ORIGINAL RESEARCH

Evaluation of *In Vitro* Cytocompatibility of New Dental Restorative Composite Resin Copolymers Containing 2π + 2π Photodimerized Cinnamyl Methacrylate Crosslinker

Murugesan Sreevarun[1], Ranganathan Ajay[2], Nasir NiloferNisha[3], Jambai S Sivakumar[4], Suthagar Abhinayaa[5], Karthigeyan Suma[6]

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Abstract

Aim: To determine the *in vitro* cytocompatibility of new dental restorative composite resin (RCR) copolymers containing photodimerized cinnamyl methacrylate (PD-CMA) crosslinker using human pulpal fibroblasts (HPF) by tetrazolium (MTT) assay.

Materials and methods: Three research groups were based on the composition of the copolymer. There was a negative control (NC) group only with the cell culture. A total of 27 disk-shaped specimens ($n = 9$ per group) were prepared. Group C0 (control) consisted of photopolymerized specimens made of base matrix-formers (B), a diluent (D), and without PD-CMA [P(BD)]; experimental groups E10 and E20 consisted of copolymers P(BD-Co-CMA) with 10 wt% PD-CMA substituting D and P(B-Co-CMA) with 20 wt% PD-CMA replacing D, respectively. The specimens were eluted, and an MTT assay was performed. The obtained optical density (OD) values in absorbance unit (AU) were subjected to statistical analysis.

Results: The mean OD of C0, E10, and E20 was 0.79, 0.92, and 1.18 AU, respectively. The difference between C0 and E10 was not significant ($p = 0.067$). The comparisons C0-E20 and E10-E20 were statistically significant ($p < 0.05$). The order of cytocompatibility was C0 = E10 < E20.

Conclusion: The new P(B-Co-CMA) was the most cytocompatible copolymer with HPF *in vitro* when compared to the P(BD-Co-CMA) and P(BD).

Clinical significance: The low-viscosity PD-CMA can eschew the toxic effects of the triethylene glycol dimethacrylate (TEGDMA) by replacing it. Nevertheless, the new copolymer P(B-Co-CMA) with hydrophobic PD-CMA crosslinker would not induce pulpal inflammation and necrosis by preserving the intracellular glutathione and preventing the formation of reactive oxygen species.

Keywords: Composite, Crosslinker, Cytotoxicity, Monomeric residuum, Triethylene glycol dimethacrylate.

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Introduction

Dental restorative materials will be in direct or indirect contact with the hard and soft tissues of the oral cavity. These restorative materials undergo compositional aberrations at the surface or core level due to constant thermo-chemico-mechanical stimuli. These stimuli cause the release of residual chemicals and their metabolites that might induce alterations in the mucosal and submucosal tissues. Among the tissue alterations, hypersensitivity changes, including oral lichenoid or anaphylactoid reactions, are common due to mucosal abuse.[1] Tissue fibrosis with the restorative materials was also reported, and it ensued due to chronic tissue contact.[2] Among the restorative materials, >12% of untoward cytotoxic reactions are engendered by restorative composite resins (RCR) alone.[3] The amount and chemical type of a leached organic constituent is accountable for dictating the RCR's biocompatibility.[4] Leached detrimental residual components from the RCR are known dentinal penetrants and diffusers that harm the pulpal tissues by deteriorating the tooth's vitality.[5]

The base matrix forming monomers (B) in the RCR including bisphenol-A-glycerolate dimethacrylate (Bis-GMA) and diurethane dimethacrylate (DUDMA) along with diluent monomers (D) triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxy-ethyl methacrylate have been studied significantly in their homo-/copolymeric forms concerning physicomechanical properties.[6,7]

[1,2]Department of Prosthodontics and Crown & Bridge, Vivekanandha Dental College for Women, Namakkal, Tamil Nadu, India

[3]Department of Conservative Dentistry and Endodontics, Faculty of Dentistry, MAHSA University, Malaysia

[4]Department of Conservative Dentistry and Endodontics, Vivekanandha Dental College for Women, Namakkal, Tamil Nadu, India

[5]Department of Prosthodontics and Crown and Bridge, KSR Institute of Dental Science and Research, Namakkal, Tamil Nadu, India

