Research article

Synthesis, characterization, and evaluation of quaternary ammonium-based polymerizable antimicrobial monomers for prosthodontic applications

Sowmya Rao a, Nandish B.T. a,*, Namitha K. Preman b, Renjith P. Johnson b, Kishore Ginjupalli c, Preethishree P. d, Ashwini Prabh u e, Ranajit Das f, Jayaprakash K a, f, Vidya Pai d

a Department of Dental Materials, Yenepoya Dental College, Yenepoya (Deemed to Be University), Mangalore, 575018, Karnataka, India
b Polymer Nanobiomaterial Research Laboratory, Smart Materials and Device Division, Yenepoya Research Centre, Yenepoya (Deemed to Be University), Mangalore, 575018, Karnataka, India
c Department of Dental Materials, Manipal College of Dental Sciences, Manipal Academy of Higher Education, Manipal, 576104, Karnataka, India
d Department of Microbiology, Yenepoya Medical College, Yenepoya (Deemed to Be University) Mangalore, 575018, Karnataka, India
e Yenepoya Research Centre, Yenepoya (Deemed to Be University), Mangalore, 575018, Karnataka, India
f Biomaterials and Research Centre, Yenepoya Dental College, Yenepoya (Deemed to be University), Mangalore, 575018, Karnataka, India

ARTICLE INFO

Keywords:
- Polymethyl methacrylate (PMMA) resin
- DHMAI
- Antimicrobial monomer
- Quaternary ammonium compounds
- Denture stomatitis (DS)
- Candida albicans
- Biomaterials
- Cytotoxicity
- Hardness
- Water sorption
- DDMAI
- Copolymerization

ABSTRACT

The present study aims to synthesize and characterize two quaternary ammonium (QAM) based monomers such as - dimethyl-hexadecyl-methacryloxyethyl-ammonium iodide (DHMAI) and 2-dimethyl-2-dodecyl-1-methacryloxyethyl ammonium iodide (DDMAI) and assess their cytotoxicity and antimicrobial properties. The study also aims to incorporate the optimized concentration of these monomers as copolymerizing monomers into conventional Polymethyl methacrylate (PMMA) denture base resin and evaluate their suitability for prosthodontic applications. DHMAI and DDMAI monomers were synthesized through a Menachutkin reaction and their chemical structure was characterized using FT-IR and 1H-NMR spectroscopy. Cytotoxicity was determined using Methyl Thiazolyl Tetrazolium (MTT) assay whereas antimicrobial activity was assessed using the agar-disc diffusion method. Subsequently, optimized concentrations of DHMAI or DDMAI, based on the cytotoxicity results, were added to conventional PMMA resin. Antimicrobial activity, cytotoxicity, surface hardness, and water sorption of PMMA denture base rein incorporated with DHMAI or DDMAI were evaluated. FT-IR and 1H-NMR results confirmed the structure of monomers and copolymerization of DHMAI and DDMAI with PMMA resin. DHMAI and DDMAI monomers were found to be cytocompatible with mouse fibroblast cells up to a concentration of 5 μg/mL and 20 μg/mL respectively. In addition, incorporation of DHMAI or DDMAI at 5 μg/mL and 20 μg/mL respectively into PMMA denture base material did not affect their cytocompatibility. PMMA denture base resin incorporated with DHMAI or DDMAI significantly reduced the adhesion of microbes. Further, an increase in the surface hardness and a reduction in the water sorption was observed. Hence DHMAI and DDMAI can be considered as potential candidates for imparting antimicrobial activity to polymeric denture base materials.

1. Introduction

Ever since PMMA was introduced to dentistry during the late 1940s, it has been widely used for the fabrication of various prosthesis such as complete dentures, partial dentures, and maxillofacial prosthesis. In addition, it also used for denture repair, relining, rebasing and for the fabrication of special trays, orthodontic retainers, occlusal splints, and obturators, etc [1]. Despite the introduction of various alternative materials, PMMA remains the choice of material for complete and partial denture fabrication due to its favorable properties such as biocompatibility, insolubility in oral fluids, lifelike appearance, and easy to process, repair, and polish. Not withstanding the popularity of this material, denture prosthesis made from PMMA exhibits inferior mechanical characteristics such as low flexural and impact strength, low surface hardness,
high water sorption and allergic reactions due to the presence of unpolymerized monomer. In addition, the surface of dental prostheses made from PMMA material inherently do not possess any antimicrobial activity. Hence the surfaces of the denture made from this material harbor various microorganisms, mainly the fungi such as *Candida albicans* ([C. albicans](#)) ([1], [2]). These microorganisms form a biofilm, subsequently leading to the development of an oral infection called denture stomatitis (DS). DS is characterized by inflammation and erythema of the oral mucosa underneath the denture-bearing areas ([3], [4]). It is common in elderly population and immunocompromised patients, causing health complications such as infectious endocarditis, malodour, pulmonary candidiasis, and aspiration pneumonia ([5], [6]). Epidemiological studies reported the prevalence of DS to be 78% ([4]). To overcome this problem, attempts have been made to impart antimicrobial activity to PMMA denture base materials by incorporating antimicrobial additives. Antimicrobial additives such as chlorhexidine, QAM-compounds, silver, and titanium dioxide nanoparticles have been investigated. However, the antimicrobial action was not long-lasting due to the leaching or cross-reactivity with the oral tissues ([1], [4]). Alternatively, in recent years, research focusing on chemically copolymerizing antimicrobial agents to PMMA resin networks is gaining attention. Such antimicrobial agents are uniformly distributed throughout the bulk of the material and provide long-lasting antimicrobial activity without leaching out from the host material ([4,7]). Copolymerizing antimicrobial monomers such as MDPP (12-Methacyryloxy -Dodecyl -Pyridinium -Bromide), MUPB (Methacyryloxy Undecyl Pyridinium Bromide), QAM (Quaternary Ammonium Monomer), DMADD (Dimethyl Amino Dodecyl Methacrylate), TBAEMA (2-Tert-Butyl Amino Ethyl Methacrylate), and AAM (Acryl Amide Monomer) have been investigated and were reported to be effective in inhibiting the microbial growth ([7], [8], [9], [10]).

