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Poly(propylene glycol) and urethane dimethacrylates improve conversion of dental composites and reveal complexity of cytocompatibility testing

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ABSTRACT

Objectives. To determine the effects of various monomers on conversion and cytocompatibility of dental composites and to improve these properties without detrimentally affecting mechanical properties, depth of cure and shrinkage.

Methods. Composites containing urethane dimethacrylate (UDMA) or bisphenol A glycidyl methacrylate (Bis-GMA) with poly(propylene glycol) dimethacrylate (PPGDMA) or triethylene glycol dimethacrylate (TEGDMA) were characterized using the following techniques: conversion (FTIR at 1 and 4 mm depths), depth of cure (BS EN ISO 4049:2009 and FTIR), shrinkage (BS EN ISO 17304:2013 and FTIR), strength and modulus (biaxial flexural test) and water sorption. Cytocompatibility of composites and their liquid phase components was assessed using three assays (resazurin, WST-8 and MTS).

Results. UDMA significantly improved conversion, BFS and depth of cure compared to Bis-GMA, without increasing shrinkage. UDMA was cytotoxic at lower concentrations than Bis-GMA, but extracts of Bis-GMA-containing composites were less cytocompatible than of those containing UDMA. PPGDMA improved conversion and depth of cure compared to TEGDMA, without detrimentally affecting shrinkage. TEGDMA was shown by all assays to be highly toxic. Resazurin, but not WST-8 and MTS, suggested that PPGDMA exhibited improved cytocompatibility compared to TEGDMA.

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Significance. The use of UDMA and PPGDMA results in composites with excellent conversion, depth of cure and mechanical properties, without increasing shrinkage. Composites containing UDMA appear to be slightly more cytocompatible than those containing Bis-GMA. These monomers may therefore improve the material properties of dental restorations, particularly bulk fill materials. The effect of diluent monomer on cytocompatibility requires further investigation.

1. Introduction

Dental composites are widely used as dental restorative materials for their high strength and excellent aesthetics. These consist primarily of: a liquid phase containing monomers and an initiator system (typically photo-activated); a filler phase (typically boro-aluminosilicate glass particles), which provides optimal mechanical and aesthetic properties; and silane, a coupling agent which enables bonding of the polymer to the filler. Bisphenol A glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA) and triethylene glycol dimethacrylate (TEGDMA) are some of the most commonly used monomers in dental composites. Upon photoinitiation, they form a cross-linked polymer network which hardens and entraps the fillers [1].

Composites are typically layered in increments and this is time-consuming for clinicians, since it requires curing each increment before proceeding with the next. This is particularly an issue in deeper posterior cavities, in which many increments may be required. As a result, *bulk fill* materials aim to overcome this issue by utilizing photoinitiators which are effective at depths of 4–5 mm, as well as monomers with low double bond concentration and, in some cases, monomers which are cleaved during polymerization.

One of the major limiting factors of composites is the close interplay between degree of conversion and other characteristics, including mechanical properties, polymerization shrinkage, water sorption and elution of toxic components. Since the level of residual monomer in a composite affects its biocompatibility, mechanical properties and aesthetics [2], high conversion is ideal for optimization of these properties. Conversely, however, high conversion is typically associated with high volumetric shrinkage. In the patient, this can result in microbial microleakage (penetration of pathogens between the composite and tooth), recurrent caries and, ultimately, failure of the restoration. Monomers with low double bond concentration and subsequent low shrinkage are therefore optimal, particularly in the case of bulk fill materials, due to the larger volume of each increment.

The aim of the present research was to improve the conversion, strength and cytocompatibility of dental composites without detrimentally affecting polymerization shrinkage or depth of cure by fully replacing Bis-GMA with UDMA and TEGDMA with PPGDMA. This is due to the greater flexibility and cross-linking density of UDMA than Bis-GMA [3,4] and the greater flexibility and significantly lower double bond concentration of PPGDMA than TEGDMA. In order to investigate the effect of each monomer on cytocompatibility, human gingival fibroblasts (HGF) were cultured in solutions of each individual liquid phase component at varying concentrations, as well as in extracts of each composite formulation. Due to the variability between different cell viability assays, which arises from the targeting of different enzymes within the cell, and the ambiguity of the widely used term 'biocompatibility' [5], three assays were compared. The null hypothesis was that replacement of Bis-GMA with UDMA and TEGDMA with PPGDMA would have no effect on these properties.

2. Materials and methods

2.1. Materials

2.1.1. Composite paste preparation

Microhybrid dental composites [1] were prepared using 10 wt% 40 nm fumed silica (Aerosil OX-50, Evonik Industries AG, Essen, Germany) and 90 wt% silane-treated barium boro-aluminosilicate glass particles of various sizes (DMG Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany). These were combined with four dimethacrylate-based liquid phases. The liquid phases consisted of bulk monomer UDMA (DMG) or Bis-GMA (Polysciences Inc., Eppelheim, Germany) combined with diluent monomer PPGDMA (Polysciences) or TEGDMA (DMG). The bulk to diluent molar ratio was 3.5:1. The liquid phases also contained 40 mM (0.58–0.61 wt%) photoinitiator camphorquinone (CQ, DMG), 60 mM (0.82–0.86 wt%) co-initiator *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA, Sigma–Aldrich, Gillingham, UK) and 100 ppm inhibitor butylated hydroxytoluene (Sigma–Aldrich). The two phases were combined to form composite pastes using a centrifugal planetary mixer (SpeedMixer, Hauschild Engineering, Hamm, Germany), in order to minimize air incorporation and ensure complete wetting of filler particles. The powder to liquid ratio (PLR) was kept constant at 40 vol% liquid (19.3–20.3 wt%, depending on liquid phase density). Dental composites were designated abbreviations based on their bulk and diluent monomer content: UP, UT, BP and BT, where U, B, P and T represent UDMA, Bis-GMA, PPGDMA and TEGDMA, respectively. Commercial composite Filtek Z250 (3M ESPE, St. Paul, MN, USA) was used for comparison.

2.1.2. Disc specimen production

Except where otherwise stated, disc-shaped specimens were moulded by applying composite pastes to metal circlips (internal diameter 10.2 mm, thickness 1 mm) and pressing them between two sheets of acetate. This prevents oxygen inhibition during polymerization and expels excess paste,

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ensuring similar specimen thickness. Specimens were photo-polymerized using a blue light emitting diode curing unit with a wavelength of 450–470 nm and power output with periodic level shifting of 1100–1330 mW/cm^2 (Demi Plus, Kerr Dental, Orange, CA, USA), in direct contact with the acetate. The curing duration for each testing method varied and is detailed in each corresponding section.

2.2. Methods

2.2.1. Handling properties and wet-point determination

The wet-point of each liquid was determined by gradually adding a small quantity of liquid phase to a known mass of filler phase and mixing, until the filler was sufficiently wetted and a cohesive paste had been formed. The quantity of liquid phase added was recorded and notes were made regarding the handling properties of each formulation. The wet-point (vol%) was then calculated from the total mass of liquid and the density of each component.

2.2.2. Polymerization properties

2.2.2.1. Degree of conversion. The conversion of each composite was determined using Fourier transform infrared spectroscopy (FTIR, System 2000, PerkinElmer, Seer Green, UK). Composite paste was applied to either a single or four stacked circlips of the same dimensions. These were placed on the diamond of an attenuated total reflectance accessory (Golden Gate ATR, Specac Ltd., Orpington, UK) and covered with a sheet of acetate. After an initial spectrum of the uncured composite had been obtained, spectra were recorded continuously for 1000 s ($n = 3$). The specimens were photo-polymerized from the top for the first 20 s. Spectra were recorded at a wavelength range of 800–1800 cm^{-1} and resolution of 8 cm^{-1}. Absorbance profiles were obtained at 1319 ± 1 cm^{-1} (C–O stretch bond) and 1334 ± 2 cm^{-1} (baseline) and used to calculate conversion at 1 mm and 4 mm depths using Eq. (1):

$$C = \left[1 - \left(\frac{A_f}{A_o}\right)\right] \times 100 \quad (1)$$

where C is conversion. A_f and A_0 are final and initial absorbance above baseline, respectively [6].

