ACP AMERICAN COLLEGE OF PROSTHODONTISTS
Your smile. Our specialty.

Antimicrobial Properties and Cytotoxicity of an Antimicrobial Monomer for Application in Prosthodontics

Rômulo Rocha Regis, DDS,[1] Maria Paula Della Vecchia, DDS,[1] Antônio Carlos Pizzolitto, MS,[2] Marco Antonio Compagnoni, DDS, PhD,[3] Pedro Paulo Chaves Souza, DDS, MS,[4] & Raphael Freitas de Souza, DDS, PhD[1]

[1]Department of Dental Materials and Prosthodontics, Ribeirão Preto Dental School, University of São Paulo, Ribeirão Preto, Brazil
[2]Department of Clinical Analyses, School of Pharmaceutical Sciences of Araraquara, São Paulo State University, Araraquara, Brazil
[3]Department of Dental Materials and Prosthodontics, Araraquara Dental School, São Paulo State University, Araraquara, Brazil
[4]Department of Physiology and Pathology, Araraquara Dental School, São Paulo State University, Araraquara, Brazil

Keywords
Local anti-infectious agents; denture bases; quaternary ammonium compounds; stomatitis under dentures; acrylic resins.

Correspondence
Rômulo R. Regis, Av. do Cafe S/N Ribeirão Preto 14050500, Brazil. E-mail: romuloregis@hotmail.com

Previously presented at the 27th Annual Meeting of the Brazilian Division of the International Association for Dental Research.

The authors would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo–FAPESP (grants no. 2007/533477-7 and 2007/05245-0) for the financial support.

Accepted July 12, 2011

doi: 10.1111/j.1532-849X.2011.00815.x

Abstract

Purpose: This study aimed to investigate the antimicrobial properties and cytotoxicity of the monomer methacryloyloxyundecylpyridinium bromide (MUPB), an antiseptic agent capable of copolymerizing with denture base acrylic resins.
Materials and Methods: The antimicrobial activity of MUPB was tested against the species *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Lactobacillus casei*, *Staphylococcus aureus*, and *Streptococcus mutans*. The minimum inhibitory and fungicidal/bactericidal concentrations (MIC, MFC/MBC) of MUPB were determined by serial dilutions in comparison with cetylpyridinium chloride (CPC). The cytotoxic effects of MUPB at concentrations ranging from 0.01 to 1 g/L were assessed by MTT test on L929 cells and compared with methyl methacrylate (MMA). The antimicrobial activity of copolymerized MUPB was tested by means of acrylic resin specimens containing three concentrations of the monomer (0, 0.3, 0.6% w/w). Activity was quantified by means of a disc diffusion test and a quantification of adhered planktonic cells. Statistical analysis employed the Mann-Whitney test for MIC and MFC/MBC, and ANOVA for the microbial adherence test ($\alpha = 0.05$).
Results: MUBP presented lower MIC values when compared with CPC, although differences were significant for *C. dubliniensis* and *S. mutans* only ($p = 0.046$ and 0.043, respectively). MFC/MBC values were similar for all species except *C. albicans*; in that case, MUPB presented significantly higher values ($p = 0.046$). MUPB presented higher cytotoxicity than MMA for all tested concentrations ($p < 0.001$) except at 0.01 g/L. Irrespective of the concentration incorporated and species, there was no inhibition halo around the specimens. The incorporation of MUPB influenced the adhesion of *C. albicans* only ($p = 0.003$), with lower CFU counts for the 0.6% group.
Conclusions: It was concluded that non-polymerized MUPB has an antimicrobial capacity close to that of CPC and high cytotoxicity when compared with MMA. The antimicrobial activity of MUPB after incorporation within a denture base acrylic resin did not depend on its elution, but was shown to be restricted to *C. albicans*.

Wearing complete dentures may have adverse effects, such as denture-induced stomatitis, a common recurring disease in edentulous patients, on the health of both oral and denture-supporting tissues.[1-3] Despite the multifactorial etiology of stomatitis, great importance can be attributed to bacterial and *Candida* spp. infections, especially when associated with poor oral hygiene.[4,5] Abu-Elteen and Abu-Alteen[6] observed that the prevalence of *C. albicans* among complete denture wearers was 78.3%, whereas only 36.8% of a group of healthy dentate subjects presented it. Acrylic resin denture bases play an important role as reservoirs of microorganisms, leading to increased risk of *Candida* colonization.[7,8] The adherence of these fungi to polymeric surfaces is implicated as the first step in the pathogenesis of associated stomatitis.[4,9] Following

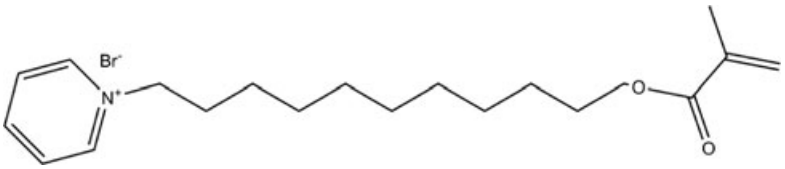

Figure 1 Structure of MUPB.

adherence, microorganisms are capable of dividing, forming microcolonies, secreting exopolymeric material, and ultimately forming a 3D biofilm.[3]

There has been increasing interest in the incorporation of biocides in polymeric dental materials. Several authors attempted to combine antifungals or antiseptics with temporary soft liners or with denture acrylic resin, such as nystatin,[10-12] miconazol,[13] ketoconazol,[13] fluconazole and itraconazole,[10] chlorhexidine,[14] triclosan,[15] titanium dioxide,[16] and zeolites.[17] However, materials that release agents may exert toxic effects or induce population shifts of microorganisms and may suffer from short-lived effectiveness and deterioration of their mechanical properties.[18] Options, such as surface treatment by glow-discharge plasma,[19] glazes,[20] incorporation of polar radicals into the polymer,[21] and the copolymerization of fluoroalkyl methacrylates[22] have been studied.

