ORIGINAL RESEARCH

Cytocompatibility of a Monomethacrylate-based Dental Copolymer Containing 1,3,5-triacryloylhexahydro-1,3,5-triazine as a Cross-linking Comonomer: An *In Vitro* Research

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Abstract

Aim: To assess the *in vitro* cytocompatibility of a monomethacrylate-based dental copolymer [P(MMA-*Co*-TATA)] containing 1,3,5-triacryloylhexahydro-1,3,5-triazine (TATA) as a cross-linking comonomer using human cells by tetrazolium (MTT) assay.

Materials and methods: The research groups were categorized based on the composition of the resultant polymer formed. Nine disk-shaped specimens ($n = 9$) per group were prepared using the thermo-polymerization technique. Group G0 (control) consists of polymerized poly(methyl methacrylate) [P(MMA)] specimens without TATA; trial groups G10 and G20 had copolymerized specimens P(MMA-*Co*-TATA) with 10 and 20% TATA cross-linker (CL), respectively. Obtained eluates from the specimens were tested for *in vitro* cytotoxicity by MTT assay in terms of cell viability percentage (CV%) on the human keratinocytes (HK) and human gingival fibroblasts (HGF).

Results: The mean CV% of G0, G10, and G20 for the HK was 72.04, 74.82, and 80.54%, respectively. Concerning HGF, the mean CV% of G0, G10, and G20 was 73.68, 80.09, and 85.00%, respectively. The descending order of cytotoxicity was G0 > G10 > G20. The CV% was statistically significant among and between the groups ($p < 0.05$) with both HK and HGF.

Conclusion: The novel P(MMA-*Co*-TATA) copolymer was nontoxic to human cells *in vitro* when compared to the P(MMA). P(MMA-*Co*-TATA) with 20 wt% TATA exhibited the highest CV%.

Clinical significance: Since TATA is an antimicrobial CL, the resultant dentures could possess improved mechanical properties along with resistance to microbial adhesion on the denture surface. This could reduce the perchance of denture-induced infections in debilitated senile geriatric patients.

Keywords: Cell viability, Cross-linker, Copolymer, Denture base, *In vitro* cytotoxicity.

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Introduction

Denture base (DB) acrylic resins are inevitable in the field of dentistry, conspicuously in prosthetic dentistry, and chemically made of conventional poly(methyl methacrylate) [P(MMA)]. Myriad prosthetic dentures and appliances are being made out of the DB. The DB's processing method and monomer-to-polymer conversion influence its physicomechanical properties and biocompatibility,[1,2] orchestrating various grades of cytotoxicity *in vitro*.[3] Heat-cured DB (HCDB) shelters less unreacted monomer residuum (MR) than its auto-cure variant.[4,5] For an auto-cure resin, there is approximately 1.5–4.5% MR has been estimated[6] whereas, for HCDB, the MR is ~0.3%.[7] However, a minimum MR concentration has also been reported between 0.045 and 0.18%.[8] Specifications by the British standards stipulate 3.5% MR as an acceptable limit for orthodontic acrylic resins.[9] According to the International Standards Organization (ISO 1567), 2.2 and 4.5% MR are acceptable for HCDB and auto-cure acrylic resins, respectively.[10] The MR and its reactive noxious metabolites or haptens (formaldehyde, phthalates, dibenzoyl peroxide, salicylates)[11] from the dentures pose inimical mucosal reactions upon contact,[12] particularly in geriatric patients with infected/inflamed/abused mucosa ensuing from contemporaneous medications or malnutrition.[13] Eluates from the DB have cytotoxic potential on oral epithelial and connective tissue cells by curbing cell growth and thwarting nuclear and metabolic activities.[14]

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Each component in the liquid monomer methyl methacrylate (MMA) can elicit irritation in the respiratory tract.[15,16] MMA also exhibits percutaneous absorption, deteriorating the digital neural myelination and conduction, which eventually leads to numbness.[17] Laboratory personnel involved in the prosthetic fabrications with DB over 3 decades have been diagnosed with peripheral neuropathy.[18,19] Nevertheless, they suffer from severe contact dermatitis and respiratory pathoses by inhaling the MMA vapors or polymeric specks of dust during prosthetic adjustments.[20,21] MMA can percolate through protective gloves and get trapped in them, which can enhance the cutaneous manifestations of prolonged intimate digital contact.[22] Also, dental students are prone to the abovementioned health hazards while manipulating the DB and other resinous materials.[23] Cutaneous eczema due to the MMA contact was reported in 2–7% of dental clinicians.[24,25]