Most polymerizable monomers are based on QAM salt that can covalently bond with the methacrylate group of the matrix-forming monomers ([7]). Such polymerizable antimicrobial agents, in general, consist of a methacrylate group to immobilize the bacterial agents, and QAM salts for antimicrobial action. The antimicrobial activity of these monomers seems to be dependent on their chain length. In general, longer chain length imparts superior antimicrobial activity, especially for monomers with chain lengths varying between C5 to C18 ([10], [11], [12]).

Novel QAM salt-based copolymerizing antimicrobial monomers, DHMAI, and DDMAI have recently been investigated for use with dental restorative resins ([11], [13]). These monomers were synthesized through a Menschutkin reaction in which different alkyl iodides such as iodohexadecane, and iodododecane were combined with polymerizable tertiary amine namely 2-dimethylamino ethyl methacrylate (DHEMA). The resultant polymerizable monomers such as DHMAI, and DDMAI with different alkyl chain lengths (C16, and C12 respectively) exhibited superior antimicrobial properties ([11]). Their antimicrobial activity is attributed to the contact killing through electrostatic interaction between the cationic/positively charged QAM-based antimicrobial monomer, and a negatively charged bacterial cell membrane causing cellular membrane damage following cell lysis, and death ([1], [6], [10]).

Although extensive research in the evaluation of various antimicrobial monomers was reported, their use in resin-based materials for the fabrication of complete denture prostheses has not been widely reported. In this regard, the use of DHMAI and DDMAI at different concentrations as copolymerizing liquid with PMMA based denture base resin increased their mechanical properties ([14]). However, it is not clear if such an addition also imparts antimicrobial activity to the denture bases. Hence the present study aims to synthesize these antimicrobial monomers, incorporate them as polymerizable monomers into heat-activated PMMA based denture base resins and evaluate its biocompatibility, antimicrobial properties, surface hardness, and water sorption.

2. Material and methods

2.1. Synthesis, and characterization of antimicrobial monomers, and modified PMMA resin

2.1.1. Synthesis of 2-Dimethyl-hexadecyl-methacryloxyethyl-ammonium iodide (DHMAI)

DHMAI monomer was synthesized through Menschutkin reaction in which the reaction between a tertiary amine, and alkyl halide results in the formation of a QAM salt. 0.06 mol of DMAEMA (9.43 g), 0.05 mol of iodohexadecane (14.81 g), and 0.05% of hydroquinone (0.025 g) were mixed in a 50 mL round bottom flask. Subsequently, the flask was placed in a silicone oil bath under a nitrogen atmosphere at 50 °C with constant stirring for 8 h (Scheme 1). At the end of the reaction time, the resultant white paste was washed 4 to 5 times with diethyl ether by centrifugation method. Subsequently, it was dried in a rotary evaporator at 40 °C for 2 h (Scheme 1).

![Scheme 1. Synthesis of DHMAI](image-url)
2.1.2. Synthesis of 1,2-dimethyl-2-dodecyl-1-methacryloxyethyl ammonium iodine (DDMAI)

DDMAI monomer was synthesized through Menschutkin reaction. 0.06 mol of DMAEMA (9.43 g), 0.05 mol of 1-Iodododecane (Dodecyl iodide) (14.81 g), and 0.05% of hydroquinone (25 mg) were taken in a 50 mL round bottom flask. Subsequently, the flask was placed in a silicone oil bath under a nitrogen atmosphere at 50 °C with constant stirring for 16 h (Scheme 2). At the end of the reaction, a white paste was formed which was washed 4 to 5 times with diethyl ether followed by drying in a rotary evaporator at 40 °C for 2 h [11].