There are other alternatives with the aim of imparting antimicrobial activity to acrylic resin, without depending on the elution of agents. Examples of these alternatives are: the incorporation of metallic silver nanoparticles[23] and copolymerizable quaternary ammonium compounds.[24-26] A possible advantage of the latter is the covalent linking of antiseptic compounds to dental polymers, which integrates them strongly.[27] An attempt to incorporate covalently bound antiseptics into denture base acrylic resin has been described.[25,28] However, it was based on a quaternary ammonium compound mixed with the polymer of an acrylic resin at high concentrations. There has been no previous description of the addition of an antiseptic methacrylic monomer, which could result in an effective material, except for a small preliminary experiment by Imazato et al.[29]

The 12-methacryloyloxydodecylpyridinium bromide (MDPB) monomer, a quaternary ammonium compound formed by a dodecylpyridinium group and a methacrylate group, is a recently developed copolymerizable biocide. When incorporated into dental composites and adhesive systems in small quantities, it maintains antiseptic effects after the curing process, without elution of the active substance.[18] Nevertheless, the synthetic route to MDPB involves the use of expensive reagents, such as 12-bromo-l-dodecanol. The substitution of the latter by more affordable reagents can result in a potentially more cost-effective antimicrobial monomer. An interesting option is the 11-bromo-l-undecanol, which would result in a new monomer, namely, methacryloyloxyundecylpyridinium bromide (MUPB).

The aim of this study was to determine the antimicrobial activity of the MUPB monomer in its non-polymerized form and following its incorporation into a denture base resin and to determine its cytotoxicity. The evaluated monomer is a quaternary ammonium compound, which, similar to MDPB, presents a methacryloyl group capable of copolymerizing with conventional acrylic resins.

Materials and methods

Structure of MUPB

Figure 1 shows the structure of MUPB, which is a compound of quaternary ammonium undecylpyridinium bromide and a methacryloyl group, synthesized as follows: 11-methacryloyloxyundecyl bromide (MUB) was synthesized by a reaction of 11-bromo-l-undecanol and methacrylic acid at 78°C for 32 hours. Purified MUB was then converted to MUPB by reaction with pyridine at 100°C for 30 minutes and purified. Configuration of the product was confirmed with ^{1}H-NMR (Bruker 400 MHz, Bruker BioSpin Corp., Billerica, MA).

Strains

C. albicans (ATCC 90028), *Candida dubliniensis* (ATCC 7987S), *Candida glabrata* (ATCC 2001), *Staphylococcus aureus* (ATCC 6538), *Streptococcus mutans* (ATCC 25175), and *Lactobacillus casei* (ATCC 393) were used. These species have involvement with denture stomatitis and are able to colonize the edentulous oral cavity by adhering to acrylic resin.[7,30,31] The fungi from stock cultures were cultivated in Sabouraud dextrose agar (SDA), and all bacteria were cultivated in brain heart infusion (BHI) agar (HiMedia, Mumbai, India). A loopful inoculum of each microorganism was transferred to 10 mL of Tryptic Soy Broth (TSB) (HiMedia) and incubated at 37°C for 24 hours before each experiment.

MIC and MFC/MBC measurements

The minimum inhibitory concentration (MIC) assay was based on the microbial growth in TSB containing different concentrations of MUPB. The initial concentration of uncured MUPB in TSB was set at 10 g/L, and serial two-fold dilutions were made into 50 μL volumes of TSB in the wells of 96-well microplates. That procedure resulted in 12 concentrations ranging from 10 to 0.005 g/L. Bacterial cultures of each microorganism were incubated overnight and adjusted to 1–5 $\times$ 10^6 CFU/mL suspensions in TSB prior to inoculating 50 μL into each well containing MUPB solution. After incubation in a candle jar for 24 hours at 37°C, the MIC value was determined by visual examination.

Subcultures were made from the wells without visible growth of microorganisms by spreading 10 μL on SDA plates (HiMedia) plates for *Candida* species and BHI agar plates for bacteria. Each plate was incubated in a candle jar for 1 to 2 days at 37°C, and the MBC value was defined as the lowest concentration of MUPB that precluded colony formation on agar. The values for all strains were compared with cetylpyridinium chloride (CPC) (Sigma, St. Louis, MO), measured by the same method.

Cytotoxicity Test

The MTT (3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide) method, which reflects mitochondrial metabolism, was carried out on L-929 mouse fibroblasts. Cells were cultivated (6×10^3 cell/well) in 96-well microplates (Costar Corp., Cambridge, MA) containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamin and incubated for 24 hours at 37°C with 5% CO_2 and 95% air.

After attachment, phosphate-buffered saline (PBS, pH = 7.1) and 200 μL of MUPB solution were added. Unpolymerized MUPB was first dissolved in dimethyl sulphoxide (VETEC Química Fina, Duque de Caxias, Brazil) (maximum: 1% v/v) and then in complete medium without fetal bovine serum. The final concentrations of MUPB were 1, 0.5, 0.25, 0.05, and 0.01 g/L. The same procedure was repeated for the methyl methacrylate (MMA) monomer (Aldrich, Stanheim, Germany), which served as a control.