The MR was reduced by the addition of cross-linkers (CL).[26,27] The CLs added in the DB not only improved the mechanical properties but also enhanced the cytocompatibility *in vitro*.[28] This kind of DB modification is not uncommon. Antimicrobial comonomers like fluoroalkyl methacrylates, methacrylic acid, methylallyl phosphate, and dimethylamino ethyl methacrylate octyl bromide were incorporated in the DB.[29] However, these comonomers were monofunctional and lacked cross-linking capability. Recently, an antimicrobial CL 1,3,5-triacryloylhexahydro-1,3,5-triazine (TATA) was successfully copolymerized with HCDB to develop a microbe-resistant denture copolymer P(MMA-Co-TATA).[30,31] However, the cytocompatibility of this copolymer has not been reported in the literature. Hence, the present research aimed to evaluate the *in vitro* cellular toxicity of the copolymer P(MMA-Co-TATA) using human keratinocytes (HK) and human gingival fibroblasts (HGF). The null hypothesis was that the copolymer P(MMA-Co-TATA) would have no cytocompatibility issues in the human cells.

Materials and Methods

The present research was done for a month at Blue Lab, Saveetha Dental College, and Hospital, Chennai, after obtaining the institutional ethical clearance (IHEC/SDC/PhD/PROSTHO-1606/21/226). P(MMA) polymeric powder (Molecular weight: 350 × 10^3 gm/mol; major ingredient in the powder composition), dibenzoyl peroxide (DBPO; initiator), MMA liquid (with ≤30 ppm mequinol inhibitor; the main ingredient in the monomer), *n*-butyl methacrylate (*n*-BMA; plasticizer), tricyclodecanedimethanol diacrylate (TCDDMDA; conventional CL), and 1,3,5-triacryloylhexahydro-1,3,5-triazine (TATA; trial CL) were procured from Aldrich Co. (Sigma-Aldrich, St. Louis, Missouri, United States of America). According to the TATA's concentration and HCDB's composition, this research's groups were categorized (Table 1). The control group G0 had no TATA in its composition, whereas the trial groups G10 and G20 had 10 and 20% TATA, respectively. This modification was executed only in the prepolymeric P(MMA) powder, and the monomer liquid's composition remained constant for all the groups. The chemical ingredients in the prepolymeric powder and the liquid monomer were proportionated following our previous research.[30,31]

The specimen's dimension and the specimen polymerization technique were followed by the methods elucidated by Ajay et al.[28] The specimen dimension was set according to the ISO 10993-12 guidelines so that the surface area of the specimen was 3 cm^2/mL.[32] A total of 27 (n = 27) polymerized specimen disks were fabricated (n = 9 per group). Human cervical keratinocytes (HeLa cell line; HK) and HGF (Fig 1) were the two cell types employed to assess metabolic enzymatic activity. HGF were available in-house at the Blue Lab, Saveetha Dental College, and Hospital. HK was procured from Pondicherry Center for Biological Sciences, Puducherry. The tetrazolium reduction (MTT) assay by elution method was performed following the guidelines stipulated by ISO 10993-5.[33]

