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Cytocompatibility of dental poly(methylmethacrylate) acrylic resin copolymerized with a spiro-acetal crosslinking comonomer on human cell lines: In vitro research

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Aim: To evaluate the *invitro*cytocompatibility of a poly(methylmethacrylate) [P(MMA)] based dental copolymer conjugated with a spiro-acetalcross-linking comonomer,3,9-bis(ethenyl)-2,4,8,10-tetraoxaspiro[5.5] undecane (BETU), on human cell lines by tetrazolium (MTT) assay.

Materials and methods: Twenty-seven (n = 27) disc-shaped specimenswere prepared by the heat-polymerization technique for 3 groups (n = 9 each group). Controlgroup G0 consists of polymerized P(MMA) specimens without BETU; Trial groups G10 and G20 had copolymerized specimens P(MMA-*Co*-BETU) with 10% and 20% BETU cross-linker (CL), respectively. The MTT assay was used to determine the specimen's*in vitro* cytotoxicity by measuring the cell viability percentage (CV%) of human oral keratinocytes (HOK) and gingival fibroblasts (HGF).

Results: The mean (standard deviation; SD) CV% of G0, G10, and G20 for the HK was 84.91 (0.95), 92.29 (1.64), and 98.94 (0.50), respectively and the mean (SD) CV% of G0, G10, and G20 for the HGF was 80.52 (0.65), 86.68 (1.07), and 96.97 (0.96), respectively. With both HOK and HGF, the CV% was statistically significant both within and between the groups ($p < 0.05$).

Conclusion: The novel P(MMA-*Co*-BETU) copolymer iscomparatively less toxic and more cytocompatible to human cells (in both 10% and 20% concentrations) *in vitro*than the P(MMA).

Keywords: Comonomer, copolymer, cross-linker, cytocompatibility, spiro-acetal.

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Introduction

The materials used in dentistry that do not cause any harm to the oral environment are considered biocompatible. Biocompatibility refers to the degree to which the surrounding tissues and the organism accept or reject artificial material.[1]In restoring dental health to its natural state, polymeric materials open up a wide range of possibilities, and the advancements in their chemical composition themselves have led to the evolution of novel materials with improved mechanical and biological properties.[2]Poly(methylmethacrylate) [P(MMA)] is one of the extensively used polymeric materials in dentistry for the fabrication of denture bases (DB), provisional restorations, and the fabrication of various prostheses.[3]Nevertheless, its unresolved, incomplete double bond conversions (DBC) during radical polymerization have led to microgel aggregation and the residual monomer causing undesirable tissue responses, despite its time-honored utility and dominance.[4]

Percutaneous absorption ofmethyl methacrylate (MMA) deteriorates the myelination and conduction of digital neurons, ultimately resulting in numbness.[5] Lab employees who have worked on DB prosthetic fabrications for more than 30 years have been diagnosed with peripheral neuropathy.[6,7]Prolonged digital contact can cause MMA to seep past the protective gloves and become stuck in them, exacerbating the cutaneous signs.[8] Additionally, while working with the DB and other resinous materials, dental students run the risk of experiencing the health risks listed above.[9] Two to seven percent of dental professionals reported developing cutaneous dermatitis as a result of their interaction with MMA.[10]

The polymer-to-monomer ratio, cycle, water immersion, storage duration, and polymerization technique are all linked to the cytotoxic effects of denture base resins.[11] Residual monomer, along with numerous other metabolic toxic chemicals such as formaldehyde, methacrylic acid, benzoic acid, dibutyl phthalate, phenyl benzoate, and phenyl salicylate, are produced on the DB surfaces during the incomplete polymerization process. Though the heat polymerization technique can reduce the residual monomer formed, it cannot be completely eradicated.