2.1.3. Characterization of antimicrobial monomers, and modified PMMA resin

FT-IR and 1H-NMR Spectroscopy were used to characterize the chemical structure of the synthesized monomers. FT-IR spectra of the monomers were recorded on a Shimadzu IR Spirit (QATR-S) spectrometer in the range of 4000–400 cm⁻¹. Briefly, the procedure involved the addition of a small amount of synthesized monomer with potassium bromide powder. Both the powders were thoroughly mixed in an agate mortar using a pestle. Subsequently, the powder was pressed into a pellet form, and the FT-IR was recorded. FT-IR spectra of DHMAI, DDMAI, and modified PMMA resin incorporated with DHMAI or DDMAI were recorded. Pure PMMA powder was considered as control.

The 1H-NMR spectra of DHMAI, DDMAI, and modified PMMA resin incorporated with DHMAI or DDMAI were recorded with the help of NMR spectrometer (Bruker Avance III 400 MHz) using deuterated dimethyl sulfoxide (DMSO d₆) as a solvent.

Cytotoxicity (%)=\frac{(\text{Absorbance of the control} - \text{Absorbance of the test compound})}{\text{Absorbance of the control}} \times 100

2.2. Cytotoxicity of antimicrobial monomers (DHMAI and DDMAI)

Cytotoxicity of antimicrobial monomers (DHMAI and DDMAI) was analyzed using MTT assay (Mosmann, 1983). Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), and 1% antibiotic-antimycotic solution was used to culture L929 mouse fibroblast cells. Cells were incubated at 37 °C, and 5% CO₂ in a humidified environment for at least three consecutive passages before being utilized in the experiments. At a seeding density of 5,000 cells per well, cells were seeded onto 96 well microtiter plates. After adherence, they were treated with different concentrations of antimicrobial monomer (DHMAI or DDMAI), viz., 1, 2, 5, 10, 20, 40, 60, 80, and 100 μg/mL concentrations. Methyl methacrylate (MMA) was used as a positive control. MTT reagent (1 mg/mL) was added to the wells after 24 h and incubated at 37 °C for 4 h. Later, dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). The percentage viability of the cells after treatment with the test compounds in triplicates was calculated in comparison to the untreated cell control. Cytotoxicity was calculated using the following formula [15].

2.3. Antimicrobial activity of monomers

2.3.1. Disc diffusion method

The antimicrobial activity of unpolymerized monomers was evaluated using an agar-disc diffusion test against the standard strains of

\[
\text{Cytotoxicity (%)} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the test compound})}{\text{Absorbance of the control}} \times 100
\]
Candida albicans (ATCC 90028), Staphylococcus aureus (ATCC 25923), and Streptococcus mutans (ATCC 25175) [1]. C. albicans was cultured on Sabouraud’s Dextrose Agar (SDA) whereas S. aureus, and S. mutans were cultured on Nutrient Agar, and Sheep Blood Agar (SBA) respectively. A loopful inoculum of each microorganism was transferred to 2 ml of Mueller Hinton Broth to make up to 0.5 McFarland Standard. Lawn cultures with a sterile cotton swab were prepared on Mueller Hinton Agar (MHA) for S. aureus, MHA with Methylene blue for C. albicans, and SBA for S. mutans. Subsequently, 6 mm diameter sterile Whatman filter paper discs (No.1) were placed on the media, loaded with 10 μg of different concentrations of DHMAI (1, 2, or 5 μg/mL) or DDMAI (5, 10, or 20 μg/mL), and incubated at 37 °C for 24 h for bacteria, and 48 h for fungal growth respectively. The zone of inhibition was measured using a caliper and was reported in millimeters (n = 3) [4].

2.4.3. Field emission scanning electron microscopy (FESEM)

The adherence of microorganisms onto the surfaces of control and test specimens was analyzed using scanning electron microscopy. PMMA samples retrieved from the microbial adherence test were further washed with phosphate buffer solution (PBS) (pH = 7) followed by immersion in 2.4% Glutaraldehyde solution at 4 °C for 2 h. Subsequently, the specimens were retrieved, washed with PBS, and dehydrated using a series of ethanol washes (70, 95, and 100%) for 10 min. These specimens were sputter-coated with gold, mounted onto aluminum stubs, and a scanning electron microscope (FESEM, Carl Zeiss) was used to examine the samples [16].

Similarly, FESEM was also used to observe the surface morphology of control and PMMA denture base resin incorporated with DHMAI or DDMAI.

2.5. Surface hardness of modified PMMA resin

Surface hardness of disk-shaped (14 mm diameter and 4 mm thick) control and test specimens was measured using a microhardness tester (Vickers hardness tester, Tecsol, India). 25 g of load was applied on the surface of each specimen for 30 s. After the dwell time, load was removed, and the area of the indentation was measured. Load divided by the area of indentation was calculated and presented as Vickers Hardness Number (VHN). Hardness on each specimen was measured at 8 different places, and an average hardness was reported [17].