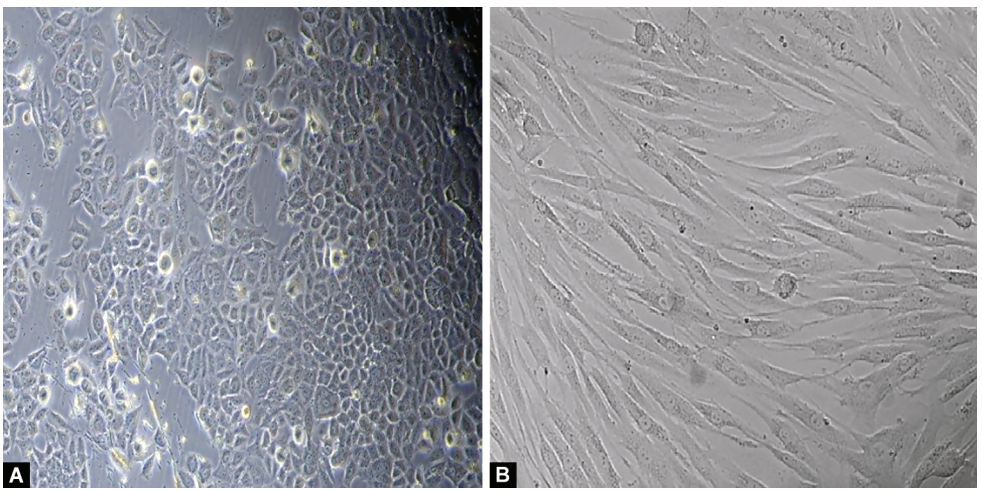
Extract Preparation

The specimens were sterilized by UV irradiation for 30 minutes and washed thrice in sterile phosphate-buffered saline (PBS). Eventually,

Table 1: Research groups with their compositional ingredients

	Composition	
Group	*Powder*	*Monomeric liquid*
G0	P(MMA) polymeric powder, 2% DBPO as initiator	MMA with 10% *n*-BMA and 10% TCDDMDA
G10	P(MMA) polymeric powder, 2% initiator, 10% TATA	
G20	P(MMA) polymeric powder, 2% initiator, 20% TATA	

Powder–monomeric liquid ratio: 3:1; the liquid composition was constant in all groups; P(MMA), poly(methyl methacrylate); DBPO, dibenzoyl peroxide; MMA, methyl methacrylate, *n*-BMA, *n*-butyl methacrylate; TCDDMDA, tricyclodecane dimethanol diacrylate; TATA, 1,3,5-triacryloylhexahydro-1,3,5-triazine



Figs 1A and B: Cells employed in the research: (A) HK; (B) HGF under inverted microscope (100× magnification)

the sterilized specimens were incubated in a 10 mm Petri dish containing Dulbecco's modified Eagle medium (DMEM) with 5% PBS and incubated at 37°C with 5% CO_2 for 24 hours. The resultant extract was filtered and used to treat the cell lines.[34,35]

Cell Culture and MTT Assay

Both the human cells were plated separately in 48-well plates (2×10^4 cells/well) containing culture media (DMEM + 1× antibiotic solution + 10% fetal bovine serum) and incubated at 37˚C with 5% CO_2 for a day. The cells were washed with 200 µL PBS; then, the cells were treated with 200 µL extract and incubated for 24 hours as above. A culture medium with only cells and no extract was considered a negative control (NC) or blank. The extract was aspirated from cells at the end of the treatment period. 0.5 mg/mL MTT prepared in 1x PBS was added and incubated at 37˚C for 4 hours. Eventually, the medium containing MTT was discarded from the cells and washed using 200 µL PBS. The soluble formazan in MTT turns into crystals of insoluble purple-blue color due to enzymatic activity. The formed crystals were dissolved with 100 µL dimethyl sulfoxide and thoroughly mixed. The absorbance was measured at 570 nm using a microplate reader. The cell viability percentage (CV%) was calculated by the equation:

$$CV\% = \frac{\text{OD570}e}{\text{OD570b}} \times 100,$$

Where OD570e and OD570b denote the mean optical density of eluates and blanks, respectively, all the experiments were performed in triplicates.

Statistical data analyses were made (Statistical Package for the Social Sciences Inc., Chicago, Illinois, United States of America; version 21.0). Kolmogorov–Smirnov and Shapiro–Wilk tests were employed to determine the normality of the data distribution and were found to be normally distributed ($p > 0.05$). Therefore, one-way analysis of variance (ANOVA) and *post hoc* Bonferroni tests were employed for data analyses. The differences among and between the groups were significant only when the $p < 0.05$.

Results

Apparently, the NC group exhibited 100% CV. The mean [standard deviation (SD)] CV% of G0, G10, and G20 for the HK was 72.04 (0.78), 74.82 (0.43), and 80.54 (0.69), respectively. The mean (SD) CV% of G0, G10, and G20 for the HGF was 73.68 (0.37), 80.09 (0.61), and 85.00 (0.50), respectively (Fig. 2). The differences were statistically significant among and between the groups ($p = 0.000$). The significant differences exhibited by the G0-G10 and G0-G20 comparisons were suggestive of their cytocompatibility. The G0 exhibited greater cytotoxicity than the other groups, and G20 exhibited the least cytotoxicity to the cells. The ascending order of cytocompatibility is G0 < G10 < G20 with an overall CV% > 70%. Hence, the novel copolymer P(MMA-*Co*-TATA) is cytocompatible with human cells *in vitro*.

