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In vitro embryotoxicity assessment with dental restorative materials

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Summary *Objectives*. Resin (co)monomers may be released from restorative dental materials and can diffuse into the tooth pulp or the gingiva, and can reach the saliva and the circulating blood. Genotoxic potential of some dental composite components has been clearly documented. The genotoxic effects of xenobiotics can represent a possible step in tumor initiation and/or embryotoxicity/teratogenesis. A modified fluorescent mouse embryonic stem cell test (R.E.Tox®) was used to test the embryotoxic potential of following dental restorative materials: Bisphenol A glycidylmethacrylate (BisGMA), urethanedimethacrylate (UDMA), hydroxyethylmethacrylate (HEMA), and triethyleneglycoldimethacrylate (TEGDMA), as well as some of their metabolic intermediates 2,3-epoxy-2-methyl-propionicacid-methylester (EMPME), methacrylic acid (MA), and 2,3-epoxy-2-methylpropionic acid (EMPA).

Methods. Mouse embryonic stem (ES) cells stably transfected with a vector containing the gene for the green fluorescent protein under control of the cardiac α-myosin heavy chain promoter were differentiated in the presence of various concentrations of the test compounds for 12 days. Fluorescence was measured using the TECAN Safire® and values were expressed as percent of control values. To distinguish between cytotoxic and embryotoxic effects, all compounds were tested in a standard MTT assay.

Results. HEMA, TEGDMA and EMPME did not influence the differentiation process of ES cells towards cardiac myocytes. No cytotoxic effects were observed at any of the concentration levels tested. Exposure to BisGMA resulted in a 50% decrease in cell

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survival and a very strong inhibition of cell differentiation at 10^{-5} M ($p<0.01$). Embryotoxic effects were also present at 10^{-6} and 10^{-7} M ($p<0.05$). EMPA induced a decrease in ES cell differentiation at 10^{-5} M ($p<0.01$) without cytotoxic effects. No embryotoxic effects were induced at lower concentrations. Exposure to UDMA resulted in a slight decrease of cell differentiation at 10^{-5} M ($p<0.05$). Exposure of cells to MA resulted in an increase of cardiac differentiation up to 150% ($p<0.05$) at 10^{-5} M without cytotoxic effects.

Conclusions. BisGMA induced a significant high embryotoxic/teratogenic effect over a large range of concentration. Therefore attention should be focused on this dental monomer, which should be investigated further by in vivo experiments.

Introduction

In permanent teeth, dental resin composites are the most commonly used tooth-colored filling materials. Dental resin composites consist of the organic resin matrix and inorganic compounds. The resin matrix includes one or more so-called 'heavy' monomer systems (e.g. bisphenol A glycidylmethacrylate, BisGMA; urethanedimethacrylate, UDMA) and 'light' co-monomer systems (e.g. triethyleneglycoldimethacrylate, TEGDMA; hydroxyethylmethacrylate, HEMA) to reduce viscosity of monomers and to enhance bond strength to dentine. Initiators are used for free radical polymerization and stabilizers for maximizing storage stability. The inorganic filler consists of particles such as glass, quartz, and/or fused silica. A coupling agent, usually an organo-silane, chemically binds the reinforcing filler to the resin matrix.

TEGDMA is a component of most dental composite filling materials, luting agents and bonding resins. The TEGDMA content varies from 25 to 50% and HEMA is also used in some bonding resins in amounts from 30 to 55%.[2] It has been demonstrated directly that unconverted (co)monomers can be released from resin composites into an adjacent aqueous phase[1] and therefore can enter the body in humans.

Numerous cytotoxic responses to dental composite resins and their components have been described.[3-10] For example, TEGDMA induced large deletions in the hprt gene of V79 cells.[6] HEMA and TEGDMA decreased the glucose formation from pyruvate in rat kidney cells (metabolic effects).[7] In previous studies the uptake, distribution and excretion of radiolabelled 14C-TEGDMA and 14C-HEMA were examined in vivo in guinea pigs. Low fecal levels (about 2% of the dose) and urinary levels of about 15% after 24 h were noted with both compounds.[11,12] Direct measurement of exhaled CO_2 showed that about 70% of the administered 14C-TEGDMA or 14C-HEMA dose left the body via the lungs.[13-15] The existence of two pathways has been postulated for the metabolisms of TEGDMA and HEMA.[14,15] It is likely that toxic, probably genotoxic/cancerogenic intermediates are formed from TEGDMA and from HEMA in vivo.[14,15] Indeed, in further studies a genotoxic/cancerogenic potential for TEGDMA, HEMA and BisGMA in human lymphocytes could be found.[10,26] Genotoxic effects of xenobiotics are a possible step in tumor initiation and/or embryotoxicity/teratogenesis.