2.6. Water sorption of modified PMMA resin

Disk-shaped (5 mm diameter and 0.5 mm thick) control and test specimens were prepared and immersed in distilled water for 24 h before the test. Later, the samples were desiccated under vacuum at 37 °C for 23 h and again stored in another desiccator containing freshly dried silica gel at 23 °C for 1 h until a constant weight was observed (m1). After the measurement of weight, the samples were immersed in distilled water at 37 °C and stored for 7 days. After 7 days, samples were retrieved from the water, surface water blotted with tissue paper, and the weight (m 2) and volume (V) of the samples were measured. Water uptake (μg/mm³) was determined using the following equation [1].

\[ W_{wp} = \frac{m_2 - m_1}{V} \]

2.7. Statistical analysis

The cytotoxicity of DHMAI and DDMAI monomers, cytotoxicity, surface hardness, and water sorption data of PMMA with and without antimicrobial monomers, were analyzed using One-Way ANOVA followed by Tukey post hoc test. Cytotoxicity of modified PMMA at different time intervals, and antimicrobial activity (Zone of inhibition) of DHMAI, and DDMAI were analyzed using Two-Way ANOVA followed by Tukey post hoc test at a 5% level of significance (α = 0.05).

3. Results

3.1. FT-IR spectra analysis

The chemical structure of synthesized monomers (DHMAI, and DDMAI), and modified PMMA resins were characterized using FT-IR spectroscopy [Figure 1]. The characteristic band around 1,720 cm⁻¹ and 1,636 cm⁻¹ in FT-IR spectra revealed the presence of methacrylate groups. The characteristic peaks appeared at 2,822, and 2,771 cm⁻¹ represent C–H stretching of –N(CH₃)₂ groups, 1,720 cm⁻¹ represent C=O, and C–O–C stretching of acrylate groups, 1,459 cm⁻¹ represent...
CH\textsubscript{2} bending, and 1,149 cm\textsuperscript{-1} represents C–N stretching. FT-IR spectrum of PMMA (Control) indicated the presence of its characteristic functional groups. Because of the presence of ester carbonyl group stretching vibration, a sharp peak at 1,720 cm\textsuperscript{-1} was observed. Broad peaks between 1,260 and 1,000 cm\textsuperscript{-1} are due to C–O (ester bond) stretching vibration, whereas broadband between 950 and 650 cm\textsuperscript{-1} is due to C–H bending. From Figure 1, it is also evident that characteristic peaks present in DHMAI, DDMAI monomers, and PMMA are also present in the copolymer prepared by adding these monomers with PMMA. Thus, FT-IR spectra analysis confirms the copolymerization of PMMA with DHMAI and DDMAI monomers.

3.2. NMR spectra analysis

The chemical structure of DHMAI monomer was further confirmed by \textsuperscript{1}H-NMR spectra (Figure 2). Following corresponding peaks in the spectra, their peak position, and its multiplicity were observed: \textsuperscript{1}H-NMR (δ ppm): 6.12 (1H, CH\textsubscript{2} = C(CH\textsubscript{3}) trans, s), 5.64 (1H, CH\textsubscript{2} = C(CH\textsubscript{3}) cis, s), 4.62–4.64 (2H, N + CH\textsubscript{2}CH\textsubscript{2}OC(O), t), 4.09–4.11 (2H, N + CH\textsubscript{2}CH\textsubscript{2}OC(O),t), 3.61–3.64 (2H,N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})13CH\textsubscript{3}, t), 3.46 (6H, N+ (CH\textsubscript{3})2, s), 1.91 (3H, CH\textsubscript{2} = C(CH\textsubscript{3}), s), 1.73–1.76 (2H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})13CH\textsubscript{3}, m), 1.21–1.31 (26H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})13CH\textsubscript{3}, m), 0.82–0.85 (3H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})13CH\textsubscript{3}, t). These NMR spectra findings are in accordance with the previously reported study [11].

Similarly, the chemical structure of DDMAI monomer was also confirmed by \textsuperscript{1}H-NMR spectra (Figure 3). Following characteristics peaks, peak position, and its multiplicity were observed: \textsuperscript{1}H-NMR (δ ppm): 6.16 (1H, CH\textsubscript{2} = C(CH\textsubscript{3}) trans, s), 5.68 (1H, CH\textsubscript{2} = C(CH\textsubscript{3}) cis, s), 4.67 (2H, N + CH\textsubscript{2}CH\textsubscript{2}OC(O), t), 4.14 (2H, N + CH\textsubscript{2}CH\textsubscript{2}OC(O), t), 3.63–3.66 (2H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})7CH\textsubscript{3}, t), 3.50 (6H, N+(CH\textsubscript{3})2, s), 1.95 (3H, CH\textsubscript{2} = C(CH\textsubscript{3}), s), 1.76–1.79 (2H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})7CH\textsubscript{3}, m), 1.25–1.35 (14H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})7CH\textsubscript{3}, m), 0.86–0.89 (3H, N + CH\textsubscript{2}CH\textsubscript{2}(CH\textsubscript{3})7CH\textsubscript{3}, t). The characteristic features observed in NMR spectra for DDMAI are similar to those reported earlier [11].