Discussion

In the current research, the HCDB was modified by the addition of the antimicrobial CL agent, the TATA. This modification led to the formation of a new copolymer P(MMA-*Co*-TATA). This trial copolymer exhibited better cytocompatibility with both the human cell types than the control P(MMA). Intriguingly, the trial groups exhibited >74% CV. However, the control group showed inferior cytocompatibility than the trial groups with 72–74% CV. Hence, the null hypothesis was accepted. This can be attributed to the monomer-polymer conversion (MPC) and MR release. In the previous research, the new copolymer P(MMA-*Co*-TATA) showed higher MPC than the conventional P(MMA), and therefore, it can be inferred that there is minimal or negligible MR with the new copolymer.[31] Nevertheless, the TATA is a trifunctional CL with three reactive acrylate terminals for polymerization. The high CV% with the trial groups (G10 and G20) can also be attributed to the acrylate moieties, which generally have a higher reactivity rate than their corresponding methacrylates. Soon after the first methacrylate group's reaction, the second or other pendant groups' reactivity decreases by 7.5–10 times.[36] Therefore, in the present research, TATA in P(MMA-*Co*-TATA) copolymer increased the MPC, reduced the MR release quantity, and eventually exhibited good cytocompatibility with the cells.

The current research results can also be corroborated with previous studies that demonstrated higher *in vitro* cytocompatibility with di-/multifunctional CLs. Tricyclodecane dimethanol diacrylate (TCDA) is a difunctional cycloaliphatic CL incorporated in HCDB to form P(MMA-*Co*-TCDA) copolymer that exhibited good cytocompatibility to the mouse fibroblasts when compared to

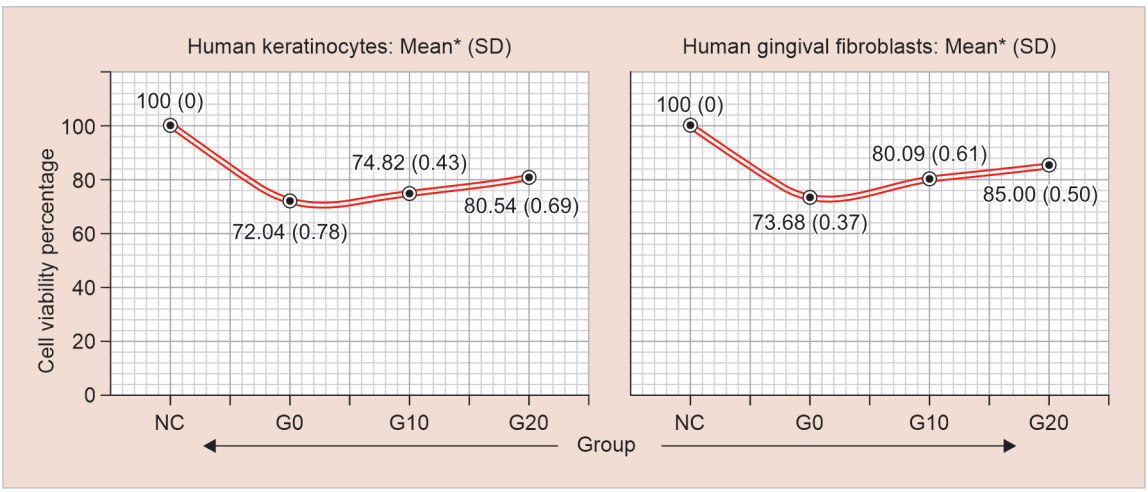


Fig. 2: Mean CV% for HK and HGF; *significant differences among and between the groups ($p < 0.05$)

regular P(MMA).[28] 3,9-dimethylene-1,5,7,11-tetraoxaspiro(5,5) undecane (DMTOSU), a double ring-opening anti-shrinking CL, substituted in the HCDB to form a novel P(MMA-Co-DMTOSU) copolymer showing low cytotoxicity to the HK and HGF when compared to P(MMA).[34] Dipentaerythritol penta-/hexa-acrylate, a multifunctional CL, when added to the restorative composite resin, exhibited minimal or negligible cytotoxicity to the mouse fibroblasts.[35] Hence, from the above context, P(MMA-*Co*-TATA) copolymer substituted with 10 and 20% TATA with a positive cross-linking capacity showed higher CV% than the control P(MMA) group.