[6]Department of Prosthodontics and Crown and Bridge, Government Dental College, Cuddalore, Tamil Nadu, India

Corresponding Author: Ranganathan Ajay, Department of Prosthodontics and Crown & Bridge, Vivekanandha Dental College for Women, Namakkal, Tamil Nadu, India, Phone: +91 8754120490, e-mail: jrangclassiq@gmail.com

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Leached monomeric residuum and their noxious metabolites [methyl ester of 2,3-epoxy-2-methyl-propanoic acid, methacrylic

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acid, 2,3-epoxy-2-methylpropionic acid, and bis-phenol-A-bis-(2,3-dihydroxypropyl) ether] are responsible for intracellular reactive oxygen species production resulting in inflammatory responses triggered by cytokines and other cytomediators.[8,9]

Several studies reported that the TEGDMA in polymerized RCR is the predominant comonomer released into aqueous media. It possesses a high cytotoxic potential and curbs cellular growth upon leaching.[10] Other unpolymerized comonomers (Bis-GMA and DUDMA) impose *in vitro/in vivo* cytotoxicity and mutations on cells.[11,12] The resin monomer, especially Bis-GMA, induces estrogen-like activity,[13–15] genetic toxicity,[16,17] and immune response alterations.[18–20] However, the clinical pervasiveness of these inopportune tissue responses remains debatable and contentious.[21] Several contemporary research studies have been conducted concerning new polymerization chemistries for RCR. There is a sharp rise in the development of RCRs, with novel compositions sprouting to augment physicomechanical properties, esthetics, and hard tissue adhesions.[22] However, the assessment of the biocompatibility of such newly developed RCRs is unclear, especially when looking for prolonged clinical serviceability. Nevertheless, the cytotoxic effects of comonomers constituting organic matrix are still undetermined. Hence, this warrants the search for a new comonomer that, upon copolymerization with conventional comonomers, enhances the properties of RCR.

A cinnamic acid ester photo-crosslinker, cinnamyl methacrylate (CMA), has been identified recently as an RCR crosslinker with low viscosity that could substitute or replace TEGDMA. Cinnamic acid ester monomers photodimerized (PD) by ultraviolet (UV) irradiation lead to the formation of a cyclobutane-ringed core structure, which is employed in the production of photo-crosslinked polymers.[23–25] Structurally, cinnamyl compounds contain an aromatic ring at the β-position of an α,β-unsaturated carbonyl group. Conspicuously, polymerization at the C=C moiety would yield biopolymers with a linear saturated structural rigid backbone with alternative aromatic rings and ester moieties as side chains. Such polymers are potential multifunctional biopolymers that combine polystyrene and acrylic resin features with high heat resistance owing to the aromatic side chains and the ditacticity interaction.[26] Recently, PD-CMA was incorporated as a crosslinker in the RCR (substituting partially or completely TEGDMA), which underwent copolymerization with the other conventional comonomers, resulting in the formation of new P(BD-Co-CMA) and P(B-Co-CMA) copolymers.[27] However, there are no studies concerning their *in vitro* cytocompatibility. Hence, the current research aimed to evaluate the cytocompatibility of these new RCR copolymers using human dental pulpal primary fibroblasts (HPF). The null hypothesis was that the new copolymer P(B-Co-CMA) without TEGDMA would have no effect on the cytocompatibility.

Materials and Methods

The research was conducted at Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai. Institutional ethical clearance was obtained (Approval No.: VMMC/DENT/2022/003). The matrix-formers, camphorquinone (CQ), dimethylaminoethyl methacrylate (DMAEMA), yttrium-stabilized zirconia (YSZ) nanoparticles, and barium oxide (BaO) were procured from Sigma-Aldrich (Sigma-Aldrich Co., St Louis, Missouri, United States of America). CMA (Polysciences, Inc., Warrington, Pennsylvania, United States of America) and barium fluoride (BaF_2; Sisco Research Laboratories Pvt. Ltd., Maharashtra, India) were also procured. The CMA was PD by exposing it to UV-C light (275 nm; Mateminco MT07 UVC torch) at a distance of 20 mm in a dark room for half an hour.