The mouse ES cell test (EST) is a useful method to test the embryo toxic potentials of xenobiotics in vitro.[16] The test is based on the ability of ES cells to differentiate in vitro into various tissues of all three germ layers, with cardiac tissue already described by Doetschman et al.[17] Exposure of ES cells to embryotoxic and teratogenic compounds during this process can interfere with correct differentiation and will alter the resulting amount of a given tissue, e.g. of cardiac tissue. The expression of a green-fluorescent-protein-reporter-gene (GFP) under control of a tissue specific promoter can be used to quantify the amount of a certain cell type or tissue by determining the amount of fluorescent cells. This was done using FACS analysis by Bremer et al.[18] to establish the fluorescent embryonic stem cell test. However, this method is not suitable for large numbers of compounds because it requires relatively laborious preparation of single cell suspensions and costly data handling. The method was therefore modified by 'Axiogenesis' as described in Section 2 to obtain a reliable and easy-to-follow protocol for quantification of fluorescent cells (R.E.Tox® assay). The assay was developed using 20 reference compounds recommended by ECVAM for the validation of in vitro assays for embryotoxic effects.[19] The results of the internal validation of the R.E.Tox assay corresponded to the classification of the validation compounds by ECVAM based on former in vitro and in vivo studies (Axiogenesis AG, data not shown).[19] Therefore, cardiac differentiation of mouse ES cells

using the R.E.Tox assay is considered to be an appropriate and sufficient model for testing embryotoxic effects of xenobiotics. The R.E.Tox assay is inexpensive, easy to handle and quick (test period within 12 days) and above all avoids the use of laboratory animals. Therefore, it is recommended that this test should be routinely used for novel dental materials (and others currently in use).

In these studies the possible capacity of dental restorative materials and some of their metabolic intermediates to induce embryotoxicity/teratogenesis was investigated using the R.E.Tox assay.

Materials and methods

Following compounds were purchased: hydroxyethylmethacrylate (HEMA), triethyleneglycoldimethacrylate (TEGDMA), urethanedimethacrylate (UDMA), and bisphenol A glycidylmethacrylate (BisGMA) from ESPE (Dental AG, Seefeld, Germany); 2,3-epoxy-2-methyl-propionicacid-methylester (EMPME) from Aldrich (Steinheim, Germany), methacrylic acid (MA) from Fluka (Neuchatel, Switzerland), and retinoic acid (RA) from Sigma (Steinheim, Germany). 2,3-Epoxy-2-methylpropionic acid (EMPA) was synthesized at the Walther-Straub-Institute, Munich, Germany, according to the method described by Yao and Richardson.[20] Test compounds were dissolved in DMSO (Sigma, Steinheim, Germany)(final concentration of DMSO: 0.1%). All chemicals and reagents were of the highest purity available.

Mouse ES cells (D3, ATCC CRL 1934) were stably transfected with the pαMHC-GFP vector containing the gene of the green fluorescent protein under control of the cardiac α-myosin heavy chain (α-MHC) promoter. To obtain this vector, a 5.4 kb fragment containing the promoter region of the mouse α-myosin heavy chain gene (GenBank U71441) was introduced into the polylinker of the pEGFP-1 vector (Clontech Laboratories, Heidelberg, Germany).

