Macrophage response to methacrylate conversion using a gradient approach

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Abstract

Incomplete conversion, an ongoing challenge facing photopolymerized methacrylate-based polymers, affects leachables as well as the resulting polymer network. As novel polymers and composites are developed, methods to efficiently screen cell response to these materials and their properties, including conversion, are needed. In this study, an in vitro screening methodology was developed to assess cells cultured directly on cross-linked polymer networks. A gradient in methacrylate double bond conversion was used to increase the experimental throughput. A substrate of 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl] propane (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) was prepared with a conversion ranging from 43.0% to 61.2%. Substrates aged for 7 days had no significant differences in surface roughness or hydrophilicity as a function of conversion. Leachables were detectable for at least 7 days using UV absorption, but their global cytotoxicity was insignificant after 5 days of aging. Thus, RAW 264.7 macrophage-like cells were cultured on aged substrates to evaluate the cell response to conversion, with possible contributions from the polymer network and local leachables. Conversions of 45% and 50% decreased viability (via calcein/ethidium staining) and increased apoptosis (via annexin-V staining). No significant changes (p > 0.05) in tumor necrosis factor-α and interleukin-1β gene expression, as measured by quantitative, real-time reverse transcription-polymerase chain reaction, were seen as conversion increased. Thus, conversions greater than 50% are recommended for equimolar BisGMA/TEGDMA. The ability to distinguish cell response as a function of conversion is useful as an initial biological screening platform to optimize dental polymers.

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1. Introduction

Polymers based on methacrylates and dimethacrylates are used in a variety of biomedical applications, including bone cements, intraocular lenses, contact lenses, tissue engineering hydrogels and dental restorations. Favorable cell–material interactions are critical to the success of these polymers. One material property that can affect these interactions is reaction conversion. The polymerization of typical dimethacrylate monomers produces a complex cross-linked polymeric network. Incomplete conversion is an ongoing challenge associated with these polymer systems [1,2], particularly in dental materials. The residual unsaturation in the polymer network consists of a mixture of unreacted free monomers and covalently attached pendant methacrylate groups. The level of conversion has been shown to influence the amount and type of extractable components [3] and the corresponding biological response [4,5].
Many studies have focused on evaluating leachables from dental polymers. Extraction studies have used techniques including gas chromatography/mass spectrometry and high-performance liquid chromatography to demonstrate that 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy)-phenyl] propane (BisGMA) [3,6], triethylene glycol dimethacrylate (TEGDMA) [3,6,7] and camphorquinone [6,7] are among the components extracted. Researchers have examined the cytotoxic effects of multicomponent extracts from cured dental materials, either by adding extracts to cells or by culturing cells in the vicinity of the samples [5,8–10]. Cell response varied with the selected material, indicating a need to test each polymer. Individual chemical components that leach out of cured dental composites, such as the monomers BisGMA and TEGDMA [11], have also been evaluated by direct addition to culture medium [12–16]. BisGMA and TEGDMA monomers reduced cell proliferation [16] and induced a dose-dependent apoptotic response [13,17] in vitro. These and other studies have provided a fundamental knowledge base for assessing cytotoxicity of leachables from dental polymeric networks.

Another aspect that may affect the cell response is the polymer network itself. Even though the extractable components may negatively affect cell viability and proliferation, the material bulk and surface properties could also do so, perhaps to a greater degree. Cells may be directly exposed to polymeric materials when composite restorations are in contact with marginal gingival tissue. Moreover, these same dimethacrylate monomers are being considered for other applications, such as tissue engineering scaffolds [18] and bone cements [19–21], where favorable cell–material interactions are crucial to success.

Macrophages present throughout the body, including the oral tissues, are involved in inflammation and antigen presentation during reaction with infectious and foreign agents. They also amplify the response by signaling other cells. Activated macrophages secrete pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These cytokines are primary mediators in the inflammatory response and have been shown to play a role in disease states including pulpal inflammation and periodontal tissue destruction [22,23]. The effects of dental monomers and polymer extracts on apoptosis and cytokine secretion of macrophages and monocytes have been investigated [4,5,14,15], but these cells have not been cultured directly on the polymers, a situation that can occur in vivo. Culturing cells directly on the materials would allow for the simultaneous evaluation of the local leachable microenvironment as well as the polymer network itself.