Three research groups were based on the composition of the copolymer. There was a negative control (NC) group, too. The research groups comprised of control (C0) and experimental groups (E10 and E20). The RCR matrices for the control and experimental groups with filler loading were proportionated and mixed by adhering to the directions elucidated by Aydınoğlu and Yoruç.[28] A total of 27 ($n = 9$ per group excluding the NC where there was only incubated cell culture) disk-shaped specimens were light-cured for 40 seconds (Guilin Woodpecker Medical Instrument Co., Ltd.; Guangxi, China; 420–480 nm, 650–800 mW/cm^2). The composition of the research groups (control: C0; experimental: E10 and E20) and the resultant copolymers were described in Table 1.

A tetrazolium (MTT) assay using the elution method was employed to assess the cytocompatibility of the resultant copolymers. Preparation of the eluates and the step-by-step protocol for MTT assay (Table 2) strictly adhered to the protocol

Table 1: Composition of control and experimental matrices and resultant copolymer

Matrix	*Composition and copolymer*
C0	• Base matrix formers: Bis-GMA (50 wt%) + DUDMA (30 wt%)—B. • Diluent: TEGDMA (20 wt%; D). • Copolymer: P(BD).
E10	• Base matrix formers: Bis-GMA (50 wt%) + DUDMA (30 wt%)–B. • PD-CMA (10 wt%) photo-crosslinker; diluent: TEGDMA (10 wt%; D). • Copolymer: P(BD-Co-CMA).
E20	• Base matrix formers: Bis-GMA (50 wt%) + DUDMA (30 wt%)–B. • PD-CMA (20 wt%) photo-crosslinker; diluent: absent. • Copolymer: P(B-Co-CMA).

Filler ingredients, BaO (30 wt%), BaF_2 (30 wt%), YSZ (40 wt%); the matrix-filler ratio was 30:70 wt%; the total CQ:DMAEMA (1:2) is 1 wt%; these were constant for all the groups

Table 2: MTT assay—sequential procedure

Time (hour)	*Procedure*
00:00	The HPFs were dispensed in the DMEM to form a cell-medium mixture (1×10^4 cells/mL); a 96-well culture plate was inoculated with 100 µL/well of cell-medium mixture (1,000 cells per well) followed by incubation
24:00	The culture medium was removed after observing cellular monolayer and rinsed with phosphate-buffered saline (PBS); 100 µL of eluates (e) were dispensed to pre-labeled wells followed by incubation; NC served as reagent blank (b) without adding the eluate
48:00	Morphological aberrations were observed under a microscope; the eluates were pipetted out, and the cells were treated with PBS; 50 µL of tetrazolium was dispensed into the wells and incubated in a dark environment for 3 hours
51:00	After aspirating the MTT solution, 100 µL of dimethyl sulfoxide was added to each well and undulated for 30 minutes until blue-colored formazan crystals dissolved
51:30	The culture plate was placed in a spectrophotometer to read the absorbance

For reproducibility, the abovementioned procedure was executed thrice in triplicate[30]

described by Sivakumar et al.[21] For the eluate preparation, the test specimens (n = 3 per group) were placed in 9 mL of culture medium [Dulbecco's Modified Eagle Medium (DMEM) + 5% fetal bovine serum + 100 IU/mL penicillin and 100 µg/mL streptomycin + 1% l-glutamine] contained in sterilized cell-culture petriplates and incubated at 37 ± 1°C for a day in 5% CO_2. For NC, a culture medium without specimens was incubated. The eluates were sterilized using thin filter paper disks and transferred into sterilized vials for refrigeration after proper sealing and labeling until further use.

The MTT assay was carried out employing HPF and is based on the quantity of formazan crystals formation and deciphered as optical density (OD) in absorbance unit (AU) at 570 nm. The cell viability compared to the blank was calculated by using the equation:

$$\text{Cell viability\%} = \left(\frac{OD570_e}{OD570_b}\right) \times 100$$

The ODs of 570_e and 570_b represent the average of obtained OD values of the experimental eluates/extracts and blanks, respectively. When the fibroblastic viability of the specimen is decreased to 70% of the blank, it is deemed cytotoxic. The specimen dimension (3 cm^2/mL total surface area per specimen) and the elution technique were followed according to the International Organization for Standardization (ISO) 10993-12 and ISO 10993-5 guidelines, respectively.[29,30]

Statistical Analysis

The data obtained were submitted to the Shapiro–Wilk and Kolmogorov–Smirnov normality test [SPSS; version 29.0, Chicago, Illinois, United States of America]. Based on the results ($p > 0.05$), one-way analysis of variance (ANOVA) with *post hoc* Tukey honestly significant difference multiple comparison tests were used to analyze the differences among and between the groups. A value of $p < 0.05$ was taken for statistical significance.