To initiate differentiation, ES cells were cultured in suspension on a rocking table (GFL 3006, GFL, Braunschweig, Germany) in Iscove's medium with 20% FCS (Invitrogen, Karlsruhe, Germany) at 37 °C, 5% CO_2, 95% humidity for 24 h to form aggregates (embryoid bodies, EB). Fifty EB were transferred to 10 cm bacterial Petri dishes (Greiner, Darmstadt, Germany) and test compounds were added at concentrations indicated in the figures (solvent: DMSO, final concentration of DMSO: 0.1%). Each compound concentration was tested in triplicates in three individual experiments. On day 5, half of the medium was replaced by fresh medium and fresh test compounds were added. On day 12, EB on each plate were counted, lysed in lysis buffer (20 mM Tris-HCl/0.5% TritonX 100) and fluorescence in the lysates was measured using a Tecan Safire® (Tecan, Crailsheim, Germany) at a wavelength of 476/508 nm. Because not all EB introduced into the test survive the procedure, the values were normalized to a supposed number of 100 EB. Normalized values were then expressed as percent of control values (0.1% DMSO only). Both, a significant decrease and a significant increase of values, compared to controls, represent an embryotoxic effect. To distinguish between cytotoxic and embryotoxic effects, all compounds were also tested in a standard MTT assay[21] using mouse ES cells and an incubation time with the compounds of 12 days like in the R.E.Tox assay. Compounds were judged as embryotoxic, if a significant alteration in differentiation was seen without cytotoxic effects at the same concentration, or if the cytotoxic effect was clearly less pronounced than the differentiation effect. The effect of the retinoic acid (RA) served as positive control and was included in every experiment.

To illustrate the inhibiting effect of RA treatment on the expression of GFP, EBs from one experiment (either differentiated in the presence of DMSO or 10^{-7} M RA) were photographed using a Zeiss Axiovert 200 M with a 10× Achroplan objective with a HQ-filterset for GFP (AF Analysentechnik, Tübingen, Germany) and a Sensicam 12bit cooled imaging system (PCO Imaging, Kelheim, Germany).

Calculations and statistics

The data are presented as means ± SEM. The statistical significance of the differences between the experimental groups was checked by the *t*-test, corrected according to Bonferroni-Holm.[22]

Results

All values from compound-treated EB are expressed as percentages of control values (mean ± SEM). To illustrate the outcome of the R.E.Tox assay, the differentiation of EB in the presence of the known embryotoxic RA is shown in the Fig. 1a, b and c. Incubation of EB with RA leads to dose-dependent inhibition of cardiac development (Fig. 1a). At 10^{-7} M, differentiation is diminished to 54.2 ± 2.1% of controls ($p < 0.01$),