The addition of crosslinkers to the DB has shown decreased*invitro*cytotoxicityin addition to improving the mechanical properties.[12]3,9-*bis*ethenyl-2,4,8,10-tetraoxaspiro[5,5]undecane (BETU) is a bicyclic organic molecule having a central quaternary carbon atom (a spiro atom), which,when appended to polymers, the instinctive propensity of the spiro-acetal group present in BETU has manifested satisfactory adhesive properties, with improved flexibility and tensile strength.[13] Nevertheless, there is no published information about the cytocompatibility of the P(MMA-*Co*-BETU) copolymer. Therefore, employing human keratinocytes (HOK) and human gingival fibroblasts (HGF), the current study aims to assess the copolymer P(MMA-*Co*-BETU)'s *in vitro*cytocompatibility,null-hypothesizing that there would be no cytocompatibility problems with the copolymer P(MMA-*Co*-BETU) in human cells.

Materials and methods

After receiving institutional ethical clearance (IHEC/SDC/PhD/ENDO-2214/23/164), the current study was conducted for one month at Blue Lab, Saveetha Dental College and Hospital, Chennai. The chemicals utilized in this

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research were procured from Aldrich Co. (Sigma-Aldrich, St. Louis, MO, USA). The groups in this study were classified based on the BETU concentration and the composition of the HCDB. The chemicals, functions, phases, and group-wise composition were tabulated in Table 1.

Table 1: Chemicals, Functions, Phases, and Group-wise Composition.

Chemical	Function	Phase	Contents in the groups		
			G0	G10	G20
Poly(methyl methacrylate) (Mw ~350,000 g/mol)	Main polymeric matrix (prepolymer)	Powder	94.5 wt.%	94.5 wt.%	94.5 wt.%
Dibenzoyl peroxide (DBPO)	Free-radical initiator	Powder	0.5 wt.%	0.5 wt.%	0.5 wt.%
Alumina (Al_2O_3) + YSZ (1:1)	Inorganic filler for strength, toughness	Powder	5 wt.%	5 wt.%	5 wt.%
γ-Methacryloyloxypropyltrimethoxysilane (γ-MPTS)	Coupling agent (for filler–matrix bonding)	-	On the fillers' surface	On the fillers' surface	On the fillers' surface
Methyl methacrylate (MMA)(≤30 ppm mequinol)	Primary liquid monomer	Liquid	Present	Present	Present
n-Butyl methacrylate (n-BMA)	Intrinsic-reactive plasticizer	Liquid	10 wt.% of liquid	10 wt.% of liquid	10 wt.% of liquid
Ethylene glycol dimethacrylate (EGDMA)	Conventional cross-linker	Liquid	10 wt.% of liquid	10 wt.% of liquid	10 wt.% of liquid
3,9-bis(ethenyl)-2,4,8,10-tetraoxaspiro[5.5] undecane (BETU)	Spiro-acetal cross-linker (experimental)	Added separately after mixing	Absent	10 wt.% of total mix	20 wt.% of total mix
For a 3:1 powder-liquid ratio, the BETU wasmixedinto the powder-liquid mixture.					

While the trial groups G10 and G20 included 10% and 20% BETU, respectively, the control group G0 did not contain any BETU. The composition of the monomer liquid was the same for each group. Following our earlier research, the chemical components of the liquid monomer and prepolymeric powder were proportioned.[14,15]For a 3:1 powder-liquid ratio, the BETU wasmixedinto the powder-liquid mixture.