The \textsuperscript{1}H-NMR spectra of modified PMMA resin incorporated with DHMAI or DDMAI were also recorded (Figure 4). Figure 4, shows \textsuperscript{1}H-NMR spectra of PMMA modified with DHMAI, where the signals of PMMA and DHMAI were overlapped resulting in a broad spectrum compared to DHMAI spectra. Similarly, in \textsuperscript{1}H-NMR spectra of PMMA modified with DDMAI, signals of PMMA, and DDMAI were overlapped leading to broad spectra compared to DDMAI spectra (Figure 5).

3.3. Disc diffusion method

Results of the disc diffusion test of antimicrobial monomers were shown in Figure 6. DHMAI and DDMAI monomers showed antimicrobial activity in a dose-dependent manner. DHMAI showed significant antimicrobial activity against \textit{S. aureus} at 5 μg/mL concentration showing a zone of inhibition of 20 ± 0.6 mm (P = 0.06) whereas the zone of inhibition for \textit{S. mutans}, and \textit{C. albicans} was found to be 18 ± 0.6 mm (P = 0.01), and 19 ± 00 mm (P’<0.0001) respectively. DDMAI showed significant antimicrobial activity against \textit{S. aureus} at 20 μg/mL concentration, showing a zone of inhibition of 19 ± 0.6 mm (P = 0.01) whereas the zone of inhibition for \textit{S. mutans}, and \textit{C. albicans} was found to be 16 ± 00

![Figure 1. FT-IR spectra of PMMA, DHMAI, DDMAI, PMMA modified with DHMAI, and PMMA modified with DDMAI monomer.](image1)

![Figure 2. \textsuperscript{1}H-NMR spectra of DHMAI monomer.](image2)
mm (P = 0.01), and 18 ± 0.6 mm (P = 0.03) respectively at this concentration.

3.4. Cytotoxicity

Figure 7, shows cytotoxicity of DHMAI, and DDMAI at 1, 2, 5, 10, 20, 40, 60, 80, and 100 μg/mL concentrations. From Figure 7, DHMAI up to 5 μg/mL, and DDMAI up to 20 μg/mL exhibited significantly less cytotoxicity compared to other concentrations (P < 0.0001). This indicates that both DHMAI and DDMAI monomers are biocompatible at lower concentrations. However, the cytotoxicity of these monomers seems to be dose-dependent indicating that the use of higher concentrations can decrease the cell viability.

Furthermore, based on the cytotoxicity results of unpolymerized monomers, suitable concentrations of antimicrobial monomers were incorporated into PMMA material, and cytotoxicity of the modified PMMA resin was measured. Figure 8, depicts the cytotoxicity of modified PMMA resin (containing DHMAI at 1, 2, or 5 μg/mL, and DDMAI at 5, 10, or 20 μg/mL). PMMA incorporated with antimicrobial monomer exhibited significantly lower cytotoxicity at DHMAI at 1 μg/mL (P = 0.9184), and DDMAI at 5 μg/mL (P = 0.5201) concentrations compared to control. Whereas statistically significant difference in cytotoxicity was
observed between control and other concentrations of DHMAI (2, or 5 μg/mL) and DDMAI (10, or 20 μg/mL) monomer, indicating its higher degree of toxicity towards L-929 cell line.

Figure 9, shows cytotoxicity of modified PMMA resin, and control (PMMA) specimens stored in artificial saliva at regular intervals of time [1 day, 1 week, and 1 month]. From Figure 9, it can be observed that the control group exhibited the lowest cytotoxicity (highest cell viability) at different time intervals compared to the experimental groups. The addition of antimicrobial monomers to PMMA denture base resin increased their cytotoxicity in a dose-dependent manner.

3.5. Antimicrobial activity

The results of disc diffusion test of PMMA discs incorporated with various concentrations of DHMAI or DDMAI monomer did not show any zone of inhibition. However, estimation of CFU using the direct contact test revealed that the incorporation of antimicrobial monomers into PMMA polymer inhibited the adhesion of microorganisms to its surface, especially S. mutans. From Table 1, it can be observed that the addition of both the monomers reduced adhesion of S. mutans completely. However, no reduction in the adhesion of S. aureus was observed with both the monomers although, the adhesion of C. albicans decreased in a dose-dependent manner for both the monomers tested.

The adhesion of microorganisms to the surface of both experimental, and control groups was also observed under FESEM and is presented in Figure 10a to e. Figure 10a shows adherence of S. aureus in the control group whereas Figures 10b, and 10c show no adherence of S. mutans on modified PMMA resin. Figures 10d, and 10e show adherence of S. mutans, and C. albicans in the control group respectively.

3.6. Surface hardness

Figure 11, shows the surface hardness of PMMA resin with, and without antimicrobial monomers. From Figure 11, it was observed that the addition of antimicrobial monomers increased the surface hardness of PMMA resin. However, the increase in the surface hardness was...
statistically significant only at 5 \( \mu \text{g/mL} \) for DDMAI monomer (\( P < 0.05 \)). No significant differences in surface hardness were observed at other concentrations of DHMAI, and DDMAI.