Combinatorial libraries and arrays are advantageous for examining the effects of physicochemical properties upon cell response by allowing for the systematic variation of composition or processing conditions [24–32]. These cell-surface studies are critical in examining the biological response to a material, as surface chemistry can affect the cell response either directly or indirectly through protein adsorption. However, the tools to carry out in situ measurements of biological response on combinatorial samples are still being developed and require adapting bioassays from single-sample analysis. Such an approach to correlate conversion and cell response has significant advantages, including increased experimental throughput, similar processing for each data point and the ability to screen more thoroughly the effects of any conversion within the tested range. The use of conversion gradient substrates to analyze mechanical properties [33] and the photopolymerization kinetics [34] of dental polymers has been previously reported, but the effects of conversion on adherent cell populations have not been previously characterized for BisGMA/TEGDMA dental copolymers.

This investigation had three overall objectives related to the goal of developing a methodology to investigate material performance following standard protocols. The objectives were (i) to develop an experimental approach with increased throughput to prepare and test polymer substrates with variable physical, chemical, and structural properties, (ii) to modify bioassays to quantify cell response and screen methacrylate-based polymers for cytotoxicity, and (iii) to quantify threshold limits or regions of interest for further investigation and reduce the number of experiments, time and materials involved in formulating novel materials. Methacrylate double-bond conversion was the property of interest selected for the study due to the incomplete conversion and potentially undesirable biological effects often associated with photo-polymerized methacrylate-based polymers. The experimental hypothesis was that lower conversions would result in increased apoptosis and cell death and that the screening methodology would be able to detect these differences quantitatively as a function of conversion. Cells cultured directly on dental polymers were analyzed using bioassays adapted for continuous gradient substrates to evaluate the combined effects of the polymer network and the local leachables resulting from the incomplete conversion.

Conversion gradient substrates were prepared, and near infrared (NIR) spectroscopy was used to measure the conversion profile. Substrates were aged to remove the initial, toxic levels of leachables. Aged substrates were evaluated using atomic force microscopy (AFM) and water contact angle measurements to determine if variations in surface features were present and possibly contributing to the cell response. Materials extracted during the aging process were analyzed using FBS as a source of protein biomarkers and the local leachables resulting from the incomplete conversion.

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2. Materials and methods

2.1. Preparation of conversion gradient substrates

An equimolar mixture of BisGMA and TEGDMA (64:36 mass fraction, Esstech, Inc., Essington, PA) was prepared containing a photoinitiator system consisting of camphorquinone (CQ, 0.1% mass fraction, Aldrich Chemicals, Milwaukee, WI) and ethyl 4-N,N-dimethylamino benzoate (4EDMAB, 0.3% mass fraction, Aldrich Chemicals). The co-monomers were added to a Teflon mold (55 mm × 12 mm × 1.5 mm), clamped between two standard glass slides, and cured in a visible-light curing unit (Triad 2000, Dentsply International, York, PA) for a total of 40, 60 or 100 s, with the time equally divided between the two sides of the sample. The Triad unit contained a tungsten halogen light bulb (250 W, 120 V) and was used to produce the variation in conversion. The sample was placed 90 mm from the bulb with one edge of the sample aligned with the center of the stage (Fig. 1a). A Cure Rite visible curing light meter (Dentsply) was used to measure the light intensity as a function of position. The power density from the bulb decreased exponentially from 209 ± 6 mW cm$^{-2}$ at the center position (90 mm from the bulb) to 6 ± 1 mW cm$^{-2}$ at 55 mm from the center (105 mm from the bulb). These two positions represent the two edges of the gradient substrates. For some assays, gradient substrates (whole substrates) were cut into five sections (substrate sections), each piece encompassing a different portion of the conversion range (Fig. 1b). Table 1 lists the characterization techniques and bioassays used in our in vitro screening platform.

2.2. Conversion measurements using NIR

Conversion was measured as a function of position on the whole gradient substrates using NIR [36]. Spectra were collected before and 24 h after curing using a Nexus 670 FT-IR ESP (Thermo Nicolet Corp., Madison, WI) and OMNIC 6.1 software (Thermo Nicolet Corp.). Measurements were taken at 5 mm increments across the gradient substrates and consisted of 64 scans from 7500 to 4000 cm$^{-1}$ with a 2 cm$^{-1}$ resolution. Conversions were calculated as the reduction in the vinyl peak (=$\equiv$C=H, 6164 cm$^{-1}$) normalized to the aromatic peak (−C=−C=−, 4623 cm$^{-1}$) [37]. After evaluating the effect of cure time on the resulting conversion range, an exposure time of 40 s was selected for all subsequent characterization and bioassays.