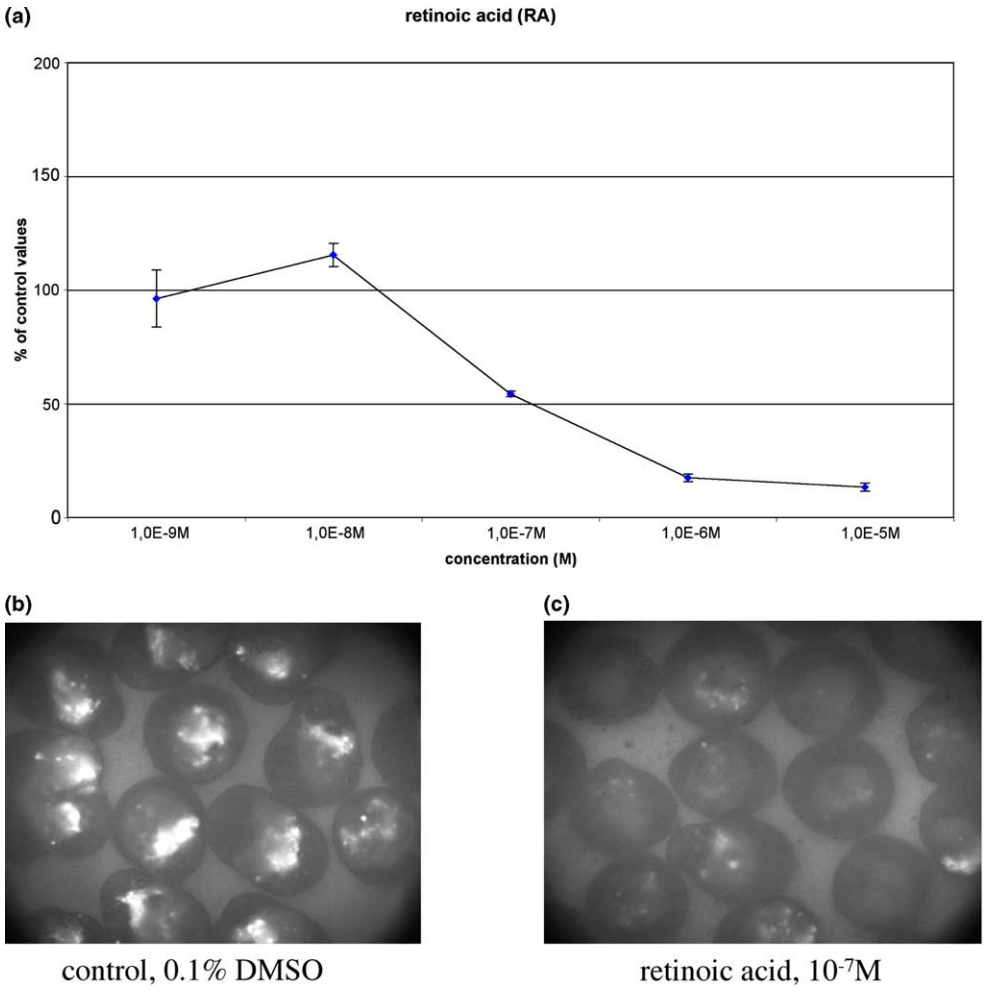

Figure 1 Mouse embryonic stem cells stably transfected with a vector containing the gene for the GFP under control of the cardiac α-myosin heavy chain promoter were aggregated into embryoid bodies (EB) and differentiated in the presence of various concentrations of retinoic acid for 12 days. Cells were lysed and fluorescence was measured as described. Values of treated EB were expressed as percent of control values (0.1% DMSO, Fig. 1a). Photomicrographs of control EB (Fig. 1b) and EB differentiated in the presence of 10^{-7} M retinoic acid (Fig. 1c) illustrate the inhibiting effect of RA treatment on the expression of GFP. Total magnification: 20×.

resulting in decreased fluorescence intensity of given EB (Fig. 1b and c).

In the present experiments, mean fluorescence intensity of all controls was 3433.4±187.2 (random units, $n=59$).

Exposure of mouse ES cells to HEMA, TEGDMA and EMPME (10^{-8}-10^{-5} M final concentration) did not result in cytotoxic effects and did not influence the differentiation process towards cardiomyocytes significantly (HEMA 110.4±7.7%; TEGDMA 82.8±11.6%; EMPME 92.9±11.5%; all at 10^{-5} M).

Exposure of cells to BisGMA resulted in a very strong inhibition of differentiation at 10^{-5} M (16.3±3.5% of controls, $p<0.01$), whereas in the MTT assay 54.7±0.9% of the ES cells survived the exposure. At lower concentrations, no cytotoxic effect was observed, but embryotoxic effects were also present at 10^{-6} and 10^{-7} M ($p<0.05$; Fig. 2a).

Exposure of cells to UDMA resulted in a slight decrease of differentiation capacity at 10^{-5} M, but with a high variation (64.9±14.0%, $p<0.05$). No cytotoxic effects were observed with UDMA at this concentration (Fig. 2b).

Exposure of cells to EMPA resulted in a decrease of ES cell differentiation at 10^{-5} M (47.5±5.7%, $p<0.01$), but no cytotoxic effect was observed at this concentration. No embryotoxic effect was induced at lower concentrations (Fig. 2c).

Exposure of cells to MA resulted in an increase of cardiac differentiation up to 152.2±22.8% ($p<0.05$) at 10^{-5} M. A high variance of these values was found at all concentration levels. No cytotoxic effect was observed in cells after exposure to MA with the MTT assay (Fig. 2d).