3.7. Water sorption

Figure 12, shows results of water sorption of modified PMMA resin. The addition of DHMAI reduced the water sorption of PMMA resin only at 1 \( \mu \text{g/mL} \) concentration. In case of DDMAI monomer, both 5 and 10 \( \mu \text{g/mL} \) discernibly reduced the water sorption of PMMA resins. However, no statistically significant difference was observed between PMMA (control) and DHMAI at 2 and 5 \( \mu \text{g/mL} \) or DDMAI at 20 \( \mu \text{g/mL} \).

3.8. FESEM analysis

The surface morphology of modified PMMA resin incorporated with DHMAI or DDMAI, and control (PMMA), were visualized using FESEM analysis. The microscopic appearance was comparable to the polymeric materials. However, modified PMMA resin samples (Fig. 13b, and c) seem to be more porous compared to the control (Figure 13a).

4. Discussion

Heat-activated PMMA is widely used as denture base material. Although it exhibits excellent aesthetics, and adequate mechanical
The antimicrobial activity of these monomers can be attributed to the action against the tested microorganisms in a dose-dependent manner. Concentrations indicating that these monomers exhibited antimicrobial activity, adherance of microorganisms, hardness, and water sorption of the resultant PMMA polymer have been investigated. In the Agar-disc diffusion test, a clear inhibitory zone was observed with DHMAI at 1, 2, or 5 \( \mu \text{g/mL} \), and DDMAI at 5, 10, or 20 \( \mu \text{g/mL} \) concentrations indicating that these monomers exhibited antimicrobial activity against the tested microorganisms in a dose-dependent manner. The antimicrobial activity of these monomers can be attributed to the presence of positively charged QAM salts with negatively charged bacterial cell membrane. DhamaI with a C12 alkyl chain and DDMAI with a C12 alkyl chain penetrate the bacterial cell and break the cell membrane, allowing cytoplasmic substances to seep out, resulting in bacterial cell death [11].

The antimicrobial activity of these monomers was also assessed after adding them to PMMA. Interestingly, the addition of these monomers to PMMA did not show any zone of inhibition against any of the microorganisms tested. This may seem to indicate that modified PMMA resin does not exhibit any antimicrobial action. However, it is to be noted that in disc diffusion testing, it is essential for the antimicrobial additive to leach, and diffuse into the agar medium for it to exhibit antimicrobial action [19]. The copolymerization of antimicrobial monomers added to PMMA entrapped them in the PMMA matrix making it difficult for them to leach out. Pure monomers, on the other hand, could easily diffuse into the agar medium, and hence exhibited antimicrobial action [1].

This was further corroborated by the results of the adherence test in which a significant reduction in the number of CFU was observed especially for \( S. \text{mutans} \). This indicates that the added monomers are present within and on the surface of the PMMA which prevented the adhesion of microorganisms which is also corroborated by microscopic images of the specimens containing antimicrobial monomers. Both antimicrobial monomers used in the present study significantly reduced the adhesion of \( S. \text{mutans} \) compared to \( S. \text{aureus} \), and \( C. \text{albicans} \) [13]. In \( S. \text{mutans} \), Guanylate-binding proteins (GBP) a glycan-binding protein promotes the growth of extracellular polymeric substances (EPS) in biofilm whereas, in \( S. \text{aureus} \), few enzymes such as proteases, nuclease, teichoic acids (an integral part of the cell wall) play a role, indicating that the EPS production is species-specific. Hence the monomers interfere with different types of proteins differently. Thus, the rate of adhesion, and also inhibition by the test compound may vary among the bacteria [20].

Although, antimicrobial activity is highly desired, for its successful long-term use in clinical conditions, biological compatibility is equally important. Fully polymerized PMMA denture base resins are highly biocompatible. However, the leaching of unpolymerized monomers from the PMMA matrix, especially in self-cure, may lead to adverse reactions such as tissue irritation, allergic reaction, and inflammation [21, 22]. In the present study, cytotoxicity of two polymerizable antimicrobial monomers viz., DHMAI, and DDMAI were investigated after incorporating into PMMA material. DHMAI and DDMAI monomers were found to be cytocompatible in polymerized, and unpolymerized forms. Although dose-dependent cytotoxicity was observed for unpolymerized monomers, the same was not observed upon adding these monomers to PMMA denture base resin. Assessment of cytotoxicity of modified PMMA resin stored in artificial saliva at different time intervals also did not show increased cytotoxicity. For both the monomers, more than 70% of cells were viable even after one month at all the concentrations tested. This suggests that modified PMMA material is cytocompatible, and satisfies the requirements of cytotoxicity according to ISO (1999)10993-5 standard [23].

An increase in surface hardness of PMMA resin was observed with the addition of antimicrobial monomers although it was not statistically significant. An improvement in the hardness could be due to the copolymerization and cross-linking of the monomers with the PMMA matrix. A reduction in the water sorption can be attributed to the hydrophobic nature of the antimicrobial monomers added to PMMA resin. Such hydrophobic nature reduces the tendency of denture base resin to absorb less water and hence superior hardness and less water sorption.