Specimen preparation and cell cultures

The techniques described by Ajay et al.[12] were applied to the specimen's dimensions and specimen polymerization process.The specimen was sized to have a surface area of 3 cm^2/mL following ISO 10993-12 criteria.[16]A total of twenty-seven (n = 27) polymerized specimen discs (n = 9 each group) were processed. The two cell types used to measure metabolic enzymatic activity were primary human oral keratinocytes (HOKs) and gingival fibroblasts (HGFs).HOKs were harvested from the palatal tissue of a healthy impacted molar with informed consent at Saveetha Dental College. The HOKs were processed and cultured following the methods described by Chen et al.[17] In brief, the harvested tissue was cleansed with phosphate-buffered saline (PBS) with Gentamicin, chopped into tiny pieces, and incubated overnight at 4 °C.The separated epithelium from the connective tissue was placed into a sterile centrifuge tube containing trypsin-EDTA complex and incubated at 37 °C for 10 min. Trypsin activity was inhibited, and the cell suspension was centrifuged and resuspended in a serum-free keratinocyte basal medium. Cells were seeded (≈ 3 × 10^6 cells/flask) and incubated at 37 °C in a humidifying atmosphere of 5% CO_2 in the air. The 70 – 80% confluent cells were passaged in the Trypsin-EDTA complex and resuspended in the growth medium. The third passage cells were employed in the present research. HGFs were available in-house at the Blue Lab, Saveetha Dental College and Hospital. The extraction method of the tetrazolium reduction (MTT) assay was carried out following ISO 10993-5[18] requirements, and a comprehensive strategy was previously reported by Ajay et al.[19] and Sivakumar et al.[20]

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Extract preparation

The polymerized specimens were treated with three rounds of sterile PBS washings after being sterilized under UV light for half an hour and then incubated for a day at 37 °C with 5% CO_2 in a 10 mm petri dish containing Dulbecco's modified Eagle medium (DMEM) with 5% PBS. From this mixture, the extracts were filtered through sterile filter paper and stored in sterile glass vials until further use.[19,20]

MTT assay

In 48-well plates (2×10^4 cells/well) supplemented with growth material (DMEM + 1x antibiotic solution + 10% fetal bovine serum), the two human cells were plated independently and incubated for a day at 37˚C with 5% CO_2. Following a 200 µL PBS wash, the cells were treated with 200 µL extract and incubated for a day as previously described. A negative control (NC) or blank culture media included only cells and no extract. At the end of the treatment period, the extract was aspirated from the cell cultures. After adding 0.5 mg/mL MTT with 1x PBS to the cell cultures, the mixture was incubated for 4 h at 37 °C. Ultimately, the MTT-containing media was removed from the cells and cleansed with 200 µL PBS. Enzymatic action converts soluble formazan in MTT to insoluble purple-blue crystals. To dissolve the generated crystals, 100 µL of dimethyl sulfoxide was added, and the mixture was stirred thoroughly. The absorbance was measured at 570 nm with a microplate reader. The cell viability percentage (CV%) was estimated using the equation: $CV\% = \frac{OD57}{OD570b} \times 100$, where OD570e and OD570b represent the mean optical density (OD) with extracts and blanks. All tests were carried out in triplicate.

Results

For both cell lines, 100% CV was observed in the NC. The mean (standard deviation; SD) CV% of G0, G10, and G20 for the HOK was 84.91(0.95), 92.29 (1.64), and 98.94 (0.50), respectively. The mean (SD) CV% of G0, G10, and G20 for the HGF was 80.52 (0.65), 86.68 (1.07), and 96.97 (0.96), respectively. The differences were statistically significant concerning HGF between and among the groups (p = 0.000; Figure: 1).

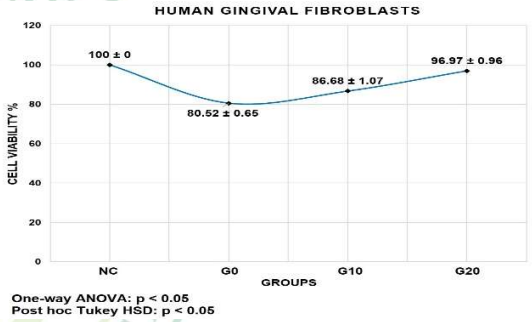


Figure 1: Cell viability of groups on Human gingival fibroblasts.

Concerning HOK, the differences were statistically significant among and between the groups (p = 0.000), except for NC-G20 (p = 0.1237; Figure: 2).