2.2.2.2. Polymerization shrinkage.

2.2.2.2.1. Shrinkage based on conversion. The volumetric shrinkage of composites was calculated from the conversion data using Eq. (2) [7], which is based on the finding that methacrylate esters typically undergo volumetric shrinkage of s = 22.5 cm^3/mol upon polymerization [8,9]:

$$S = 100 \times \left(smC\rho \sum_i \left(\frac{n_i x_i}{W_i}\right)\right) \quad (2)$$

where S, m, C, ρ, Σ, i, n, x and W are shrinkage (vol%), monomer mass fraction within composite, conversion, composite density, sum of all monomers in liquid phase, each monomer in liquid phase, number of C=C bonds per molecule, mass fraction of monomer in liquid phase and molecular mass, respectively.

2.2.2.2.2. Shrinkage based on volume change. The shrinkage of composites was also obtained by measuring the density of polymerized and unpolymerized specimens according to BS EN ISO 17304:2013 [10]. This technique uses an analytical balance equipped with a density determination apparatus (AG 204 & MS-DNY-43, Mettler Toledo, Beaumont Leys, UK) and is based on Archimedes' principle. Disc specimens were cured for 40 s from each side to ensure complete conversion. Specimen edges were polished to remove loose chips. The mass of three cured and three uncured specimens of each formulation were measured in air and under deionized water. Each value for mass under water was averaged from 10 readings. The shrinkage of composites and their SD were calculated using the equations provided in the standard.

2.2.2.3. Depth of cure. The depth of cure of composites ($n = 3$) was measured according to BS EN ISO 4049:2009 [11]. Briefly, composite paste was applied to a brass split-mould (internal diameter 4 mm, height 6 mm) and photo-polymerized for 20 s from the top. The specimen was removed from the mould and a plastic spatula was used to remove any uncured material from the bottom. The depth of cured material was measured using digital callipers to an accuracy of ±0.01 mm and the reading was halved, as required by the standard, in order to give a value for depth of cure.

2.2.2.4. Water sorption. In order to determine water sorption, disc specimens were cured for 40 s from each side to ensure maximum conversion. The mass of composites before and after immersion in 10 mL deionized water for one week was then determined using an analytical balance.

2.2.3. Mechanical properties

Composite disc specimens ($n = 10$) were cured for 40 s from each side. Specimens were stored dry for 24 h, before placement in 10 mL deionized water at 37 °C for one week. They were then tested using a biaxial flexural test (Autograph AGS-X, Shimadzu, Milton Keynes, UK), with a 2 kN load cell and ball-on-ring jig at a cross-head speed of 1 mm/min, until specimen failure. Biaxial flexural strength (BFS, MPa) and modulus of tensile elasticity (E, GPa) were calculated using Eqs. (3) and (4), respectively,

$$BFS = \frac{P}{t^2}\left[(1+\upsilon)\left(0.485 \ln\left(\frac{a}{t}\right) + 0.52\right) + 0.48\right] \quad (3)$$

$$E = \left(\frac{\Delta P}{\Delta w_c}\right) \times \left(\frac{\beta_c a^2}{t^3}\right) \quad (4)$$

where BFS, P, t, υ and a are biaxial flexural strength, failure load (N), specimen thickness (mm), Poisson's ratio (0.3) and jig support radius (4 mm), respectively, and E, ($\Delta P/\Delta W_c$) and β_c are modulus, gradient of elastic region and center deflection function (0.5024) [12], respectively.

2.2.4. Cytocompatibility

2.2.4.1. Cell culture. Primary HGF were obtained from a commercial source (ScienCell Research Laboratories, Carlsbad, CA, USA). HGF were cultured under standard conditions (37 °C, 95% air, 5% CO_2, 95% relative humidity) in Dulbecco's

modified Eagle medium (DMEM, Gibco, Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin/streptomycin (PAA Laboratories, GE Healthcare, Chalfont St. Giles, UK). Passage numbers 4–8 were used for cytocompatibility studies.

2.2.4.2. *Preparation of test solutions*. Test solutions were prepared in serum-free DMEM. As is typical in biomaterials testing, serum-free medium was used in order to prevent adsorption of serum proteins to material components. Controls consisted of serum-free DMEM.

2.2.4.2.1. *Liquid phase components*. Five ten-fold serial dilutions of each component were prepared in serum-free DMEM, ranging from 0.01 to 100 mM for DMAEMA, UDMA, PPGDMA and TEGDMA, and 0.001 to 10 mM for CQ and Bis-GMA, due to their lower solubility. The solutions were stored for ~30 min at 60 °C and then stirred using a sterile spatula, in order to aid dissolution of components with low solubility, particularly the bulk monomers.

2.2.4.2.2. *Composite extracts*. In order to prepare specimens for extract testing, a 1 mm thick circlip atop a sheet of acetate was filled with composite and covered with acetate. A further three circlips were stacked on top and filled with composite and covered with acetate. The resultant 4 mm deep stack was then photo-polymerized for 20 s from the top. The bottom 1 mm thick section was removed from the mould and incubated in 650 μL serum-free DMEM at 37 °C. This provided an extraction ratio of 1 mL/3 cm^2 surface area, as required by ISO 10993-12:2009 [13]. Specimens ($n = 3$) were agitated at 100 rpm during extraction (orbital shaker, Stuart Scientific, Stone, UK). After 24 h, specimens were transferred to fresh medium and incubated for a further 6 days, yielding extracts from 1 and 7-day time-points.

2.2.4.3. *Cytocompatibility assays*. HGF were seeded at a density of 30,000 cells/cm^2 in 96 well plates ($n = 3$ per specimen, time-point and assay). After an initial 24 h seeding period, cell culture medium was replaced with 100 μL of the specimen (component solution, composite extract or control). The solutions were thoroughly mixed using a vortex mixer (Stuart SA8, Bibby Scientific, Stone, UK) in order to ensure even dispersion of dissolved components. Cytocompatibility was assessed after a further 24 h of culture (48 h time-point), in accordance with ISO 10993-5:2012 [14]. The specimen was then replaced with serum-free DMEM and cultured for a further 24 h recovery period (72 h time-point). Three water-soluble cell metabolic activity assays were used to assess cytocompatibility: resazurin (alamarBlue Cell Proliferation Assay, AdB Serotech, Bio-Rad Laboratories Inc., Hemel Hempstead, UK); water-soluble tetrazolium salt-8 (WST-8, Cell Counting Kit-8, Sigma–Aldrich); and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS, CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay, Promega, Southampton, UK). Medium was aspirated from the cells and replaced with 100 μL cell culture medium containing the corresponding substrate (Table 1). After incubation at 37 °C, fluorescence (FLx800, BioTek, Potton, UK) or absorbance (Infinite M200, Tecan, Männedorf, Switzerland) was measured. Standard curves were obtained by seeding a range of cell concentrations in 96 well plates, 2 h prior to each assay.

Table 1 – Cytocompatibility assay parameters.