In summary, QAM-based monomers with antimicrobial activity were synthesized and characterized. Optimized concentrations of these monomers were added to conventional heat-activated denture base materials either by blending or copolymerizing the antimicrobial agents to the PMMA resin matrix. In recent years, copolymerizing of antimicrobial monomers to biomedical polymers has gained interest due to their long-lasting antimicrobial action. In the present study, two monomers based on QAM salts were synthesized and added to conventional PMMA denture base materials, and their effect on biocompatibility, antimicrobial activity, adherence of microorganisms, hardness, and water sorption of the resultant PMMA polymer has been investigated. In the Agar-disc diffusion test, a clear inhibitory zone was observed with DHMAI at 1, 2, or 5 \( \mu \text{g/mL} \), and DDMAI at 5, 10, or 20 \( \mu \text{g/mL} \) concentrations indicating that these monomers exhibited antimicrobial activity against the tested microorganisms in a dose-dependent manner. The antimicrobial activity of these monomers can be attributed to the presence of positively charged QAM salts with negatively charged bacterial cell membrane. DhamaI with a C12 alkyl chain and DDMAI with a C12 alkyl chain penetrate the bacterial cell and break the cell membrane, allowing cytoplasmic substances to seep out, resulting in bacterial cell death [11].

The antimicrobial activity of these monomers was also assessed after adding them to PMMA. Interestingly, the addition of these monomers to PMMA did not show any zone of inhibition against any of the microorganisms tested. This may seem to indicate that modified PMMA resin does not exhibit any antimicrobial action. However, it is to be noted that in disc diffusion testing, it is essential for the antimicrobial additive to leach, and diffuse into the agar medium for it to exhibit antimicrobial action [19]. The copolymerization of antimicrobial monomers added to PMMA entrapped them in the PMMA matrix making it difficult for them...
materials, and their properties were investigated. The incorporation of antimicrobial monomers imparted antimicrobial action and reduced the adhesion of microorganisms to the modified denture base material in a dose-dependent manner without significantly affecting other properties.

Figure 10. Adherence of microorganisms on control and experimental groups. Scanning electron microscopy images of (a) *S. aureus*-control; (b) *S. mutans*- DHMAI 5 μg/mL; (c) *S. mutans*- DDMAI 20 μg/mL; (d) *S. mutans*-control; (e) *C. albicans* – control.

Figure 11. Vickers hardness of modified PMMA resin containing antimicrobial monomer (a) DHMAI (1, 2, or 5 μg/mL), and (b) DDMAI (5, 10, or 20 μg/mL).
However, the results of the present study cannot be extrapolated to the clinical performance of the materials due to the difference in the controlled testing conditions of in vitro and oral cavity. In addition, the long-term biocompatibility of PMMA denture base material incorporated with antimicrobial monomers as well as long-term antimicrobial activity was not investigated in the present study. Further, the study investigated the effect of antimicrobial monomers with only a single commercially available PMMA material. In this regard, future studies are warranted to analyze the effect of antimicrobial monomers on different types of PMMA denture base materials, and the effect of processing methods to provide a better understanding of their suitability for clinical usage.

5. Conclusions

It can be concluded based on the findings of this investigation, that the addition of DHMAI and DDMAI monomers to conventional PMMA-based denture base resins significantly reduced the surface adhesion of microorganism to the denture surfaces. Further, the addition of these monomers, increased surface hardness, and reduced water sorption of the denture base resins. Hence DHMAI and DDMAI can be considered as potential candidates for imparting antimicrobial activity to polymeric denture base materials to combat DS.

Declarations

Author contribution statement

Sowmya Rao; Nandish B.T; Kishore Ginjupalli: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Namitha K. Preman; Renjith P. Johnson; Preethishree; Ashwini Prabhu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ranajit Das: Analyzed and interpreted the data.

Jayaprakash K; Vidya Pai: Conceived and designed the experiments.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors thank Dr. Murari M S (Scientific Officer, DST- PURSE Program Mangalore University, Mangalagangotri) for his help in FESEM imaging.
References

[1] A. Mirizadeh, M. Atai, S. Ebrahimi, Fabrication of denture base materials with antimicrobial properties, J. Prosthodont. Dent 119 (2) (2018) 292–298.

[2] J. Marra, A.G. Paleari, I.S. Rodriguez, A.R. Leite, A.C. Pero, M.A. Compagnoni, Effect of an acrylic resin combined with an antimicrobial polymer on biofilm formation, J. Appl. Oral Sci. 20 (6) (2012) 643–648.

[3] M.J. Lee, M.J. Kim, S.H. Oh, J.S. Kwon, Novel dental poly (methyl methacrylate) containing phytocidic for antifungal effect and inhibition of oral multispecies biofilm, Materials 13 (2) (2020) 371–383.

[4] R.R. Regin, M.P. Vecchia, A.C. Pizzolitto, M.A. Compagnoni, P.P. Souza, R.F. Souza, Antimicrobial properties and cytotoxicity of an antimicrobial monomer for application in prosthodontics, J. Prosthodont. 21 (4) (2012) 283–290.