Furthermore, *n*-acetyl cysteine[37] and silver nanoparticles[38] modified DBs were cytocompatible with the rat and mouse fibroblasts, respectively. Carbon-graphite fibers[39] and MMA-elemental iodine conjugation[40] incorporated in the DB were noncytotoxic. Biosurfactants' incorporation aimed to impede biofilm formation on the DB were also cytocompatible.[41] However, concerning methacryloyloxydecyl pyridinium bromide[42] and polyoxymethylene[1] additions in the DB, the antithesis is true with the CV%. The CL concentration affects the MPC and MR, which can be extrapolated to adjudicate the cytocompatibility of the DB. An increased ethylene glycol dimethacrylate concentration with reduced MPC was reported by Horie et al.[43] A marked reduction in MR with CLs such as triethylene glycol dimethacrylate or tetrahydrofurfuryl methacrylate substituted in the DB at 12 or 17% concentrations was reported by Moharram et al.[27] An increased MPC and lowered MR with a dendritic CL substituted in DB was reported by Viljanen et al.[44] Hence, in this current research, the maximum concentration of TATA in HCDB was 20%. Therefore, the type, structural chemistry, and concentration of the comonomer added/substituted determines the cytocompatibility of the DB with selected cell lines or primary cells.

In vitro, the cytotoxicity of the DB has been explicitly analyzed in terms of the polymerization type and method through its evaluations. An eluate is a mixture of MR and its metabolites along with free radicals, rendering difficulty in interpreting the results of the previous studies. Hence, it becomes imperative to discriminatorily assess the cytotoxicity of MMA monomer, which was not the objective of this study. The *in vivo* ambience is entirely distinct from the *in vitro* cellular culturing environment. In the body, the MR from polymerized DB could be washed away by saliva and expelled out through urine. However, the released MR might often amass between the oral mucosa and the denture with direct contact and become cytotoxic, which could be strongly influenced by the individual's rate of salivary flow and immune responses to MR.[45]

A denture contacts primarily the first layer of oral cells, the keratinocytes, with or without being protected by a layer of keratin. Hence, any noxious substances from the DB first should cross this barrier to enter the subjacent connective fibroblasts, especially with abused/ulcerated tissues either by the dentures or infections. Since dentures remain in close adaptation with the mucosa, leached substances <100 kDa could readily percolate to reach the fibroblasts.[46] Numerous studies concerning the cytotoxicity of DB have been conducted on mouse fibroblasts.[47] However, cells of human origin would be more suitable for mimicking and quadrating *in vivo* responses with a magnified clinical coherence than other mammalian cells. Therefore, in the current research, HK and HGF were utilized, adhering to the Japanese guidelines for evaluating the biological repercussions of dental biomaterials.[48]

The current research's results might not be extrapolated to the *in vivo* conditions. However, with annihilating factors minimized, *in vitro* research is often considered to be a pertinent affirmation in assessing the cytocompatibility of DBs.[49] Nevertheless, the DBs are subjected to thermomechanical stimuli during clinical usage by the patients. Therefore, *in vivo* environment simulation is a phenomenal limitation while extrapolating the results of *in vitro* studies to existing clinical conditions. This research did not conduct mechanical stressing by cyclic flexural loads and thermal aging by thermocycling. Nonetheless, assessing DBs' cytotoxicity by MTT assay is quite easy to demonstrate with palatable reproducibility and reliability at a low cost. Based on the above limitations, it is necessary to conduct *in vivo* research to study and appreciate the tissue changes by the P(MMA-*Co*-TATA) copolymer in the future.

Conclusion

Following the research guidelines and considering the limitations, it was concluded that the novel P(MMA-Co-TATA) dental copolymer was nontoxic to human cells *in vitro* when compared to the P(MMA). Therefore, a P(MMA-*Co*-TATA) copolymer shall be used to construct DB with antimicrobial properties for patients who lack dexterity in maintaining denture hygiene due to senile motor activities.

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