Substrate	Incubation time (min)	Measurement
Resazurin, 10%	90	*Fluorescence* – excitation: 560 nm, emission: 590 nm
WST-8, 10%	80	*Absorbance* – 460 nm (reference: 650 nm)
MTS, 20%	60	*Absorbance* – 490 nm (reference: 630 nm)

2.2.5. *Statistical analyses*

One-way analysis of variance (ANOVA) and post-hoc Tukey's tests were used to determine significance ($p \leq 0.05$) between composite formulations (UP, UT, BP, BT and Z250). Two-way ANOVA and post-hoc Tukey's tests were also used in order to determine the significance of the effects of multiple factors, e.g. UDMA vs. Bis-GMA and PPGDMA vs. TEGDMA. For all techniques, the standard deviation (SD) of each formulation was displayed on graphs, except in the case of polymerization shrinkage, where the SD was averaged for each technique, due to the high variability of the volume change method. Statistical significance is presented on graphs ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ or $^{****}p < 0.001$).

3. Results

3.1. *Handling properties and wet-point*

All experimental formulations had wet-points of 33.3 ± 0.4 vol% liquid. At this level, formulation pastes containing Bis-GMA felt considerably less malleable during specimen moulding than those produced with UDMA. At 40 vol% liquid, all formulations were slightly more malleable, but formulations containing Bis-GMA still exhibited lower malleability than those containing UDMA. Z250 had handling properties between those of Bis-GMA- and UDMA-containing composites. No discernible difference in handling properties was observed between PPGDMA and TEGDMA.

3.2. *Polymerization properties*

3.2.1. *Conversion*

Experimental composites containing UDMA had, on average, 1.2 and 1.3 times higher conversion at 1 and 4 mm depth, respectively, than those containing Bis-GMA (average p-value of both depths = 0.001) (Fig. 1a). Replacement of TEGDMA with PPGDMA further increased conversion by 1.1 and 1.2 times at 1 and 4 mm, respectively (average p-value of both depths < 0.01). UP had the highest conversion (68% and 65% at 1 and 4 mm). At both depths, UP's high conversion was highly statistically significant compared to BP, BT and Z250 ($p < 0.001$) and at 1 mm, it was significantly higher than that of UT ($p < 0.05$). Z250 had the lowest (50% and 39% at 1 and 4 mm) and was comparable to BT. Composites containing Bis-GMA (BP, BT and Z250), showed a significant reduction in conversion upon increasing depth from 1 to 4 mm.

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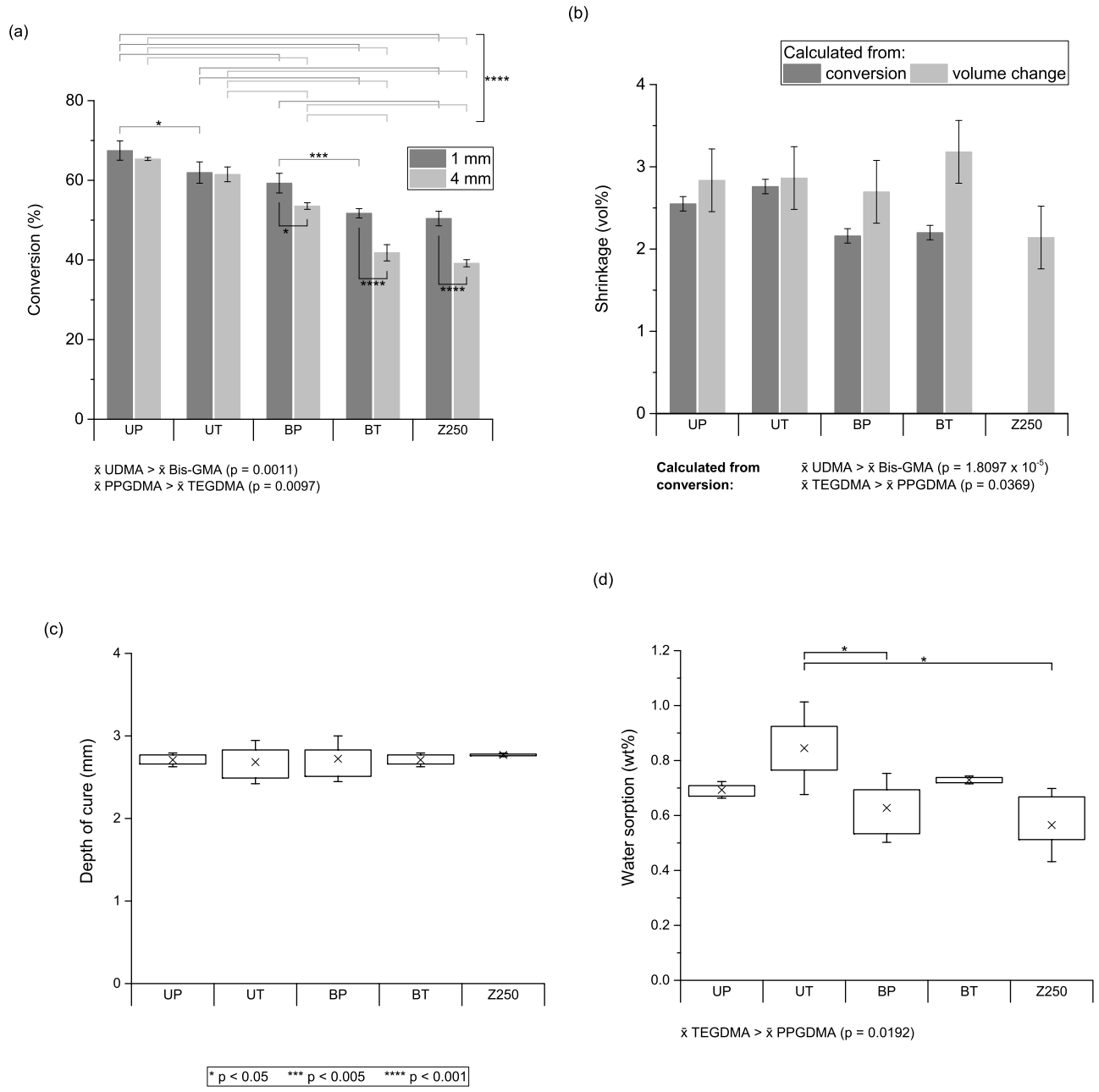

Fig. 1 – (a) Conversion of composites at 1 and 4 mm depth. Columns represent mean, error bars represent SD. (b) Shrinkage of 1 mm thick composite discs, calculated from conversion or volume change. Columns represent mean, error bars represent mean SD of the corresponding technique (±0.05 and ±0.23 vol% for conversion and volume change, respectively). (c) Depth of cure of composites. (d) Water sorption of composites after immersion in deionized water for one week. (c, d) Crosses represent mean, boxes represent 25–75 percentiles, error bars represent SD.

3.2.2. *Shrinkage*

Based on conversion at 1 mm depth, the shrinkage of UDMA-containing composites was predicted to be 1.2 times higher, on average, than those containing Bis-GMA ($p < 2 \times 10^{-5}$) (Fig. 1b). Composites containing TEGDMA were estimated to have 1.1 times higher shrinkage than those containing PPGDMA ($p < 0.04$). When shrinkage was determined by volume change, however, no statistically significant effect of monomer could be observed. This was due to the high standard deviation of the technique. The shrinkage of Z250 could not be calculated using conversion data, since its exact composition was unknown. The measured shrinkage, however, of Z250 was comparable to all experimental formulations.

3.2.3. *Depth of cure*

The depth of cure (Fig. 1c) was highly comparable for all composites (2.7 ± 0.25 mm), regardless of monomer composition ($F = 0.96$).

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3.2.4. *Water sorption*

The water sorption of formulations containing TEGDMA was, on average, 1.2 times higher than that of those containing PPGDMA ($p < 0.02$) (Fig. 1d). On average, UDMA induced ~1.1 times higher water sorption than Bis-GMA, although this was not statistically significant. Z250 had the lowest water sorption, comparable to that of BP. UT had the highest water sorption, significantly higher than that of BP and Z250 ($p < 0.05$).