[5] D.T. De Castro, M.L. Valente, J.A. Agnelli, C.H. da Silva, E. Watanabe, R.L. Siqueira, O.L. Alves, R.D. Holtz, A.C. Dos Reis, In vitro study of the antibacterial properties and impact strength of dental acrylic resins modified with a nanomaterial, J. Prosthodont. Dent 115 (2) (2016) 238–246.

[6] Y. Zhang, B. Ren, X. Zhou, H.H. Xu, Y. Chen, Q. Han, B. Li, M.D. Weir, M. Li, M. Feng, L. Cheng, Effect of antimicrobial denture base resin on multi-species biofilm formation, Int. J. Mol. Sci. 17 (7) (2016) 1033.

[7] S. Rao, B.T. Nandish, G. Kishore, K. Jayaprakash, A review on poly (methyl methacrylate) denture base materials with antimicrobial properties, Trends Biomater. Artif. Organs 13 (3) (2021) 316–322.

[8] A.R. Cocco, W.L. da Rosa, A.F. da Silva, R.G. 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.

[9] S. Imazato, J.H. Chen, S. Ma, N. Izutani, F. Li, Antibacterial resin monomers based on quaternary ammonium and their benefits in restorative dentistry, Japanese Dental Science Review 48 (2) (2012) 115–125.

[10] P. Mallvandi, R. Jameledin, M. Jabbari, N. Nikfarjam, A. Borzaczchiello, Antibacterial quaternary ammonium compounds in dental materials: a systematic review, Dent. Mater. 34 (6) (2018) 851–867.

[11] J. He, E. Soderling, M. Osterhald, P.K. Vallittu, L.V. Lassila, Synthesis of methacrylate monomers with antibacterial effects against S. mutans, Molecules 16 (11) (2011) 9755–9763.

[12] Y.H. Xiao, J.H. Chen, M. Fang, X.D. Xing, H. Wang, Y.J. Wang, F. Li, Antibacterial effects of three experimental quaternary ammonium salt (QAS) monomers on bacteria associated with oral infections, J. Oral Sci. 50 (3) (2008) 323–327.

[13] F.Z. Cherchali, M. Mouzali, J.B. Tommasino, D. Decorret, N. Attik, H. Aboulleil, D. Seux, B. Grosgeot, Effectiveness of the DHMAI monomer in the development of an antibacterial dental composite, Dent. Mater. 33 (12) (2017) 1381–1391.

[14] S. Rao, B.T. Nandish, K. Ginjupalli, K. Jayaprakash, Effect of copolymerizing antimicrobial monomers on mechanical properties of PMMA heat cure denture base resin, Trends Biomater. Artif. Organs 36 (1) (2022) 56–58.

[15] K.G. Odedirin, H. Yilmaz, S. Yilmaz, In vitro evaluation of cytotoxicity of soft lining materials on 1529 cells by MTT assay, J. Biomed. Mater. Res. B Appl. Biomater. 90 (1) (2009) 82–86.

[16] A.S. Takamiya, D.R. Monteiro, L.F. Gorup, E.A. Silva, E.R. de Camargo, J.E. Gomes-Filho, S.H. de Oliveira, D.B. Barbosa, Biocompatible silver nanoparticles incorporated in acrylic resin for dental application inhibit Candida albicans biofilm, Mater. Sci. Eng. C 118 (2021), 111341.

[17] R.R. Regis, A.P. Zanini, M.P. Della Vecchia, C.H. Silva-Lovato, H.F. Oliveira Paranhos, R.F. de Souza, Physical properties of an acrylic resin after incorporation of an antimicrobial monomer, J. Prosthodont.: Implant, Esthetic Reconst. Dentistry 20 (5) (2011) 372–379.

[18] J. He, E. Soderling, P.K. Vallittu, L.V. Lassila, Preparation and evaluation of dental resin with antibacterial and radio-opaque functions, Int. J. Mol. Sci. 14 (3) (2013) 5445–5466.

[19] M. Balouiri, M. Sadiki, S.K. Hnsouda, Methods for in vitro evaluating antimicrobial activity: a review, J. Pharmaceutical Analysis 6 (2) (2016) 71–79.

[20] Z. Khatoon, C.D. McTiernan, E.J. Suuronen, T.F. Mah, E.I. Alarcon, Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention, Heliyon 4 (12) (2018), e01067.

[21] P.A. Leggat, U. Kedjarune, Toxicity of methyl methacrylate in dentistry, Int. Dent. J. 53 (3) (2003) 126–131.

[22] Z. Razewski, Influence of polymerization method on the cytotoxicity of three different denture base acrylic resins polymerized in different methods, Saudi J. Biol. Sci. 27 (10) (2020) 2612–2616.

[23] International Organization for Standardization, ISO 10993-5: Biological Evaluation of Medical Devices - Part 5: Tests for in Vitro Cytotoxicity, ISO, Geneva, 1999,