Results

The mean (±standard deviation) OD (cell viability percentage) of the NC, C0, E10, and E20 were 1.26 ± 0.12 AU (100%), 0.79 ± 0.09 AU (62.9%), 0.92 ± 0.09 AU (73.3%), and 1.18 ± 0.11 AU (93.5%), respectively. There was a statistically significant difference among the groups ($p < 0.05$). When compared between the groups, the comparisons NC-E20 ($p = 0.391$) and C0-E10 ($p = 0.067$) showed no significant differences. All the other comparisons were statistically significant ($p < 0.05$). Figure 1 shows the OD values with the cell viability% of the groups and the comparisons between them. Figure 2 shows the HPF of the groups under the inverted microscope (100× magnification; Labomed Inc., Los Angeles, United States of America). The order of cytocompatibility was C0 = E10 < E20. Hence, it can be inferred that the copolymer P(B-Co-CMA) without TEGDMA diluent is more cytocompatible than the P(BD-Co-CMA) with TEGDMA diluent. The control group C0 P(BD) without PD-CMA showed the least cytocompatibility. Therefore, the incorporation of PD-CMA in the RCR matrix decreases the cytotoxicity *in vitro*.

Discussion

The incorporation of crosslinking comonomers that can copolymerize with the conventional matrix-forming monomers is a proven method to enhance the monomer-to-polymer conversion. However, this conversion is never complete in the polymer chemistry and results in unreacted monomer residuum release, which jeopardizes the cytocompatibility. Therefore, the cytocompatibility of the new copolymers P(BD-Co-CMA) and P(B-Co-CMA) containing PD-CMA crosslinker was assessed by the eluate's effect on the HPF. It is substantially believed that highly crosslinked copolymers are not susceptible to degradative processes due to the restricted space availability in the polymeric network for solvent percolation within the network.[31] Therefore, in the present research, PD-CMA crosslinker was used to prepare new RCR copolymers.

In the present research, the matrix-forming comonomers' composition was modified by either substituting the diluent TEGDMA with 10 wt% PD-CMA [E10: P(BD-Co-CMA)] or replacing TEGDMA with PD-CMA [E20: P(B-Co-CMA)]. The experimental group E20 was more cytocompatible than C0 and E10. Therefore, the null hypothesis was not rejected. This is attributable to the absence of TEGDMA in E20. Though the OD value of E10 is slightly higher than the C0, the difference between them was insignificant, which is attributable to the presence of 10 wt% TEGDMA. Therefore, the results of the present research suggest that TEGDMA was the chief culprit inducing cytotoxicity to HPF. Nonetheless, the high cytocompatibility of the E20 P(B-Co-CMA) copolymer can also be attributed to its high monomer-to-polymer conversion, thereby reducing the unreacted monomeric residuum release.[27]

The release of TEGDMA residuum in elution media was significantly higher (0.04–2.3%) than Bis-GMA (0.03–0.07%).[1,32,33] TEGDMA is more vulnerable to enzymatic hydrolytic degradation than Bis-GMA.[34,35] Enzymatic hydrolysis of dimethacrylates by cholesterol esterase and pseudocholinesterase yields various metabolites, chiefly formaldehyde (oxidative byproduct) and methacrylic acid. A dialcohol 2,2-bis[4-(2,3-hydroxypropoxy)phenyl]propane,[34,36–40] 2,3-epoxymethacrylic acid (2,3-EMA), and ethoxylated bisphenol A[41,42] are formed out of hydrolyzed or esterified Bis-GMA. The biodegradation metabolites from TEGDMA, like triethylene glycol methacrylate, 2,3-EMA, triethylene glycol, and methacrylic acid, cause minimal to significant cytotoxicity.[43] Kinetic research