3.3. *Mechanical properties*

Experimental composites containing UDMA had significantly higher strength (153–158 MPa) than those containing Bis-GMA ($p = 1 \times 10^{-7}$). The strength of BP was significantly higher than that of BT (127 and 99 MPa, respectively; $p < 0.05$) (Fig. 2a). The modulus of experimental formulations containing PPGDMA was, on average, slightly lower than that of formulations containing TEGDMA (6.15 and 6.75 GPa, respectively; $p = 0.009$) (Fig. 2b). Z250 had the highest strength (173 MPa) and lowest modulus (5.3 GPa).

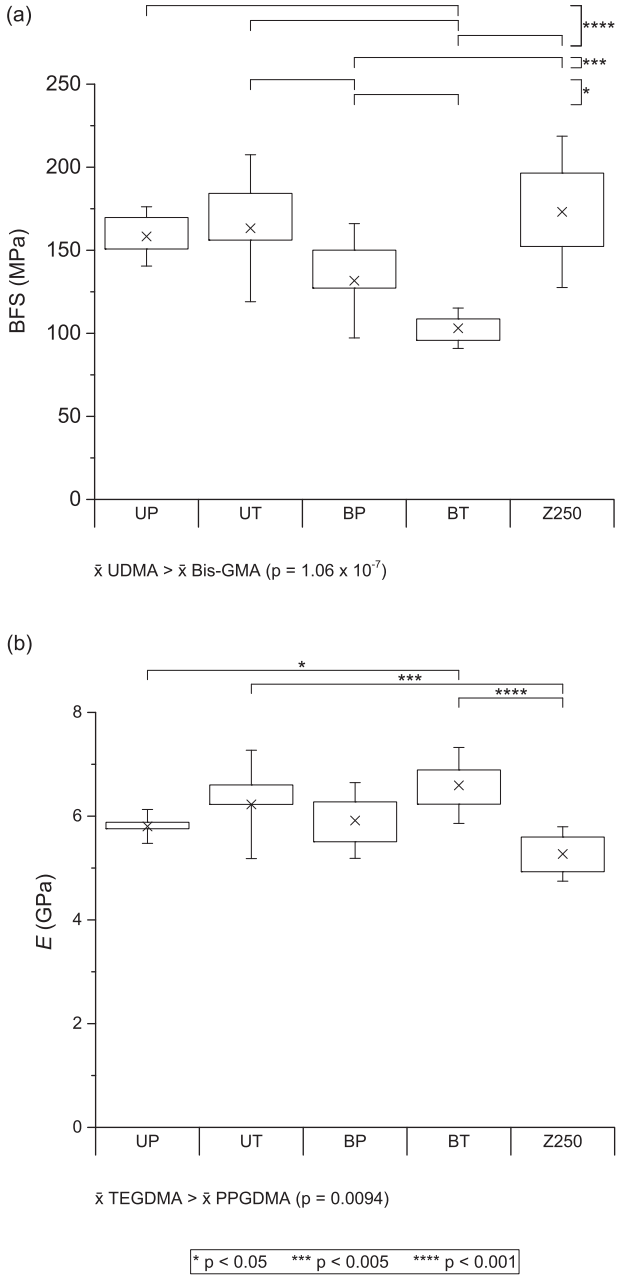

Fig. 2 – (a) Biaxial flexural strength and (b) modulus of tensile elasticity of composites after storage in deionized water at 37 °C for one week. Crosses represent mean, boxes represent 25–75 percentiles, error bars represent SD.

3.4. *Cytocompatibility*

3.4.1. *Comparison of resazurin, WST-8 and MTS assays*

The apparent cell density of HGF after 48 and 72 h culture in serum-free DMEM is shown in Fig. 3. Resazurin and WST-8 assays reported a similar increase in apparent cell density from 30,000 to ~49,000 cells/cm^2 between 0 and 48 h. MTS reported a lesser increase in apparent cell density (from 30,000 to 37,000 cells/cm^2 after 48 h). After 72 h in culture, the resazurin assay reported cell density to have increased to 113,000 cells/cm^2, whereas the WST-8 assay reported cell density to be 74,000 cells/cm^2. MTS again reported only a small increase in apparent cell density (45,000 cells/cm^2). Since the assays reported different cell densities in the controls at different time-points, subsequent data are normalized to the control for the corresponding assay and time-point and are reported in terms of relative metabolic activity.

3.4.2. *Composite component cytocompatibility*

The relative metabolic activity of surviving HGF following exposure to serial dilutions of liquid phase components, as well as following a subsequent recovery period, is presented in Fig. 4. The dashed lines represent the mean value obtained for the DMEM control for each corresponding time-point and assay. All components caused a typical, concentration-dependent reduction in cell number as concentration was

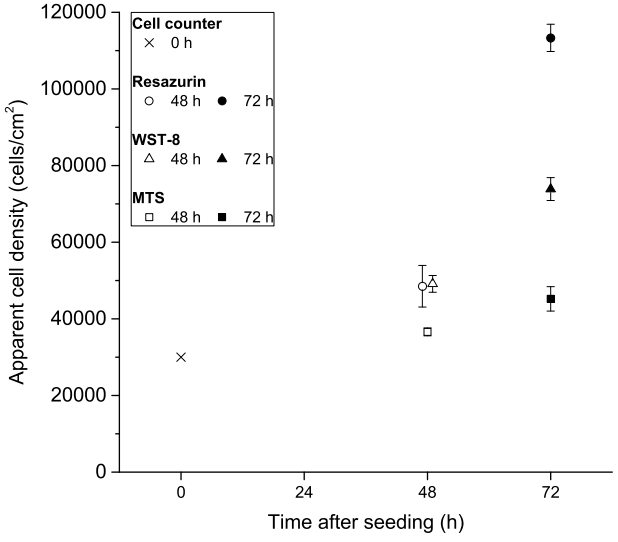

Fig. 3 – Initial seeding density of HGF determined by cell counting (0 h, cross), and apparent density after culture in DMEM for 48 h (unfilled symbols) and 72 h (filled symbols), as assessed by resazurin (circle), WST-8 (triangle) and MTS (square) assays. Error bars represent SD.