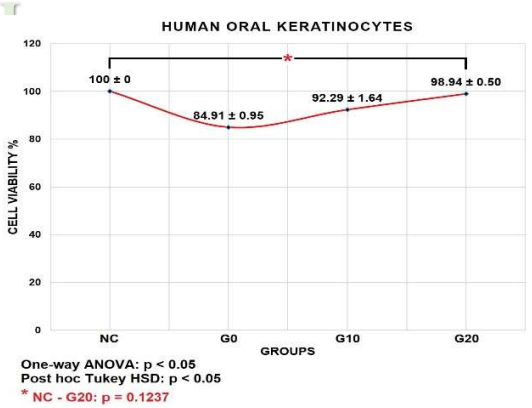


Figure 2: Cell viability of groups on Human oral keratinocytes.

The G20 group showed the least amount of cytotoxicity to the cells, whereas the G0 group showed the most cytotoxicity. With an overall CV% > 80%, the cytocompatibility order obtained for HGF and HOK, respectively, is as follows: G0 < G10 < G20 < NC and G0 < G10 < G20 = NC. Hence, the novel copolymer P(MMA-*Co*-BETU) is cytocompatible with human cells *in vitro*.

Discussion

Integrating crosslinking comonomers that can copolymerize with standard matrix-forming monomers is a widely recognized strategy for augmenting the DBC. However, this conversion is seldom entirely secured in polymer chemistry, resulting in the liberation of unreacted monomer residuum (URM) that compromises cytocompatibility. As such, the cytocompatibility of the newly developed copolymer P(MMA-Co-BETU), which features the BETU crosslinker, was scrutinized based on the effects of the eluate on HOK and HGF. It is generally believed that copolymers with high crosslink density are less vulnerable to degradative phenomena due to the limited spatial orientation within the polymeric network, which hinders solvent infiltration.[21] Accordingly, in this study, the BETU crosslinker (CL) was utilized to fabricate novel denture copolymer P(MMA-*Co*-BETU),demonstrating superior cytocompatibility with human cell types, achieving spectacular >85% CV in the trial groups, compared to 80–84% CV in the control P(MMA). Hence, this supports the acceptance of the null hypothesis, attributed to the effects of DBC and URM release. A possible explanation for the results can be discerned froma previous research, where the new copolymer P(MMA-*Co*-BETU) demonstrated a higher DBC compared to the conventional P(MMA). This suggests that there is minimal or negligible URM with the new copolymer.[22]

The findings of the present study are consistent with earlier research highlighting enhanced *in vitro* cytocompatibility when di- or multifunctional CLs are used. For instance, the incorporation of tricyclodecanedimethanoldiacrylate (TCD), a difunctional cycloaliphatic CL, into P(MMA) resin led to the formation of a P(MMA-*Co*-TCD) copolymer that exhibited superior cytocompatibility with mouse fibroblasts compared to conventional P(MMA).[12]Furthermore, the addition of dipentaerythritolpenta-/hexa-acrylate—a multifunctional CL—to restorative composite resin resulted in minimal to no cytotoxic effects on mouse fibroblasts.[20]Another study found that the P(MMA-*Co*-TATA) copolymer, containing 10% and 20% antimicrobial CL, 1,3,5-triacryloylhexahydro-1,3,5-triazine, exhibited a higher cell viability percentage (CV%) on human keratinocytes and fibroblasts compared to the unmodified P(MMA).[23] Similarly, 3,9-dimethylene-1,5,7,11-tetraoxaspiro(5,5)undecane (DMTOSU), a double ring-opening CL known for its anti-shrinking properties, was incorporated into P(MMA) to create a P(MMA-*Co*-DMTOSU) copolymer, which demonstrated reduced cytotoxicity towards human keratinocytes and gingival fibroblasts.[24]