After 48 hours of cell growth in either the control or test culture media, the cytotoxicity of the solutions was assessed. Each well received 20 μl of MTT solution (5 g/L in Hanks balanced salt solution), and the microplates were further incubated at 37°C for 3 hours. After the incubation period, 100 μL of acidified isopropanol (0.04 N HCl in isopropanol) was added to the cultures and mixed thoroughly to dissolve the dark blue formazan crystals. After this, cell viability was determined by spectrophotometric measurement of absorbance at 550 to 620 nm (Labsystems Multiskan Ascent, Thermo Labsystems, Vantaa, Finland). Each test was conducted four times as in previous studies.[32,33]

Inhibitory effect on microbial growth

A heat-polymerized acrylic resin (Lucitone 550; Dentsply International Inc., York, PA) was used as the control and the basis for two experimental groups. Three disc-shaped (15-mm diameter × 1-mm thick) specimens were obtained for each group and species. Metal master patterns were individually invested in high-viscosity silicone (Zetalabor; Zhermack S.p.A, Badia Polesine, Rovigo, Italy), and supported by type III dental stone (Herodent; Vigodent SA Ind Com, Rio de Janeiro, Brazil) within flasks. After the dental stone had set, the flasks were separated, and the master patterns were removed from the silicone mold. For the control group, a portion of monomer (10 mL) and polymer (21 g) was mixed for each flask, according to the manufacturer's recommendations, thus reaching a dough stage, and then the mixture was placed into the molds. For the other groups, MUPB was incorporated at 0.3% or 0.6% (w/w). The first concentration was based on a preliminary test described in the patent for MDPB; at that opportunity, acrylic resins containing 0.3% MDPB were tested with promising results against *S. mutans*.[25] In this study, 0.6% MUPB was tested to determine whether higher doses would improve antimicrobial properties.

A pneumatic press (PM-2000; Techno Máquinas Ltda, Vinhedo, Brazil) was used for packing the denture base resin, initially at 500 kgf and finally at 1250 kgf, maintained for 60 minutes. The resin was polymerized in a water bath, according to the manufacturer's instructions (73°C for 90 minutes, followed by 30 minutes at 100°C). The flasks containing acrylic resin discs were then bench cooled overnight before deflasking. The excess resin was trimmed with a bur (Maxi-Cut; Malleifer SA, Ballaigues, Switzerland). Each disc was then finished using 200-, 400-, 600-, and 1,200-grit wet/dry abrasive paper (Norton; Saint-Gobain Abrasivos Ltd, Guarulhos, Brazil) in a polishing machine (DPU-10; Panambra Ind. e Técn. S.A., São Paulo, Brazil) at 250 rpm for 60 seconds. Dimensions were confirmed with a digital caliper (Model CD-6'' CSX-B; Mitutoyo Sul Americana Ltda., Suzano, Brazil). The discs were immersed for 5 days in distilled water at 37°C and sterilized by ethylene oxide. Testing protocols were conducted at least 15 days after sterilization.

Table 1 Criteria for determining microbial growth under the specimens

Score	Status
3	Dense bacterial growth or attachment on the whole surface
2.5	Dense bacterial growth or attachment covering more than 2/3 of the surface
2	Dense bacterial growth or attachment covering 2/3-l/2 of the surface, or moderate bacterial growth or attachment on the whole surface
1.5	Dense bacterial growth or attachment covering l/2–1/3 of the surface
1	Dense bacterial growth or attachment covering 1/3–1/5 of the surface, or slight bacterial growth or attachment on the whole surface
0.5	Bacterial growth or attachment covering less than l/5 of the surface
0	No bacterial growth or attachment

For each concentration of MUPB, the bottom surface of three discs was placed onto an SDA plate inoculated with 100 μL of 1–5 × 10^6 CFU/mL of each C*andida* species or BHI agar plate for bacteria. Plates were incubated aerobically at 37°C for 24 hours, and the elution of antibacterial components was estimated from the production of inhibition zones around the discs. Discs were then removed, and microbial growth in contact with the acrylic resin was determined by means of a visual scoring system (Table 1).[34] One disc per plate was placed for each control and experimental material, and the test was performed in triplicate.

Measurement of microbial adherence

A series of cube-shaped specimens (10 mm × 10 mm × 3 mm) was obtained according to the aforementioned procedures. Ten specimens for each concentration of MUPB were placed in tubes with 10 mL of TSB inoculated with each microorganism. After aerobic incubation at 37°C for 24 hours, specimens were taken from these suspensions and washed with saline five times to dislodge loosely bound plaque. Afterwards, each specimen was immersed in 10 mL of 1 mol/L NaCl and vortexed vigorously for 1 minute and allowed to stand for 9 minutes, followed by a short vortex for 1 minute to collect the adhered cells. Dilutions (10^{-1} to 10^{-3}) were transferred to plates containing an SDA or BHI agar as appropriate (10 μL each dilution). Plates were incubated at 37°C for 48 hours, and colonies were

counted for quantification of colony-forming units per milliliter (CFU/mL).