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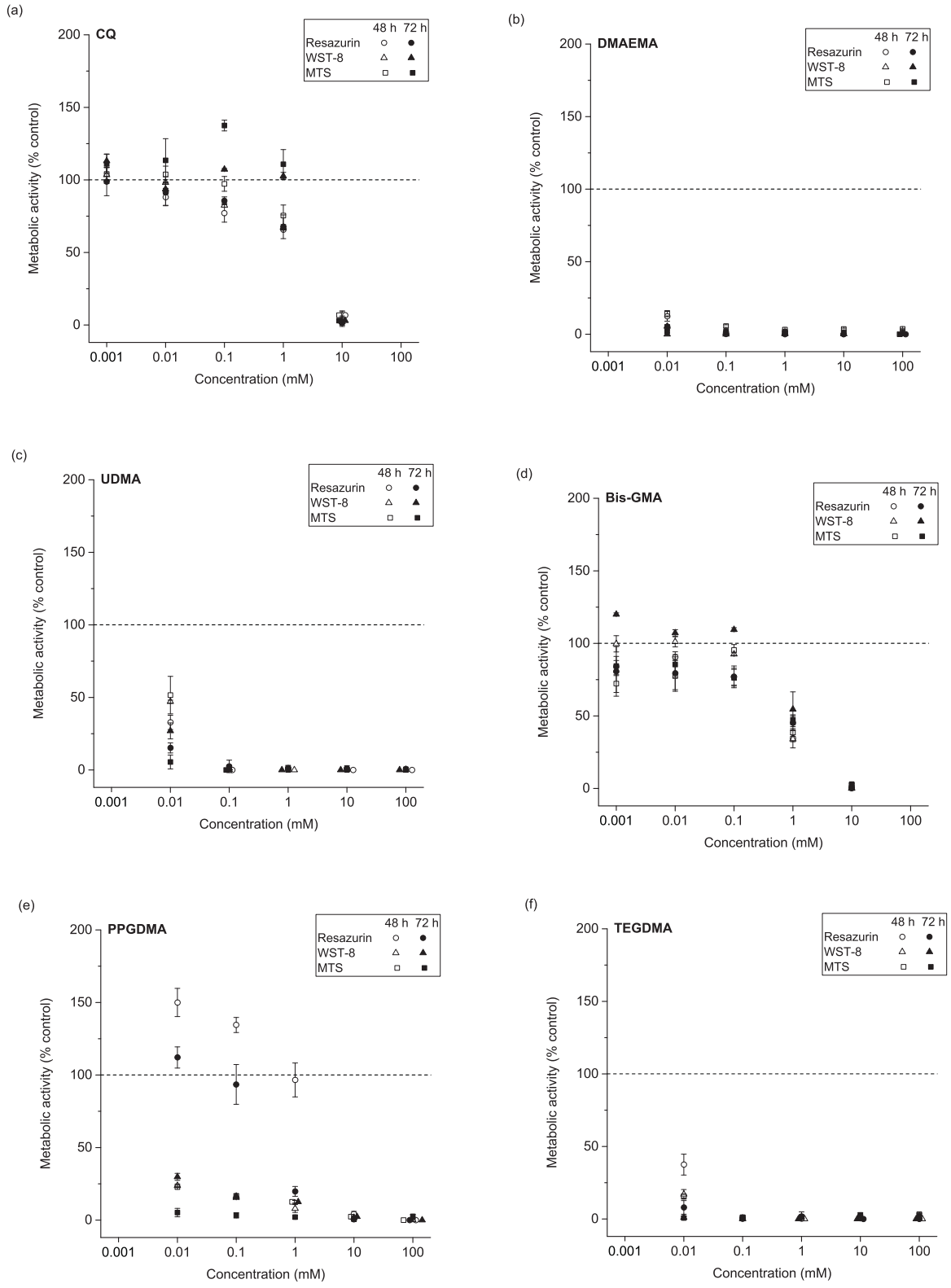

Fig. 4 – Metabolic activity (relative to the corresponding control) of surviving HGF after culture in aqueous solutions of composite liquid phase components (a) CQ, (b) DMAEMA, (c) UDMA, (d) Bis-GMA, (e) PPGDMA or (f) TEGDMA. Resazurin (circle), WST-8 (triangle) and MTS (square) assays were performed after 24 h culture in the solutions (48 h time-point, unfilled symbols), and after a subsequent 24 h recovery period in DMEM (72 h time-point, filled symbols). Component concentrations ranged from 0.001 to 10 mM (a and d) or 0.01 to 100 mM (b, c, e and f). Dashed lines represent the mean value of the control, error bars represent SD.

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increased, though the extent of this relationship depended on the assay. In the case of all components, a concentration of 10 mM was sufficient to cause close to 100% reduction in metabolic activity.

After 48 h, WST-8 indicated a direct correlation between CQ concentration (Fig. 4a) and metabolic activity, with 0.001 and 0.01 mM having no significant effect on metabolic activity. As concentration was increased to 0.1, 1 and 10 mM, metabolic activity was reduced by 17, 33 and 98%, respectively. Resazurin reported similar levels (±5%) of metabolic activity at most concentrations, whereas MTS values were up to 15% higher. After 72 h, resazurin reported similar levels of relative metabolic activity to after 48 h. WST-8, however, indicated similar levels of metabolic activity to the control (±7%) at all concentrations except ≥10 mM, at which cells did not recover. MTS showed elevated metabolic activity (10–38% greater than that of the control) at all concentrations after 72 h, except ≥10 mM.

By contrast, at 48 h, 0.01 mM DMAEMA (Fig. 4b) caused an 86–95% reduction in metabolic activity in all assays, with no recovery after 72 h. All assays indicated that concentrations ≥0.1 mM caused total inhibition of metabolic activity. Similarly, at a critical concentration of 0.01 mM or higher, UDMA (Fig. 4c) and TEGDMA (Fig. 4f) both inhibited metabolic activity in all assays.

Bis-GMA (Fig. 4d) appeared to be more cytocompatible than DMAEMA, UDMA and TEGDMA. After 48 h, WST-8 indicated that concentrations of 0.001–0.01 mM had no effect on metabolic activity. It fell, however, by 7 and 66% upon increasing concentration to 0.1 and 1 mM, respectively. The other assays showed a similar trend, but at 0.001–0.1 mM the values reported by resazurin and MTS were ~10–15% and ~23–28% lower, respectively. After 72 h, values obtained by WST-8 assay were elevated by between 7 and 20% compared to the previous time-point, except at 10 mM. At 0.001–0.1 mM, metabolic activity appeared higher than the controls. Resazurin reported similar values (±10%) after 72–48 h.

HGF exposed to PPGDMA (Fig. 4e) exhibited more complex trends. The three assays gave different results for concentration-dependent effects on relative metabolic activity. According to all assays, at ≥10 mM, PPGDMA completely inhibited metabolic activity after 48 h and this did not recover after 72 h. Upon lowering concentration, there was a small increase in activity according to the WST-8 and MTS assays, but it remained well below control values and was not enhanced by providing additional recovery time. Conversely, at 48 h and PPGDMA level below 0.1 mM, the cell activity according to the Resazurin test tended to higher values than with the control. By 72 h, however, this "excess" metabolic activity was down to control levels. Concentrations of 0.01, 0.1 and 1 mM, PPGDMA caused a reduction in WST-8 activity of 76, 85 and 92%, respectively, after 48 h. These levels remained unchanged after 72 h. MTS showed similar values to WST-8. By contrast, after 48 h, resazurin activity was elevated by 63 and 35% above the control at 0.01 and 0.1 mM, respectively, and at 1 mM was similar to the control. After 72 h, these values fell, with resazurin activity remaining 12% above the control at 0.01 mM, and being 7 and 80% lower than the control at 0.1 and 1 mM, respectively.

3.4.3. *Composite extract cytocompatibility*

The metabolic activity of HGF after culture in composite extracts is presented in Fig. 5. Significant differences were again observed between different assays. In order to facilitate elucidation of trends, data are expressed as a percentage of the control for each corresponding extract (1 or 7 days) and assay time-point (48 or 72 h).

After 24 h exposure to 1 day composite extracts (48 h time-point), a general downward trend in resazurin metabolism was observed as follows: UP > UT ≥ BP > BT ≥ Z250. Although WST-8 activity differed slightly (UP ≈ UT > BP ≈ BT > Z250), both assays confirmed that the extracts from the lower quadrant of 4 mm deep composite samples containing UDMA had less effect on the 48 h metabolic activity than those containing Bis-GMA. In the case of resazurin, this was statistically significant ($p = 0.001$). They also showed that Z250 extracts were less cytocompatible than all of the experimental formulations. MTS showed no significant differences between experimental formulations but confirmed the low cytocompatibility of Z250. Metabolic activity in all assays varied from being similar to the control for UP, down to ~50% of control for Z250. Composites containing TEGDMA caused a slight but significant reduction in resazurin activity compared to PPGDMA ($p < 0.01$), but WST-8 and MTS assays showed no clear trends with regards to the effect of diluent monomer.