In previous studies, P(MMA) modified with silver nanoparticles[25] and N-acetyl cysteine[26]demonstrated cytocompatibility with mouse and rat fibroblasts, respectively. Similarly, conjugation of MMA with elemental iodine[27]and incorporation of carbon-graphite fibers[28] resulted in non-cytotoxicP(MMA)denture bases. Biosurfactants, introduced to inhibit biofilm formation on denture surfaces, also showed favorable cytocompatibility profiles.[29] In

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contrast, the addition of methacryloyloxydecylpyridinium bromide[30] and polyoxymethylene[31] exhibited adverse effects on cell viability (CV%). The concentration of CLs plays a crucial role in influencing DBC and UMR release, which are key indicators of cytocompatibility. Moharramet al.[32] observed a significant reduction in UMR when triethylene glycol dimethacrylate or tetrahydrofurfuryl methacrylate were used as CLs at 12–17% concentrations. Likewise, Horieet al.[33] reported a decline in DBC with increasing concentrations of ethylene glycol dimethacrylate. Conversely, Viljanenet al.[34] found that the incorporation of a dendritic CL led to improved DBC and decreased UMR. Based on these findings, the current study limited the concentration of BETOSU in the P(MMA) to a maximum of 20%. These observations underscore that the cytocompatibility of denture bases is determined by the type, molecular structure, and concentration of the comonomer used, as well as its interaction with the specific cell lines or primary cells employed in testing.

The cytotoxicity of P(MMA) materials has been extensively examined in vitro, particularly concerning the type and method of polymerization. However, interpreting results from earlier studies is complicated by the nature of the eluate, which consists of UMR, their metabolites, and residual free radicals. This complexity highlights the importance of specifically evaluating the cytotoxic effects of the MMA, though this was not the focus of the present study. Moreover, it is important to note that in vivo conditions differ significantly from those of in vitro cell culture systems. Within the oral cavity, substances leached from the polymerized DB may be diluted by saliva and ultimately eliminated via urinary excretion. Nevertheless, leachable components may accumulate at the interface between the denture and the oral mucosa, resulting in localized cytotoxic effects. These effects are likely influenced by individual variations in salivary flow rate and immune responsiveness to the released materials.[35]

Denture bases are in direct contact with oral keratinocytes, which may lack protective keratin layers, especially in ulcerated or damaged mucosa. Substances leached from the denture, particularly those <100 kDa, can penetrate this barrier and reach underlying fibroblasts.[36] Although mouse fibroblasts are commonly used for cytotoxicity testing, human cells offer greater clinical relevance. Accordingly, this study utilized HOK and HGF, following Japanese guidelines for biological evaluation of dental materials.[37]Although the findings of this study provide valuable insight into the cytocompatibility of P(MMA), they may not fully reflect in vivo conditions.[38] The dental industry must explore and promote the use of eco-friendly biodegradable alternatives to reduce plastic pollution and ensure sustainability.[39]

In vitro models minimize confounding variables, making them useful for preliminary assessments; however, they do not account for clinical factors such as thermomechanical stress. This study did not incorporate thermal cycling or cyclic flexural loading, both of which are relevant to oral environments. Despite this, the MTT assay remains a simple, cost-effective, and reproducible method for evaluating cytotoxicity. Given these limitations, future in vivo studies are essential to better understand the biological impact of the P(MMA-Co-BETOSU) copolymer on oral tissues.

Conclusion

The in vitro cytotoxicity data obtained in the present study are relevant and bolstering the results of previous researches on the new copolymer P(MMA-*Co*-BETU).

Hence, it is concluded that within the research guidelines and limitations that P(MMA-*Co*-BETU) with better mechanical properties and proven cytocompatibility properties, it can be deployed in the fabrication of high calibred denture base resins.

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Data availability
Available on request

Ethics approval and consent to participate
Not applicable for in vitro studies

Competing interests
Authors declare that they have no conflict of interest

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