Statistical analysis

Comparisons by means of parametric tests were performed only for normally distributed variables that presented homogeneous variances. This way, results for MIC and MFC/MBC were analyzed by the Mann-Whitney test and those for the cytotoxicity assay by two-way ANOVA, followed by Student–Newman–Keuls test. The inhibitory effect on bacterial growth assay generated an ordinal variable; therefore, it was analyzed by Kruskal–Wallis test. As the counts of adhered cells were normally distributed after transformation (log (CFU+1)/mL), data for adherence assays were analyzed by means of one-way ANOVA followed by Tukey HSD test. Analysis was performed at a 0.05 level of significance by means of a software package (PASW 17.0.0; SPSS Inc., Chicago, IL).

Results

MIC and MFC/MBC measurements

Table 2 summarizes results for MIC and MFC/MBC of uncured MUPB and CPC. MUPB presented lower MIC than CPC for all species, although their differences were significant only for *C. dubliniensis* and *S. mutans*. Regarding MFC and MBC, a slighter difference was found between the tested compounds for almost all species. A significant difference was found only for *C. albicans*, with MUPB presenting higher values than CPC.

Cytotoxicity test

Table 3 and Figure 2 show results obtained by means of the MTT assay. Differences between monomers ($p < 0.001$) and among concentrations ($p < 0.001$) were highly significant, as was their interaction ($p < 0.001$). MUPB and MMA presented similar cytotoxicity only at the lowest concentration tested (0.01 g/L); moreover, their cytotoxic effect can be considered negligible for this concentration. MMA caused significant reduction in viable cells only at the highest concentration tested (1 g/L).

Table 2 MIC and MFC/MBC of MUPB and CPC [mean (standard deviation)] in g/L, and comparison between compounds (Mann-Whitney test)

	MIC			MFC/MBC		
	MUPB	CPC	*p*	MUPB	CPC	*p*
C. albicans	0.63 (0.00)	0.84 (0.71)	0.480	5.83 (3.82)	0.84 (0.71)	0.046*
C. dubliniensis	0.83 (0.36)	5.83 (3.82)	0.046*	6.67 (2.89)	5.83 (3.82)	0.637
C. glabrata	1.04 (0.36)	5.42 (4.39)	0.105	5.21 (4.69)	5.42 (4.39)	0.822
S. mutans	1.04 (0.36)	3.33 (1.44)	0.043*	4.17 (1.44)	5.83 (3.82)	0.637
S. aureus	1.75 (0.66)	3.57 (5.58)	0.513	5.83 (3.82)	3.59 (5.55)	0.376
L. casei	0.63 (0.00)	6.67 (5.77)	0.480	4.58 (4.73)	6.68 (5.75)	0.817

*Significant difference ($p < 0.05$).

Table 3 Absorbance at 570 nm for the MTT assay [mean (standard deviation)] in percent

	Concentration (g/L)				
	0.01	0.05	0.25	0.5	1
MMA	95.3 (4.5) ^{Aa}	93.8 (5.1) ^{Aa}	96.9 (1.8) ^{Aa}	89.2 (6.2) ^{Aa}	65.7 (9.1) ^{Ab}
MUPB	90.0 (6.0) ^{Aa}	52.3 (6.3) ^{Bb}	6.5 (0.8) ^{Bc}	7.2 (0.7) ^{Bc}	8.3 (0.5) ^{Bc}

Vertically, means with same uppercase letters are not significantly different. Horizontally, means with same lowercase letters are not significantly different (Student–Newman–Keuls test, $p < 0.05$).

Nevertheless, a change in the concentration of MUPB from 0.01 to 0.05 g/L resulted in a 42% lower absorbance. When compared with 0.01 g/L, a concentration of 0.25 g/L caused further reduction (87%), but higher concentrations caused no significant change.

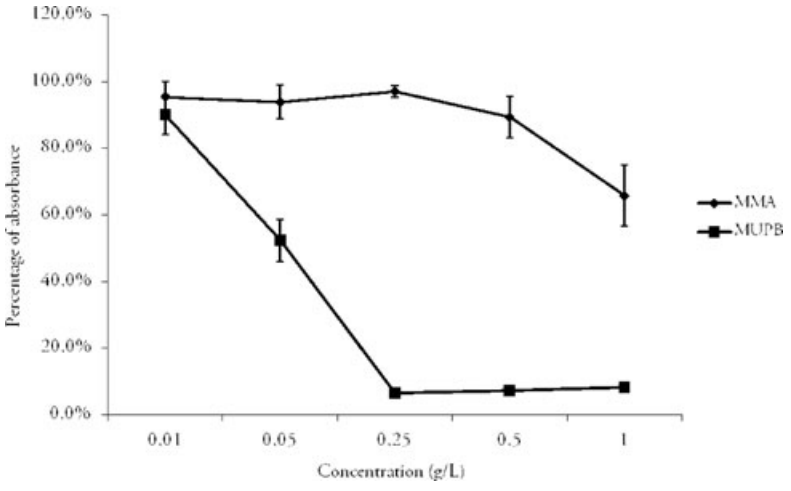

Figure 2 Mean absorbance for the MTT test in percent. Error bars represent standard deviations.