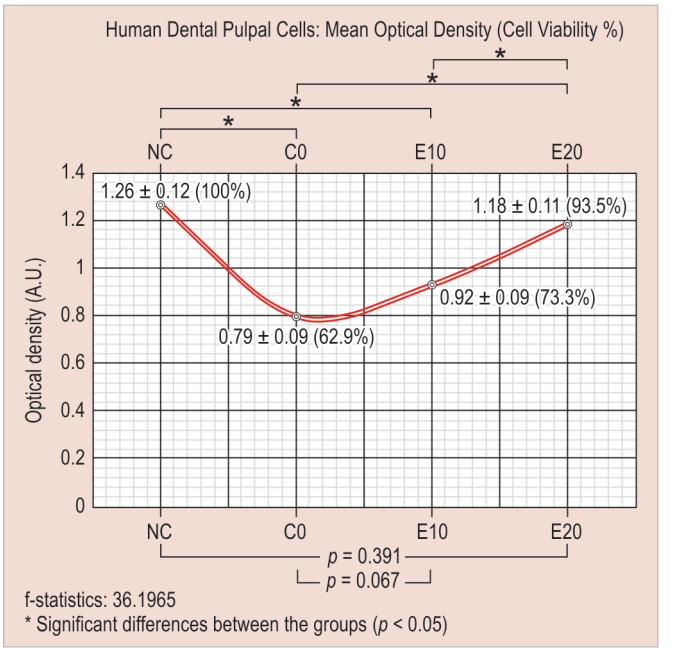
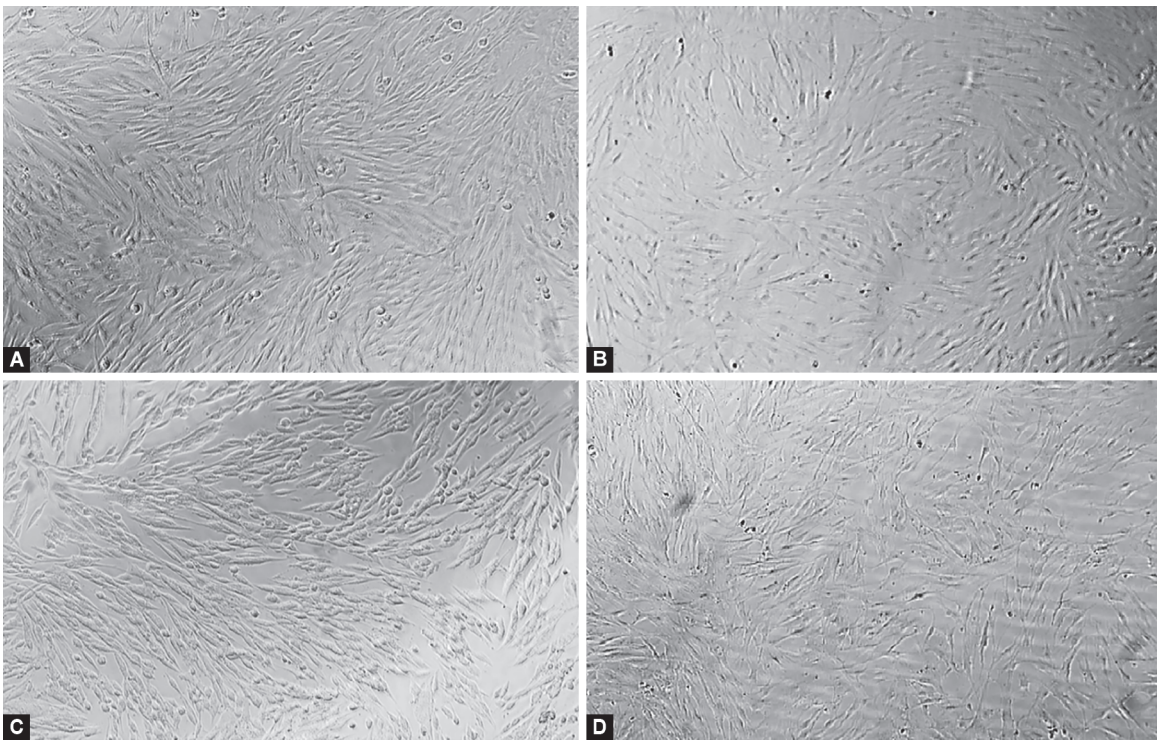