2.3. Substrate sterilization and aging

Whole gradient substrates as well as substrate sections were sterilized at room temperature using ethylene oxide gas (Anprolene Sterilization System, Andersen Products, Inc., Haw River, NC), degassed for at least 72 h, and washed twice with sterile phosphate-buffered saline (PBS, pH 7.4, Invitrogen Corp.). Validation studies confirmed that the sterilization process did not affect the conversion levels. Whole gradient substrates were aged with 6 ml of either PBS (for UV absorption measurements) or cell culture medium (to evaluate effects of leachables on cell viability) at 37 °C in four-well tissue culture plates. Substrate sections were each aged with 1.2 ml solution in 12-well tissue culture plates. Solutions were changed every 24 h for 7 days.

2.4. Surface characterization using contact angle and AFM

The surface hydrophilicity and roughness were characterized to determine if these properties changed as a function of conversion. The advancing and receding contact angles on the aged surfaces were measured at 25 °C using water as the probe fluid and a DSA 10 Mr2 (Kruss, Germany) drop shape analysis system. The standard uncertainty is represented by the standard deviation between two independent measurements every 5 mm on each of two gradient substrates that had been aged for 7 days.

Tapping-mode AFM measurements were conducted in air with a Nanoscope IV system (Digital Instruments)
operated under ambient conditions with standard silicon tips (Nanodevices; L, 125 μm; normal spring constant, 40 N m⁻¹; resonance frequency, 300–360 kHz). Root mean square (r.m.s.) roughness measurements were determined using standard Digital Instruments software, and averages and standard deviations were calculated from two measurements every 5 mm on each of two aged gradient substrates (n = 4).

2.5. Cell culture

The murine RAW 264.7 macrophage-like cell line from the American Type Culture Collection (ATCC, Manassas, VA) was maintained in growth medium consisting of Roswell Park Memorial Institute medium 1640 (RPMI, Invitrogen Corp., Carlsbad, CA) supplemented with 10% (volume fraction) heat-inactivated fetal bovine serum (FBS, Invitrogen Corp.) and 2 mM L-glutamine (FBS, Invitrogen Corp.) and 2 mM L-glutamine (FBS, Invitrogen Corp.) supplemented with 10% (volume fraction) heat-inactivated fetal bovine serum (FBS, Invitrogen Corp.) [35]. Cells were cultured in humidified incubators at 5% (volume fraction) CO₂ and 37 °C. Cells were subcultured weekly using Hanks’ Balanced Salt Solution (Invitrogen) and physical agitation to release cells. Experiments were carried out using cell passages 8–12.

2.6. Extract analysis

PBS extracts from five whole substrates and three sectioned substrates were analyzed using a UV–Vis spectrophotometer (Varian, Inc., Palo Alto, CA). Spectra for extracts in PBS and for each individual component (Bis-GMA, TEGDMA, CQ and 4EDMAB) in methanol were collected from 350 to 190 nm with a 1 nm resolution. Component spectra were qualitatively compared to spectra from extracts to identify overlapping peaks.

Extracts collected from whole and sectioned substrates aged in RPMI medium were used to evaluate the effects of the leachables on cell viability. RAW 264.7 cells were seeded (26,000 cells cm⁻²) on tissue culture polystyrene (TCPs) 12-well plates and cultured for 24 h. The growth medium was removed and replaced with the extracts supplemented with 10% (volume fraction) heat-inactivated FBS. The cells were further cultured for 24 h and then analyzed using the viability assay.

2.7. Cell seeding on substrates

After aging, substrates were washed with PBS, and a cell suspension of 5.0 × 10⁵ cells in 0.2 ml growth medium was added uniformly to each gradient substrate. After 15 min at room temperature, the substrates were completely immersed using 6 ml growth medium containing an additional 2.0 × 10⁶ cells to fill each well in the four-well plate. Gradient sections were seeded in a similar manner, with an initial seeding of 1.0 × 10⁵ cells in 40 μl medium, followed by an additional 2.5 × 10⁵ cells in 1 ml medium in a 12-well plate. Positive and negative controls, seeded to have a cell density similar to that on the gradient substrates, included cells seeded in 12-well TCPS plates (2.0 × 10⁵ cells in 1 ml growth medium per well) with and without 0.1 μg ml⁻¹ lipopolysaccharides (LPS, Sigma–Aldrich, Inc.) purified from Escherichia coli serotype O127:B8, source strain ATCC 12740. This concentration was selected based on its ability to induce Annexin-V staining while not affecting cell viability after 24 h of culture.

