.011.
[52] C. Sohlenkamp, O. Geiger, Bacterial membrane lipids: diversity in structures and pathways, FEMS Microbiol. Rev. 40 (1) (2016) 133–159, doi:10.1093/femsre/fuv008.
[53] T. Pomorski, S. Hrafnsdóttir, P. Devaux, G. van Meer, Lipid distribution and transport across cellular membranes, Semin. Cell Dev. Biol. 12 (2) (2001) 139–148, doi:10.1006/scdb.2000.0231.
[54] S. Wang, H. Wang, B. Ren, X. Li, L. Wang, H. Zhou, M. Weir, X. Zhou, R. Masri, T. Oates, L. Cheng, H. Xu, Drug resistance of oral bacteria to new antibacterial dental monomer dimethylaminohexadecyl methacrylate, 8(1) (2018) 5509 doi: 10.1038/s41598-018-23831-3Y.L.
[55] Y. Jiang, W. Qiu, X. Zhou, H. Li, J. Lu, H. Xu, X. Peng, M. Li, M. Feng, L. Cheng, B. Ren, Quaternary ammonium-induced multidrug tolerant Streptococcus mutans persisters elevate cariogenic virulence in vitro, Int J Oral Sci 9 (e7) (2017) 1–8, doi:10.1038/ijos.2017.46.