After 72 h, similar trends were observed in all three assays, with metabolic activity falling to approximately 50% of that of the control for all experimental materials. The only differences between assays were observed with Z250, where resazurin activity had remained similar to the previous time-point at ~50%, but WST-8 activity had fallen from 53 to 37% and MTS activity had fallen from 53 to 33%. These differences, however, were not statistically significant.

Resazurin and WST-8 suggested that 7-day extracts were more cytocompatible than 1 day extracts, since metabolic activity returned to control levels after 72 h. The trend of the 48 h resazurin assay after 7 days extraction was similar to the corresponding 1 day extract, but with slightly higher values. For example, 48 h assays showed that BT – the least cytocompatible experimental composite – had 75% resazurin activity relative to the control after 7 days extraction, compared to 56% after 1 day. In the case of all experimental formulations, WST-8 activity was similar to the control or slightly elevated by up to 11% after 48 h. The 7-day extract of Z250, however, still caused a significant reduction in WST-8 activity. After 72 h, the resazurin and WST-8 activity of HGF exposed to 7-day extracts had, in general, recovered. Resazurin activity of BT and Z250 remained slightly reduced at 89 and 82%, respectively. After 72 h, WST-8 activity of HGF exposed to all composite extracts was elevated above that of the control by between 10 and 31%. The MTS assay gave significantly different values compared to the other two assays for the 7-day extracts. After 48 h, MTS activity of HGF exposed to all experimental formulations was 60–75% higher than the control and remained similar after 72 h. Z250 values were similar to those reported by WST-8. UDMA was also shown to be more cytocompatible than Bis-GMA in 7-day extracts (48 h resazurin assay, $p = 0.03$; 72 h WST-8 assay, $p = 0.0008$; 72 h MTS assay, $p = 0.007$). WST-8 activity was also significantly higher in cells exposed to 7-day

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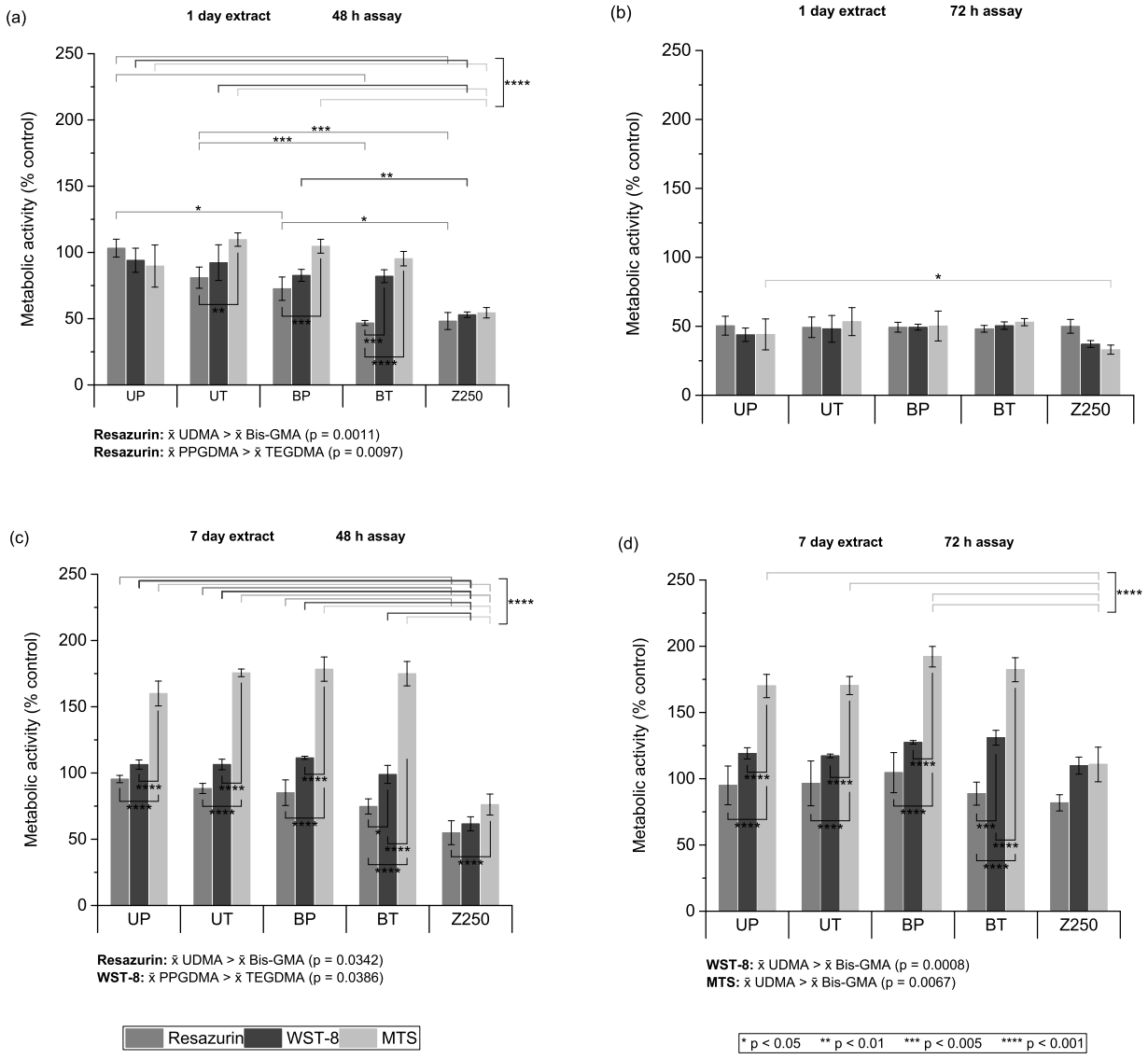

Fig. 5 – Metabolic activity (relative to the corresponding control) of HGF after 48 (a and c) or 72 (b and d) h culture in 1 (a and b) or 7 (c and d) day composite extracts, assessed by resazurin (dark gray), WST-8 (black) and MTS (light gray) assays. Dashed lines represent the mean value of the control, error bars represent SD.

extracts of PPGDMA-containing composites than TEGDMA-containing composites after 48 h ($p = 0.04$).

4. Discussion

As hypothesized, dental composites containing UDMA had significantly higher monomer conversion and slightly better cytocompatibility than those containing Bis-GMA. Similarly, composites containing PPGDMA achieved more complete conversion than those containing TEGDMA. Importantly, the use of UDMA and/or PPGDMA as the sole bulk/diluent monomer had no detrimental effect on the shrinkage or depth of cure of the materials. In addition, composites containing UDMA had improved handling properties compared to those containing Bis-GMA.

The variation in the conversion of the composites is attributed to differences in the chemical structures (Fig. 6) and physical characteristics (Table 2) of their constituent monomers. Although UDMA is a slightly smaller molecule than Bis-GMA and, as a result, has slightly higher double bond concentration (ratio of double bonds per molecule to molecular mass), its lower glass transition temperature (T_g) and greater flexibility enable more complete cross-linking [15]. The bulky aromatic groups of Bis-GMA cause steric hindrance, reflected in its very high viscosity value, which is ~80 times that of UDMA. This reduced flexibility limits the likelihood of methacrylate groups coming into contact with each other and binding. PPGDMA has more than twice the molecular mass of TEGDMA and as a result, its greater flexibility and lower double bond concentration result in improved conversion.