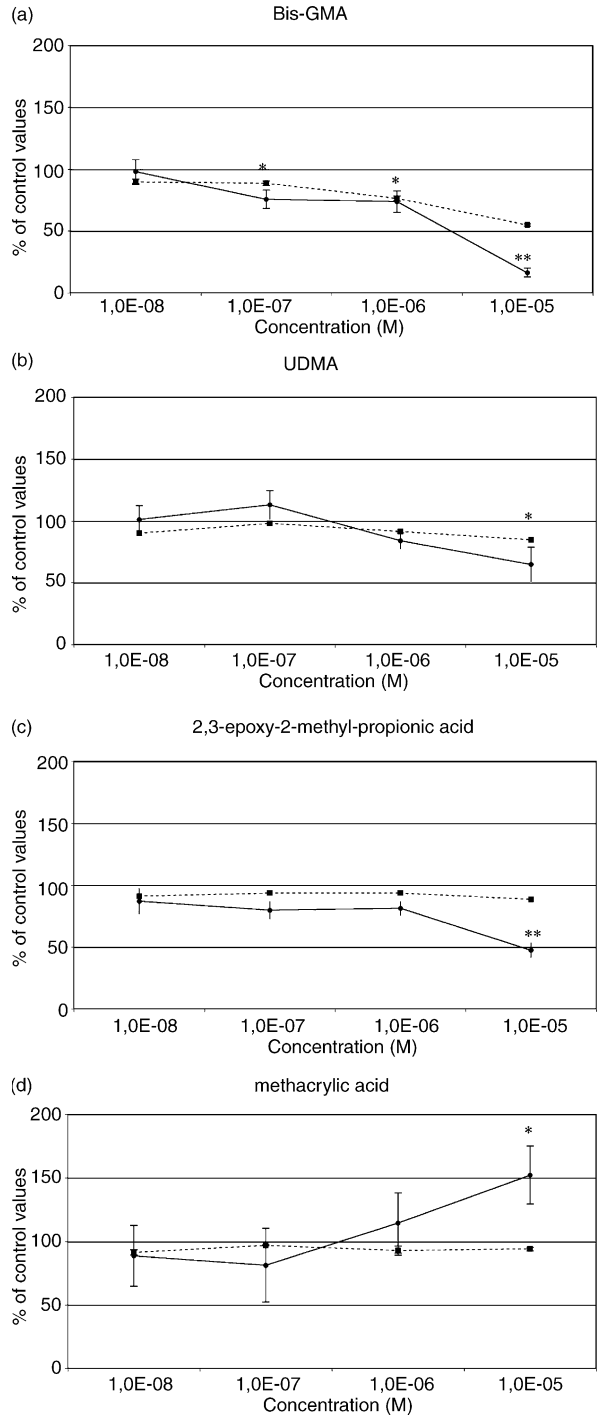

Figure 2 Mouse embryonic stem cells were cultured in suspension to start differentiation as described. Embryoid bodies were differentiated in the presence of the test compounds BisGMA (a), EMPA (b), UDMA (c), and MA (d) at concentrations indicated in the figures for 12 days. Fluorescence was measured in cell lysates using the TECAN Safire®. Values were expressed as percent of control values (0.1% DMSO only)(bold lines; mean ± SEM). To distinguish between cytotoxic and embryotoxic effects, all compounds were tested also in a standard MTT assay (dotted lines). *($p<0.05$) **($p<0.01$) significantly different to controls.

Discussion

Non-amalgam filling and bonding materials for dental cavities comprise a wide range of components, which may be released in their surrounding phases and may show biologic activity in the organism. Numerous effects (e.g. cytotoxic, cancerogenic, mutagenic, genotoxic) of tooth colored dental restorative materials have been described.[4,6,8–10] However, little data exist on teratogenic and embryotoxic effects of these compounds. Genotoxic effects of xenobiotics can represent a possible step in tumor initiation and/or embryotoxicity/teratogenesis, although conceivable tumorigenic and/or teratogenic effects may be detected only in vivo and after long time intervals.

There are a few major comonomers of filling materials, e.g. TEGDMA and HEMA, and monomers, such as BisGMA and UDMA, which are presented in many dental restorative materials. In previous studies two pathways for the postulated metabolisms of TEGDMA and HEMA were described.[14,15] It is very likely that derivatives of methacrylates, as epoxy-compounds, as EMPA and EMPME are formed from TEGDMA and HEMA in vivo.[14,15] Generally most epoxy-compounds are regarded as toxic (predominantly genotoxic/mutagenic/cancerogenic) agents.[23] Meanwhile the formation of these epoxy-compounds was found after exposure of human liver microsomes to TEGDMA.[24] Therefore, these epoxy-compounds were additionally investigated in these studies.