Table 4 Scores for the inhibitory effect of acrylic resin discs and comparison among groups, according to the studied species

		Groups			
Species	Disc	Control (0%)	0.30%	0.60%	Kruskal–Wallis test
C. albicans	1	2	1	1	KW = 1.654; $p = 0.437^{(ns)}$
	2	1	2.5	1.5	
	3	1.5	2.5	1	
C. dubliniensis	1	0	0	0.5	KW = 1.143; $p = 0.565^{(ns)}$
	2	0.5	0.5	0.5	
	3	0.5	0.5	0.5	
C. glabrata	1	1	1	1.5	KW = 0.164; $p = 0.921^{(ns)}$
	2	0	0.5	0.5	
	3	2.5	1.5	0.5	
S. mutans	1	0	0.5	0	KW = 0.800; $p = 0.670^{(ns)}$
	2	0	0	0	
	3	0.5	0.5	0.5	
S. aureus	1	0	0.5	0	KW = 0.800; $p = 0.670^{(ns)}$
	2	0	0	0.5	
	3	0.5	0.5	0	
L. casei	1	0	0	0	KW = 0.000; $p = 1.000^{(ns)}$
	2	0	0	0	
	3	0	0	0	

$^{(ns)}$No significant difference ($p > 0.05$).

Table 5 Quantification of microorganisms adhered on acrylic resin specimens [mean (standard deviation)] in log(CFU+1/mL), and comparison among groups (one-way ANOVA)

	C. albicans	*C. dubliniensis*	*C. glabrata*	*S. mutans*	*S. aureus*	*L. casei*
0%	5.6 (0.7)	6.1(0.6)	5.0 (0.4)	5.9 (0.6)	6.7(0.5)	0.9 (2.4)
0.3%	5.9 (0.4)	5.7 (0.7)	4.9 (0.3)	5.7 (0.5)	7.1 (1.1)	0.7 (2.1)
0.6%	4.9 (0.7)	6.1(0.5)	5.0 (0.5)	5.7 (0.3)	6.8 (0.4)	1.3 (2.4)
p	0.003*	$0.372^{(ns)}$	$0.880^{(ns)}$	$0.577^{(ns)}$	$0.612^{(ns)}$	-

*Significant difference ($p < 0.05$).
$^{(ns)}$No significant difference ($p > 0.05$).

Inhibitory effect on microbial growth

No inhibition zone was produced by either control or experimental discs, indicating that no antimicrobial component was eluted. Table 4 presents scores for fungal and bacterial growth under each disc. No difference was found among the three experimental groups, regardless of the species tested.

Measurement of microbial adherence

Mean values and standard deviations for microbial counts are shown in Table 5. The incorporation of 0.6% MUPB significantly inhibited the adherence of *C. albicans*, whereas no statistical difference was found between the 0.3% MUPB and control groups. Irrespective of concentration, MUPB had no influence on the other species.

No adhered *L. casei* was detectable on most specimens. The control group presented a count higher than zero (6.8 log (CFU + 1/mL)), as well as the 0.3% group (5.9 log(CFU + 1/mL)). The 0.6% group presented only two positive results (4.5 and 5.8 log(CFU + 1/mL)). For this species, comparison among groups was done by means of the Kruskal–Wallis test, and no significant difference was found (KW = 0.323; $p = 0.851$).

Discussion

This preliminary in vitro assessment found that the MUPB monomer presents antimicrobial activity in its uncured form. Results for a structurally similar monomer, MDPB, were similar, with strong antimicrobial activity against *Streptococcus* spp., *Lactobacillus* spp., and other cariogenic species.[18,24,27,35-42] Although only *C. dubliniensis* and *S. mutans* presented significant differences, MUPB showed a trend towards lower MIC than those for CPC, regardless of the species. Since CPC is a quaternary ammonium compound with a high spectrum frequently used in dentifrices and mouthrinses, results have shown that MUPB is an efficacious antimicrobial agent; however, MUPB presents slightly lower MIC values when compared with MDPB, which can be explained by a longer extension of the aliphatic chain of the latter, namely 11 versus 12 carbon atoms. The longer the aliphatic chain linked to the pyridinium group, the higher the hydrophobic interaction between the compound and cell wall's lipidic components; thus, the greater the antimicrobial activity.[24,43]

MFC and MBC values varied according to species, with MUPB presenting a significantly lower effect than CPC only for *C. albicans*. This reinforces the potential of MUPB as an antimicrobial agent, since the overall results were similar to those of the control compound; however, MDPB presented higher MBC than CPC against cariogenic species.[32,39] This difference was explained by the higher hydrophilicity of CPC and the absence of the methacryloyl group, with the latter reducing interaction with cell walls. The differences between MUPB and CPC were similar to those between MDPB and CPC with regard to MFC and MBC.[32] These results indicate similar mechanisms involved in the antimicrobial activity of the two monomers. Nevertheless, the MBC values of MDPB were slightly lower than those found for MUPB, which suggests the same bactericidal activity with lower concentrations.[32,37]

MUPB was cytotoxic at relatively low concentrations when compared with the control monomer, MMA. An IC50 close to 0.05 g/L was observed, which is nearly similar to the IC50 of MDPB on human pulp cells (0.04 g/L),[32] and 20 times lower than the value found for MMA. The cytotoxicity of MUPB can be compared with that of other monomers, such as HEMA and UDMA,[44] which are widely used in dental composites and light-polymerized denture base resins. The present results should not necessarily be viewed as unfavorable, since several monomers used in dental adhesives are more cytotoxic than this, that is, Bis-GMA, TEGDMA, and UDMA (IC50: 0.0048, 0.0357, and 0.0087 g/L, respectively).[45] Moreover, the aliquots of MUPB incorporated into the tested resin were relatively small. Because of that, amounts of MUPB may be present in extracts from polymerized resins at concentrations lower than those of MMA or dental composite monomers; however, more relevant conclusions regarding the safety of MUPB cannot be drawn until further clinically relevant assays are carried out.[32,46]