Fig. 1: Optical density (OD) of the groups and their comparisons



Figs 2A to D: Human dental pulpal primary fibroblasts under the inverted microscope (100× magnification); (A) NC; (B) C0; (C) E10; (D) E20

demonstrated that pseudocholinesterase favored hydrolysis of TEGDMA, while the esterification of Bis-GMA by cholesterol esterase was 14 times higher than the pseudocholinesterase.[39] Molecular TEGDMA possesses a profound mechanism of cytotoxicity. Lipophilic TEGDMA can effortlessly penetrate the cell membrane lipid bilayer and reach the cytosol.[44] TEGDMA induces concentration-dependent (0.5–5 mM) cytotoxicity in myriad cell lines.[45] TEGDMA at reduced concentration resulted in cellular apoptosis by its impeding effect on phosphatidylinositol-3-kinase in HPF.[46] Nevertheless, necrosis resulted from high concentrations.[47–50] TEGDMA causes reactive oxygen species release accompanied by 85 ± 15% intracellular glutathione exhaustion by lipid peroxidation and mitochondrial damage.[51,52] TEGDMA also causes significant genotoxicity or DNA damage at subtoxic concentrations by slowing the DNA repair mechanisms at the G2 phase of the cell cycle.[53] Therefore, all the abovementioned cytotoxic and genotoxic effects of TEGDMA had been either minimized in the E10 group [P(BD-Co-CMA)] where the TEGDMA was partly substituted with 10 wt% PD-CMA or eschewed in E20 group [P(B-Co-CMA)] where the TEGDMA is totally replaced by PD-CMA. Hence, E20 copolymer P(B-Co-CMA) without TEGDMA possessed the highest cytocompatibility, and the C0 P(BD) copolymer exhibited the lowest cytocompatibility. E10 copolymer P(BD-Co-CMA) showed cytocompatibility equivalent to C0 with the HPF.

In 22% of indirect pulp capping cases, composite resins cause odontoblastic morphological aberrations with inflammation, while adhesives and composite resins for direct pulp capping cause moderate-to-strong inflammatory reactions with abscess formation.[54] TEGDMA percolates the dentin and reaches the pulp at a concentration as high as 4 mmol/L[11] and affects pulpal homeostasis and healing.[10,11,22] The percolation raises with thin prevailing dentin, conspicuously <1 mm or after acid-etching.[55] The toxic concentration 50 of TEGDMA for HPF was found to be 2.6 ± 1.1 mM.[56] TEGDMA cannot induce α-tumor necrosis factor secretion from the human monocytic cell line by itself; however, it extinguishes lipopolysaccharide-induced α-tumor necrosis factor release and thereby alters the normal pulpal inflammatory response.[57] TEGDMA not only jeopardizes the normal differentiation of pulpal fibroblasts into odontoblasts but also affects their mineralization capacity at meager concentrations.[58] Hence, in the current research, HPFs were preferred and utilized for assessing the cytocompatibility of the new RCR copolymers over conventional murine fibroblasts.

In the regular clinical scenario, RCRs are dispensed into the freshly cut cavity in an uncured stage, which intends to trigger local tissue responses. The surface of the RCR should be subsequently finished and polished to remove the oxygen-inhibited surface, thereby curbing the leaching of cytotoxic components.[59] The RCR specimens with the oxygen-inhibited layer demonstrated greater cytotoxicity than the polished specimens.[60] Since the eradication of the oxygen-inhibited surface by polishing improved the cytocompatibility, the outcome of the evaluated cytotoxic potential becomes questionable. Hence, in the present research, the specimens were not polished to assess the exact apparent cytocompatibility. Since the results of this research were obtained in a controlled *in vitro* environment, *in vivo* studies are mandatory to ascertain the biocompatibility of the new RCR copolymers. Nevertheless, this is the only research assessing the cytocompatibility of the RCR containing PD-CMA. Therefore, the results of this research should be interpreted with caution and should be tested rigorously with other cell lines for the authenticity of the material.

Conclusion

Within the research constraints, it is concluded that the new RCR copolymer P(B-Co-CMA) containing PD-CMA photo-crosslinker without TEGDMA was the most cytocompatible copolymer with

the human dental pulpal primary fibroblasts. The cytocompatibility of the copolymer P(BD-Co-CMA) was equal to that of the P(BD).

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