Within the non-amalgam materials and some of their metabolic intermediates tested in the R.E.Tox assay, BisGMA showed a strong embryotoxic effect over a large range of concentration, whereas UDMA, MA and EMPME showed significant ($p<0.05$) embryotoxic effects only at 10^{-5} M, the highest concentration tested in the assay. BisGMA showed a pronounced cytotoxic effect, nevertheless, the effect observed at 10^{-5} M is not as strong as the embryotoxic effect observed at the same concentration. This suggests that apart from the overall cytotoxic effect a specific embryotoxic effect is seen with this compound in the R.E.Tox assay. No cytotoxic effects were observed with the other compounds at 10^{-5} M, the highest concentration used in the R.E.Tox assay. However, at 10^{-4} M a 50% cytotoxic effect was also seen with UDMA and with EMPA.

The strong embryotoxic effect of BisGMA over a large range of concentration could be due to the molecule structure and/or to the higher lipophilic character of BisGMA, compared to the other compounds.[25] These data indicate that BisGMA

and/or their metabolic intermediates are able to cross cell membranes possibly more effective than the other tested dental molecules and thus penetrate into the cell organelles (e.g. nucleus) and can even lead to DNA single strand breaks as has already been described for BisGMA in human lymphocytes.[26] Furthermore BisGMA represented also a higher genotoxic potential, compared to UDMA, TEGDMA and HEMA in human lymphocytes.[26]

Exposure of ES cells to HEMA, TEGDMA and EMPME in a concentration range from 10^{-8} to 10^{-5} M (final concentration) did not result in cytotoxic effects and did not influence the differentiation process towards cardiomyocytes. In previous experiments the biological clearance and the organ burden of TEGDMA, HEMA and BisGMA in guinea pigs were investigated. In these experiments the animals received radiolabelled ^{14}C-TEGDMA, ^{14}C-HEMA or 14C-BisGMA. The dose level was chosen in order to exceed the dose levels expected in humans for (co)monomers from a single composite resin restoration.[11,14,15] Organ burden found in these studies was only in the femtomolar range.[11,14,15] These data indicate that at least for TEGDMA, HEMA and BisGMA the existence of enhancement of embryotoxic effects is even more unlikely for the in vivo situation. However, data on BisGMA, UDMA and MA vary and in some studies it has been shown for BisGMA to be genotoxic and cancerogenic,[26-28] as it has shown also the highest embryotoxic effect in the present study.

During the differentiation process of mouse ES cells, not only the reduction of the amount of a certain tissue, but also the enhanced differentiation towards this tissue represents a disturbed overall development and is thus indicative of a compound's embryotoxic action. An example of a compound with well-established teratogenic effects is 'Thalidomide' which augments formation of cardiomyocytes in the R.E.Tox assay (Axiogenesis AG, data not shown). Therefore, the increase of fluorescence seen with the highest concentration of MA (10^{-5} M) represents also a significant embryotoxic effect. Additionally, high variances of these values could be observed, which are also an indicator for effects of the given compound on differentiation processes. 'Thalidomide' is an immunomodulatory agent with a spectrum of activity that is not fully characterized. 'Thalido-Thalidomide' is a useful drug for patients with erythema nodosum leprosum (therapeutical dosage: Thalidomide 50 mg/70 kg b.w.; $c_{max.(serum)}$: 2.4×10^{-6} M). Since 1998 'Thalidomide' is approved for marketing only under a special restricted distribution program approved by the food and drug -administration. Thalidomide is contraindicated in women of childbearing potential. This therapeutical thalidomide c_{max}-concentration is in that range which was found for, e.g. BisGMA that induced embryotoxic effects.

The R.E.Tox assay cannot completely represent the in vivo situation, because the metabolic activity of ES cells during the differentiation process is limited, and the maternal metabolism is obviously absent in this assay. However, positive results for compounds found in the R.E.Tox assay give evidence for the existence of an embryotoxic/teratogenic potential of these compounds. The significant effects of BisGMA, UDMA and the TEGDMA/HEMA-metabolic-intermediates MA and EMPA observed in the R.E.Tox assay show that these non-amalgam dental restorative materials and some of their metabolic intermediates have embryotoxic/teratogenic potentials, which have to be elucidated by further in vivo experiments (e.g. 'Prenatal Developmental Toxicity Study'; OECD guide line 414).[29]

Conclusion

BisGMA induced a significant high embryotoxic effect over a large range of concentration. Therefore, higher attention should be focused on this dental monomer, which should be chosen for further investigations.

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