The absence of inhibition halos was a common finding for all groups or species studied and showed that no eluted compound inhibited microbial growth, that is, uncured MUPB. This indicates that MUPB was immobilized within the polymeric chains by means of copolymerization. Similar results were found for MDPB against *A. viscosus*, *L. casei*, and *Streptococcus*

spp.,[18,34,41,47] and for TBAEMA against *Escherichia coli*.[48] As MUPB and MDPB are very similar, the same copolymerization ability can be expected for both. Such antimicrobial monomers can copolymerize their methacrylic radical with acrylic resins,[18,49,50] and the opposite molecular portion, the quaternary ammonium radical, would act by direct contact with microbial cells.[18] As long as the compounds become covalently linked to the denture base resin, the effect of incorporated MUPB is not expected to diminish over time.

The method used to assess microbial growth under specimens was based on visual scoring, and was unable to detect differences among control and experimental groups. A similar method was used to test the incorporation of MDPB into dental composites and showed reduction in the growth of *S. mutans*, *L. casei*, and *A. viscosus*.[18,24,34,47] Although the method used is not very sophisticated, it is able to detect important differences among groups; however, it is debatable whether the groups were not different, or the discrepancies were too small and would demand other methods of assessment to be detected.

C. albicans presented significantly lower adherence to specimens associated with the incorporation of 0.6% of MUPB. This agrees with the findings of another study that found lower adherence of *C. albicans* on acrylic resin containing a polymerized quaternary ammonium compound.[25,28] However, the effect was only found when much higher concentrations were incorporated, suggesting that MUPB presents strong antifungal properties against this species. Reduction of *S. aureus* was also found after the ammonium polymer had been incorporated. In this study, no reduction of the latter was found, and the possible reason for this is the different protocol used. Different concentrations of the agent, as well as the immersion in artificial saliva,[51-53] might have been responsible for the different results.

The difference among groups in terms of the adherence of *C. albicans* is an interesting finding by itself. Distinct species of the genus *Candida* differ according to their surface hydrophobicity and ability to form hyphae,[55] which are features associated with their adherence and virulence.[7] *C. albicans* is less hydrophobic than *C. glabrata*[56] and may present greater variation in response to environmental factors when compared with *C. dubliniensis*.[57] Other studies about the adherence of *Candida* spp. on acrylic resin have also shown wide variation among species.[7,56]

Incorporated MUPB was unable to affect the adherence of bacterial cells, and the explanation for this may be associated with cell morphology. Gram-positive bacteria (i.e., *S. mutans*, *S. aureus*, and *L. casei*) show less susceptibility to antimicrobial agents such as silver-based agents[23,58] and MDPB.[37] Those bacteria have more peptidoglycans in their cell walls than gram-negative bacteria, which may prevent damage to their plasma membrane caused by quaternary ammonium radicals. Nevertheless, it is possible that incorporated MUPB may present an important antimicrobial effect at higher concentrations or even be efficacious against gram-negative species at the tested proportions. Another copolymerizable quaternary ammonium compound, TBAEMA, was able to reduce adhered *S. aureus* after incorporation, but at a minimum concentration of 1.5% (w/w).[26]

Future studies would be necessary to investigate possible undesirable effects of MUPB on other properties of denture base acrylic resins, such as those found after incorporating other antimicrobial agents.[16,17,59-61] We have already assessed mechanical properties and color stability of a resin containing 0.6% MUPB, and no important deleterious change was found.[62] Despite the results of this preliminary study with planktonic cells, future research using biofilm models associated with quantitative and qualitative approaches should be encouraged.[63] Such research may provide more information about the interaction between microbial communities and the incorporated MUPB in specific applications. Based on this study, a possible speculation is that resins containing 0.6% MUPB might prevent fungal infections by means of a selective effect on *C. albicans*, though bacterial cells would still adhere as normal; however, future studies should also gather results with regard to other properties, such as the elution of components, compatibility with artificial teeth and in vivo biocompatibility, as well as other applications of the MUPB monomer, that is, as a component of denture glaze resins.

Conclusions

Uncured MUPB was comparable with CPC in terms of antimicrobial activity and nearly 20 times more cytotoxic than MMA. When incorporated into a denture base acrylic resin, MUPB was able to reduce the adherence of *C. albicans*. This action did not depend on the release of eluates into the surrounding medium.

Acknowledgements

The authors thank Dr. Izabel Ito (in memoriam) and Dr. Paula Sanitá for kindly providing the microbial strains used.