The similar depth of cure of the composites is likely to be due to the similar refractive indices of the monomers used

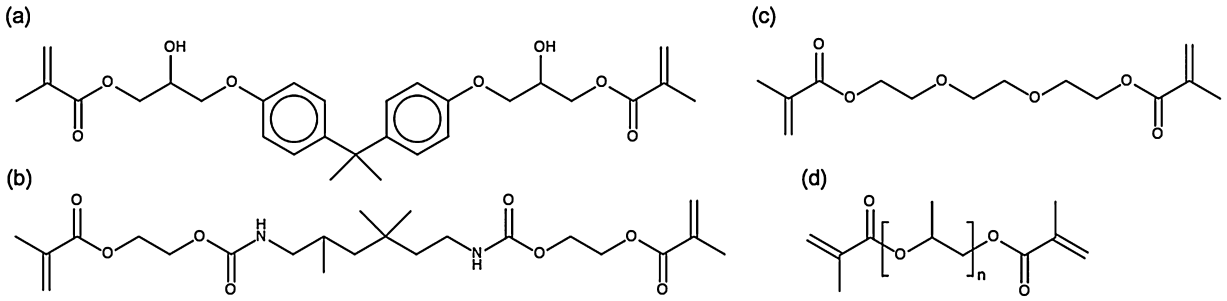

Fig. 6 – Chemical structures of bulk monomers (a) UDMA and (b) Bis-GMA and diluent monomers (c) PPGDMA, where $n \approx 7$ propylene glycol units and (d) TEGDMA.

Table 2 – Chemical and physical properties of bulk and diluent monomers. Data obtained from suppliers.

Monomer	Molecular mass (Da)	Density (g/cm^3)	Viscosity (Pa s)	Refractive index	T_g (°C)
UDMA	470.6	1.129	~8.5	1.483	−35.3
Bis-GMA	512.6	1.161	~700	1.540	−7.7
PPGDMA	600.0	1.01	0.085	1.452	−62.0[a]
TEGDMA	286.3	1.09	0.050	1.461	−83.4

[a] Estimate based on poly(ethylene glycol-400) monomethyl ether monomethacrylate.

(1.48 ± 0.04). The standard protocol, however, provides limited information. The 'scraping' method described in BS EN ISO 4049:2009 is a rough measure of composite hardness versus depth, which likely suffers from variability between different users of the standard. In recognition of this, as well as the fact that the values obtained may not accurately reflect the depth at which there is ≥50% conversion, the standard requires that the value obtained is divided by 2. This appears to result in under-reporting of depth of cure values for some composites. A value of ≥50% monomer conversion may be considered as indication that an average of one methacrylate group per molecule has reacted and that the composite is cured and cytocompatible [16]. This arises because the first double bond on dimethacrylates tends to polymerize faster than the second. Using FTIR to assess the conversion at various specified depths is advantageous and provides more detailed information than the standard depth of cure technique. With regards to the present study, the ISO test predicts that all formulations have a similar depth of cure (2.7 ± 0.25 mm). The measurement of conversion at different depths, however, confirms that UP, UT and BP have ≥50% at depths of at least 4 mm, and that BT and Z250 are insufficiently cured at this depth. The use of FTIR with ATR, a facile technique, therefore offers greater insight into the inter-related topics of monomer conversion and depth of cure.

FTIR can also be used to overcome the high margin of error associated with the use of density determination apparatus for assessment of shrinkage [17], which is highly sensitive to entrapment of air bubbles and instability of balance readings. While the use of conversion values predicted that the present UDMA-containing composites would have slightly higher shrinkage, the volume change method failed to elucidate clear differences between formulations. FTIR achieves lower variability and requires fewer replicates, making it advantageous over BS EN ISO 17304:2013. It should, however, be taken into account that Eq. (2) ($S = (mC\rho\sum_i(n_i x_i/w_i) \times 2250)$ is based on the assumption that one mole of polymerizing C=C bonds typically undergoes volumetric shrinkage of ~22.5 cm^3 [8]. This generalized value for methacrylates is based on results from Bis-GMA-based monomers and does not take into account possible differences resulting from physical properties such as flexibility or steric hindrance. The conversion method gives more reliable results and does not conflict with those obtained by the volume change method.

Although high conversion is typically associated with high shrinkage, composites containing UDMA had acceptable shrinkage of 2.55–2.86 vol% (assessed by either method), despite having slightly higher double bond concentration than Bis-GMA and conversion as high as 62–67.5%. One possible explanation for this may be that shrinkage of dimethacrylates may be less strongly affected by the conversion of the second double bond than the first. Additionally, composites containing PPGDMA had, on average, slightly lower shrinkage than those containing TEGDMA, despite having higher conversion. This is due to PPGDMA having much lower double bond concentration, which results from its molecular mass being over double that of TEGDMA.

A simplified version of BS EN ISO:2009 was used to estimate water sorption from mass increase measurements after one week storage in H_2O, which allowed for the use of less material. Bis-GMA induced slightly lower water sorption than UDMA, due to the hydrophobicity of its aromatic groups. Similarly, the propylene glycol groups of PPGDMA are more hydrophobic than the ethylene glycol groups of TEGDMA. Combined with the lower cross-linking density of TEGDMA, this resulted in higher water sorption in TEGDMA-containing composites.

The correlation between conversion and BFS of experimental formulations is likely due to improved entrapment of filler particles in composites with greater cross-linking.

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Additionally, the interaction between the monomers and the short, aliphatic silane (3-(trimethoxysilyl)propyl methacrylate) coating on the fillers, which has relatively low hydrophobicity, is stronger with aliphatic groups (UDMA, PPGDMA and TEGDMA) than aromatic groups (Bis-GMA). The higher BFS of Z250 is likely due to differences in the fillers used, the composition of the liquid phase and the filler to liquid ratio. In the present paper, a *molar* ratio of bulk to diluent monomer was used, in order to enable direct comparison of conversion and cytocompatibility. The relatively low ratio of diluent monomer used in this study is likely the reason that Z250 had significantly higher strength than BP and BT.

Although the densities of the monomers do not vary significantly, variations in molecular mass and viscosity result in noticeable differences in composite handling properties. Bis-GMA, in particular, is highly viscous and difficult to handle, due to its bulky aromatic rings. UDMA, despite its relatively high viscosity, is significantly less viscous than Bis-GMA, making it more facile to handle during clinical use and composite manufacture. Although PPGDMA has higher viscosity than TEGDMA, its molecular mass is more than double that of TEGDMA. This means that, when comparing composites with the same *molar* ratio of bulk to diluent monomer (as opposed to *wt%* ratio), handling properties were similar.

Despite the low solubility of most of the liquid phase components analyzed in this study, it was possible to dissolve them in cell culture medium at up to 10 mM (CQ and Bis-GMA) or 100 mM (DMAEMA, PPGDMA, TEGDMA and UDMA). Most components remained fully dispersed in the medium, although CQ and TEGDMA did settle at high concentrations. In order to ensure complete dispersal of the components, the test solutions were thoroughly mixed by vortexing immediately prior to adding them to each well.

Due to the limitations of cytocompatibility assays and variability between the different enzymatic substrates available [5], the present study utilized three different assays (resazurin, WST-8 and MTS) to assess the cytocompatibility of composite liquid phase components and composite extracts. It is important to take into consideration that the values obtained by these assays report not the true cell density, as may be determined by cell counting. They are, instead, an estimate of cell density based on the metabolic activity of the corresponding enzyme. The nature of these assays relies on the assumption that relative metabolic activity remains constant with time after seeding. It can be seen in Fig. 3, however, that different assays reported different cell densities over time in the controls, despite having the same initial seeding density. For this reason, the cytocompatibility of the composite components and extracts was reported in terms of metabolic activity as a percentage of the corresponding control, for each assay and time-point. The use of serum-free medium did not noticeably limit the proliferative potential of HGF over the short duration of these assays (up to 48 h), since apparent cell density values fell within the expected range for HGF based on typical culturing conditions with foetal bovine serum.