References

1. Carlsson GE: Clinical morbidity and sequelae of treatment with complete dentures. J Prosthet Dent 1998;79:17-23
2. Marchini L, Tamashiro E, Nascimento DF, et al: Self-reported denture hygiene of a sample of edentulous attendees at a university dental clinic and the relationship to the condition of the oral tissues. Gerodontology 2004;21:226-228
3. Ramage G, Tomsett K, Wickes BL, et al: Denture stomatitis: a role for *Candida* biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;98:53-59
4. Ohman SC, Osterberg T, Dahlen G, et al: The prevalence of *Staphylococcus aureus*, *Enterobacteriaceae* species, and *Candida* species and their relation to oral mucosal lesions in a group of 79-year-olds in Göteborg. Acta Odontol Scand 1995;53:49-54
5. Paranhos HF, Silva-Lovato CH, Souza RF, et al: Effects of mechanical and chemical methods on denture biofilm accumulation. J Oral Rehabil 2007;34:606-612
6. Abu-Elteen KH, Abu-Alteen RM: The prevalence of *Candida albicans* populations in the mouths of complete denture wearers. New Microbiol 1998;21:41-48
7. He XY, Meurman JH, Kari K, et al: In vitro adhesion of *Candida* species to denture base materials. Mycoses 2006;49:80-84
8. Compagnoni MA, Souza RF, Marra J, et al: Relationship between *Candida* and the nocturnal denture wear: quantitative study. J Oral Rehabil 2007;34:600-605

9. Nikawa H, Nishimura H, Makihira S, et al: Effect of serum concentration on *Candida* biofilm formation on acrylic surfaces. Mycoses 2000;43:139-143
10. Chow CKW, Matear DW, Lawrence HP: Efficacy of antifungal agents in tissue conditioners in treating candidiasis. Gerodontology 1999;16:110-119
11. El-Charkawi H, El-Said EA, Safouh HM, et al: Effect of addition antimicrobial agents to denture reliners. Egypt Dent J 1994;40:785-790
12. Urban VM, Souza RF, Arrais AG, et al: Effect of the association of nystatin with a tissue conditioner on its ultimate tensile strength. J Prosthodont 2006;15:295-299
13. Quinn DM: The effectiveness, in vitro, of miconazole and ketoconazole combined with tissue conditioners in inhibiting the growth of *Candida albicans*. J Oral Rehabil 1985;12:177-182
14. Wilson SJ, Wilson HJ: The release of chlorhexidine from modified dental acrylic resin. J Oral Rehabil 1993;20:311-319
15. Lefebvre CA, Wataha JC, Cibirka RM, et al: Effects of triclosan on the cytotoxicity and fungal growth on a soft denture liner. J Prosthet Dent 2001;85:352-356
16. Shibata T, Hamada N, Kimoto K, et al: Antifungal effect of acrylic resin containing apatite-coated TiO_2 photocatalyst. Dent Mat J 2007;26:437-444
17. Casemiro LA, Martins CHG, Pires-de-Souza FC, et al: Antimicrobial and mechanical properties of acrylic resins with incorporated silver–zinc zeolite—part I. Gerodontology 2008;25:187-194
18. Imazato S, Torii M, Tsuchitani Y, et al: Incorporation of bacterial inhibitor into resin composite. J Dent Res 1994;73:1437-1443
19. Yildirim MS, Hasanreisoglu U, Hasirci N, et al: Adherence of *Candida albicans* to glow-discharge modified acrylic denture base polymers. J Oral Rehabil 2005;32:518-525
20. Sesma N, Laganá DC, Morimoto S, et al: Effect of denture surface glazing on denture plaque formation. Braz Dent J 2005;16:129-134
21. Park SE, Periathamby AR, Loza JC: Effect of surface-charged poly(methyl methacrylate) on the adhesion of *Candida albicans*. J Prosthodont 2003;12:249-254
22. Cunha TR, Regis RR, Bonatti MR, et al: Influence of incorporation of fluoroalkyl methacrylates on roughness and flexural strength of a denture base acrylic resin. J Appl Oral Sci 2009;17:103-107
23. Monteiro DR, Gorup LF, Takamiya AS, et al: The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. Int J Antimicrob Agents 2009;34:103-110
24. Imazato S, Russell RR, McCabe JF: Antibacterial activity of MDPB polymer incorporated in dental resin. J Dent 1995;23:177-181
25. Pesci-Bardon C, Fosse T, Madinier I, et al: In vitro new dialysis protocol to assay the antiseptic properties of a quaternary ammonium compound polymerized with denture acrylic resin. Lett Appl Microbiol 2004;39:226-231
26. Seyfriedsberger G, Rametsteiner K, Kern W: Polyethylene compounds with antimicrobial surface properties. Eur Polymer J 2006;42:3383-3389
27. Imazato S, Kinomoto Y, Tarumi H, et al: Antibacterial activity and bonding characteristics of an adhesive resin containing antibacterial monomer MDPB. Dent Mater 2003;19:313-319
28. Pesci-Bardon C, Fosse T, Serre D, et al: In vitro antiseptic properties of an ammonium compound combined with denture base acrylic resin. Gerodontology 2006;23:111-116
29. Imazato S, Torii M, Tsuchitani Y, et al, inventors; Kuraray Co., Ltd., assignee. Antimicrobial polymerizable composition, the polymer and article obtained from the same. US Patent 5,408,022. 1995 Apr 18.
30. Carlsson J, Soderholm G, Almfeldt I: Prevalence of *Streptococcus sanguis* and *Streptococcus mutans* in the mouth of persons wearing full-dentures. Arch Oral Biol 1969;14:243-249
31. Marsh PD, Percival RS, Challacombe SJ: The influence of denture-wearing and age on the oral microflora. J Dent Res 1992;71:1374-1381
32. Imazato S, Ebi N, Tarumi H, et al: Bactericidal activity and cytotoxicity of antibacterial monomer MDPB. Biomaterials 1999;20:899-903
33. Campanha NH, Pavarina AC, Giampaolo ET, et al: Cytotoxicity of hard chairside reline resins: effect of microwave irradiation and water bath postpolymerization treatments. Int J Prosthodont 2006;19:195-201
34. Imazato S, Ehara A, Torii M, et al: Antibacterial activity of dentine primer containing MDPB after curing. J Dent 1998;26:267-271
35. Ebi N, Imazato S, Noiri Y, et al: Inhibitory effects of resin composite containing bactericide-immobilized filler on plaque accumulation. Dent Mater 2001;17:485-491
36. Imazato S, Kinomoto Y, Tarumi H, et al: Incorporation of antibacterial monomer MDPB into dentin primer. J Dent Res 1997;76:768-772
37. Imazato S, Torii Y, Takatsuka T, et al: Bactericidal effect of dentin primer containing antibacterial monomer methacryloyloxydodecylpyridinium bromide (MDPB) against bacteria in human carious dentin. J Oral Rehabil 2001;28:314-319
38. Imazato S, Walls AW, Kuramoto A, et al: Penetration of an antibacterial dentine-bonding system into demineralized human root dentine in vitro. Eur J Oral Sci 2002;110:168-174
39. Imazato S, Kuramoto A, Takahashi Y, et al: In vitro antibacterial effects of the dentin primer of Clearfil Protect Bond. Dent Mater 2006;22:527-532
40. Imazato S, Ohmori K, Russell RR, et al: Determination of bactericidal activity of antibacterial monomer MDPB by a viability staining method. Dent Mater J 2008;27:145-148
41. Korkmaz Y, Ozalp M, Attar N: Comparison of the antibacterial activity of different self-etching primers and adhesives. J Contemp Dent Pract 2008;9:57-64
42. Yoshikawa K, Clark DT, Brailsford SR, et al: The effect of antibacterial monomer MDPB on the growth of organisms associated with root caries. Dent Mater J 2007;26:388-392
43. Xiao YH, Chen JH, Fang M, et al: Antibacterial effects of three experimental quaternary ammonium salt (QAS) monomers on bacteria associated with oral infections. J Oral Sci 2008;50:323-327
44. Geurtsen W, Lehmann F, Spahl W, et al: Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. J Biomed Mater Res 1998;41:474-480
45. Ratanasathien S, Wataha JC, Hanks CT, et al: Cytotoxic interactive effects of dentin bonding components on mouse fibroblasts. J Dent Res 1995;74:1602-1606
46. Imazato S, Tarumi H, Ebi N, et al: Cytotoxic effects of composite restorations employing self-etching primers or experimental antibacterial primers. J Dent 2000;28:61-67
47. Imazato S, Imai T, Russell RR, et al: Antibacterial activity of cured dental resin incorporating the antibacterial monomer MDPB and an adhesion-promoting monomer. J Biomed Mater Res 1998;39:511-515