CQ was shown to have good cytocompatibility and, given that it is present at low concentrations in the material, is unlikely to cause a toxic effect. DMAEMA was demonstrated to be highly cytotoxic. Unlike some other initiators, such as *N,N*-dimethyl-*p*-toluidine, however, DMAEMA contains a methacrylate group which enables it to become incorporated within the polymer and likely minimizes its release.

A wealth of studies have shown that dental monomers such as Bis-GMA, UDMA and TEGDMA cause cytotoxic, genotoxic and apoptotic responses in a variety of cell types [18–22]. Presence of excessive reactive oxygen species have previously been shown to arrest cell cycle at different phases in different fibroblast types upon exposure to these monomers [20,23–27]. As well as delaying cell cycle progression, these monomers also impact on proliferation and survival and cause inflammation and/or necrosis. TEGDMA has also been shown to act as a vasorelaxant [28–32], to cause apoptosis and necrosis, which was associated with a reduction in cdc2, cyclin B1 and cdc25C expression and increase in p21 expression [33] and upregulation of caspases [34]. Bis-GMA induces inflammation and necrosis by upregulating expression of prostaglandin, tumour necrosis factor-a and various surface antigens [35–37]. Although Bis-GMA is widely used in dental composites, some researchers have concerns over its ability to degrade to form bisphenol A [38]. Furthermore, toxic monomers can be released by hydrolytic and enzymatic degradation, as well as mechanical abrasion [22].

It was hypothesized that by increasing the length of the diluent monomer by using PPGDMA instead of TEGDMA, it would be possible to alleviate oxidative stress to some degree [39]. In the present short-term study, HGF metabolized resazurin significantly more efficiently after exposure to PPGDMA, either in solution or via composite extracts, than after exposure to TEGDMA or UDMA. HGF failed, however, to efficiently metabolize WST-8 and MTS after exposure to any of these components, even at low concentrations.

Bis-GMA had a lesser effect on metabolic activity of HGF than the other monomers, up to a concentration of at least 0.1 mM. Its TC_{50} value (the concentration at which 50% of cells survived) was in the range of ~0.5–1 mM, consistent with previous findings in HGF and HaCaT (keratinocyte cell line) [40] and an order of magnitude higher than observed in some other cell lines (e.g. L929) [41,42]. This highlights the potential for variability, which can arise between different cell types and methods of material preparation, culture duration, seeding density, etc. Although neither the longer-term cytotoxic effects nor the genotoxic effects of Bis-GMA were investigated, previous studies have demonstrated that the use of Bis-GMA in dental materials is cause for concern. The use of alternative monomers with excellent mechanical properties and relatively high viscosity, but lower viscosity and with better handling properties than Bis-GMA, would therefore be ideal. The TC_{50} of UDMA and TEGDMA could not be directly compared to previous findings, since even at the lowest concentration tested, fewer than 50% of cells were viable after 48 h. The TC_{50} values of UDMA (≤0.01 mM) and TEGDMA (<0.01 mM) were notably lower, however, than previously observed (~0.06–4 mM [24,40,42] and ~1–4.1 mM [40,42], respectively).

UDMA is also widely used in dental composites, typically in combination with Bis-GMA and/or similar high viscosity monomers. The use of UDMA has been shown to result in significantly higher conversion, more complete cross-linking and lower leaching of uncured monomer than Bis-GMA [4], as well as higher flexural strength and hardness [43]. It has

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therefore been suggested that UDMA may be used as the sole bulk monomer in orthodontic adhesives [3] and bone cements [44]. In the present study, the use of UDMA as the sole bulk monomer in a dental composite was investigated. It was hypothesized that its higher conversion would result in improved cytocompatibility due to reduced leaching of uncross-linked monomers and initiators (such as the highly toxic DMAEMA). Despite UDMA being more cytotoxic to HGF than Bis-GMA in solution, the results of the 48 h resazurin and WST-8 assays suggest that 1 day extracts of UDMA-containing composites were more cytocompatible than those of Bis-GMA-containing composites. After a subsequent recovery period, however, all three assays showed similar metabolic activity after exposure to all materials, suggesting that UDMA is similarly cytotoxic but has a delayed action. These findings may result from a combination of the greater presence of Bis-GMA and diluent monomers in the extracts, due to Bis-GMA's poorer conversion, and UDMA's higher cytotoxicity.

Although water-soluble assays offer a convenient insight into the cytocompatibility of biomaterials, they have the drawback of being unable to differentiate between cells that are actively proliferating and cells that are in a quiescent state [45]. This is reflected in the over-estimation of cell density by the MTS assay in the present study. Overestimation and/or underestimation can also result from interference caused by the compound being analyzed. Although resazurin [46], WST-8 [47] and MTS [48] have all been previously reported to overestimate and/or underestimate cell number, MTS appears to be particularly affected. In the present study, the MTS assay's significant underestimation of cell density in the control was most likely the cause of its overestimation of metabolic activity per cell.

Further complications arise from the fact that different studies utilize varying cell types. Given their differing phenotypes and susceptibility to cytotoxic and genotoxic effects [49], direct comparisons cannot be made. In addition, short-term cytocompatibility studies do not take into account possible genotoxic and mutagenic effects. Although it was beyond the scope of this study, future research into the longer-term cytotoxic and genotoxic effects of a wide range of commonly used composite components would be highly beneficial in elucidating the causes of the complex trends observed in this and other studies. Although an extensive range of such studies has been performed, the different parameters investigated between different research groups (material concentration, material preparation, extraction technique, cell type, assay, genes analyzed, duration of study) make it difficult to directly compare results and definitively interpret them. Better understanding of the fundamental effects of each individual component is required in order to improve the biocompatibility of novel dental composites. A more systematic and wide-ranging approach would be beneficial in establishing a library which comprehensively documents the effects of each component.

More comprehensive studies are therefore required to elucidate the longer-term effects of monomers and composite extracts on a wide range of markers of biocompatibility in a variety of relevant cell types. This includes analysis of expression of cell surface markers and genes implicated in oxidative stress and apoptotic response, in parallel with multiple metabolic activity assays and quantification of total DNA. This would enable more targeted design of novel composites with improved biocompatibility for improved treatment of dental caries.

5. Conclusions

It can be concluded that UDMA significantly improved conversion, BFS and depth of cure compared to Bis-GMA, due to its greater flexibility and lower T_g. Its higher conversion also slightly improved cytocompatibility, likely due to reduced monomers leaching. PPGDMA improved conversion relative to TEGDMA. The use of UDMA and PPGDMA did not cause a detrimental increase in polymerization shrinkage. Although the resazurin assay suggested that PPGDMA be more cytocompatible than TEGDMA, WST-8 and MTS assays proved inconclusive. Longer-term cytocompatibility and genocompatibility testing is therefore required.

Furthermore, MTS significantly over-estimated metabolic activity. Additionally, FTIR is a facile technique which provides greater information about the depth of cure of composites and appears to accurately predict polymerization shrinkage.

These results suggest that careful consideration should be given to the polymerization behaviour of the monomers used in dental composites, as well as their cytocompatibility. This is particularly true in the case of bulk fill materials, which aim to expedite the treatment of carious lesions by reducing the number of composite layers required, since the effect of monomer on conversion of the present composites was more pronounced at 4 mm than at 1 mm. Furthermore, the cytocompatibility test, which demonstrated that all formulations affect metabolic activity to different degrees, utilized specimens from the bottom 1 mm from a 4 mm stack. The deepest section of a restoration receives the lowest intensity of light during curing and as a result, can suffer from poor conversion if the constituent monomers lack flexibility. Given that this is also the most likely region of the composite to be in close proximity to pulp tissue, cytocompatibility is of particular importance for bulk fill materials.

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