48. Lenoir S, Pagnoulle C, Galleni M, et al: Polyolefin matrixes with permanent antibacterial activity: preparation, antibacterial activity, and action mode of the active species. Biomacromolecules 2006;7:2291-2296
49. Li F, Chen J, Chai Z, et al: Effects of a dental adhesive incorporating antibacterial monomer on the growth, adherence and membrane integrity of *Streptococcus mutans*. J Dent 2009;37:289-296
50. Thomassin JM, Lenoir S, Riga J, et al: Grafting of poly[2-(tert-butylamino)ethyl methacrylate] onto polypropylene by reactive blending and antibacterial activity of the copolymer. Biomacromolecules 2007;8:1171-1177
51. Moura JS, da Silva WJ, Pereira T, et al: Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. J Prosthet Dent 2006;96:205-211
52. Pereira-Cenci T, Del Bel Cury AA, Crielaard W, et al: Development of *Candida*-associated denture stomatitis: new insights. J Appl Oral Sci 2008;16:86-94
53. Pereira-Cenci T, Deng DM, Kraneveld EA, et al: The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces. Arch Oral Biol 2008;53:755-764
54. Ramage G, Vandewalle K, Wickes BL, et al: Characteristics of biofilm formation by *Candida albicans*. Rev Iberoam Micol 2001;18:163-170
55. Samaranayake YH, Wu PC, Samaranayake LP, et al: Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelial and denture acrylic surfaces. APMIS 1995;103:707-713
56. Luo G, Samaranayake LP: *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. APMIS 2002;110:601-610
57. De Souza RD, Mores AU, Cavalca L, et al: Cell surface hydrophobicity of *Candida albicans* isolated from elder patients undergoing denture-related candidosis. Gerodontology 2009;26:157-161
58. Kawahara K, Tsuruda K, Morishita M, et al: Antibacterial effect of silver-zeolite on oral bacteria under anaerobic condition. Dent Mater 2000;16:452-555
59. Akiba N, Hayakawa I, Keh ES, et al: Antifungal effects of a tissue conditioner coating agent with TiO_2 photocatalyst. J Med Dent Sci 2005;52:223-227
60. Dhir G, Berzins DW, Dhuru VB, et al: Physical properties of denture base resins potentially resistant to *Candida* adhesion. J Prosthodont 2007;16:465-472
61. Park SE, Chao M, Raj PA: Mechanical properties of surface-charged poly(methyl methacrylate) as denture resins. Int J Dent 2009;2009:841431
62. Regis RR, Zanini AP, Della Vecchia MP, et al: Physical properties of an acrylic resin after incorporation of an antimicrobial monomer. J Prosthodont 2011;20:372-379.
63. Chandra J, Mukherjee PK, Ghannoum MA: In vitro growth and analysis of *Candida* biofilms. Nat Protoc 2008;3:1909-1924