2.8. Calcein/ethidium staining for viability analysis

After 24 h of culture, cell viability on whole gradient substrates, substrate sections and TCPS was evaluated to assess the acute toxicity associated with the conversion level. The growth medium was removed, and the cells were washed with fresh growth medium and incubated (10 min, 37 °C) with growth medium containing 2 μmol l⁻¹ calcein acetoxyethyl ester (calcein AM, Invitrogen Corp.) and 2 μmol l⁻¹ ethidium homodimer-1 (Invitrogen Corp.). Cells on TCPs were imaged using a Nikon Eclipse TE300 inverted epifluorescence microscope (Nikon Corp., Japan) and a Nikon Coolpix 990 digital camera (Nikon Corp.). Cells on gradient substrates were imaged using a Leica DMI upright microscope with epifluorescence capabilities (Leica Microsystems AG, Wetzlar, Germany). At least two images were captured for each location using a digital camera (Hamamatsu Photonics KK, Hamamatsu City, Japan) and Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Live and dead cells on whole gradient substrates (n = 4) were quantified.
2.9. Annexin V staining to assess apoptosis

Apoptosis evaluation was used to complement the viability staining. Annexin V staining allows for a more sensitive study of overall cell health, as compared to the viability stain. Apoptosis in the non-adherent, floating cell population was evaluated on whole and sectioned gradient substrates that had been aged for 7 days. At 24 h after seeding, the growth medium containing the non-adherent cells was removed and supplemented with 1 µl each of commercially prepared solutions containing 7-aminoactinomycin D (7-AAD) and Annexin V-fluorescein isothiocyanate (Guava Nexin Apoptosis Assay, Guava Technologies, Hayward, CA). Using these dyes, viable cells remained unstained, early apoptotic cells stained with the Annexin V conjugate, late apoptotic cells stained with both dyes, and dead cells stained with 7-AAD. This assay does not identify the mode of death for cells that have already died. Cell count and the fluorescence intensity from each dye were measured using a Guava PCA flow cytometer (Guava Technologies). Triplicate readings were taken from each sample, and at least three individual polymer samples were analyzed for the whole substrates and substrate sections. The total number of non-adherent cells was represented as a fraction of the number of initially seeded cells. Viable, early apoptotic, late apoptotic and dead cells were expressed as a percentage of the total non-adherent cell population.

Apoptosis in adherent cells was determined using Annexin V staining and epifluorescence microscopy. Aged, whole substrates were seeded, cultured for 24 h, washed twice with cold annexin binding buffer (ABB, 10 mmol l\(^{-1}\) HEPES sodium salt, 140 mmol l\(^{-1}\) NaCl, 2.5 mmol l\(^{-1}\) CaCl\(_2\), pH 7.4), and incubated with staining solution for 15 min at room temperature. The staining solution consisted of ABB with 10% (volume fraction) Annexin V-Alexa Fluor 488 (Invitrogen Corp.) to stain apoptotic cells, 2 µmol l\(^{-1}\) ethidium homodimer-1 (Invitrogen Corp.) to stain cells with compromised membranes, and 10 µg ml\(^{-1}\) Hoechst 33342 (Invitrogen Corp.) to stain all cell nuclei. The cells were washed with ABB and imaged.

2.10. Quantitative real-time RT-PCR to measure gene expression

Stimulated macrophages can initiate an inflammatory reaction, so gene expression levels for two inflammatory cytokines, IL-1β and TNF-α, were measured to determine the effects of conversion level on inflammation in the RAW 264.7 cells. At 24 h post-seeding, aged whole substrates were removed from the growth medium, cut with a razor blade into small pieces of known conversion, and immediately placed in lysis buffer supplied in the RNeasy Mini Kit (Qiagen, Valencia, CA). Each piece encompassed a 3% range in conversion. Cell lysates were homogenized using QIAshredder spin columns (Qiagen), and total RNA was isolated following the RNeasy kit protocol including on-column DNase digestion. The RNA was eluted with water, treated with RNA Secure (Ambion, Austin, TX) and stored at \(-80^\circ C\).

RNA samples from three individual, gradient substrates were each assayed in triplicate using quantitative, real-time RT-PCR with an iCycler (Bio-Rad Laboratories, Hercules, CA) and the QuantiTect SYBR Green RT-PCR Kit (Qiagen) as previously described [35]. The 18 S ribosomal subunit served as the internal reference, and plasmids containing cDNA inserts for 18 S, IL-1β and TNF-α (ATCC) were used for the standard curves. Primers (MWG-Biotech Inc., High Point, NC) are listed in Table 2. The RT-PCR protocol began with reverse transcription (30 min, 50 °C) and activation (15 min, 95 °C). The cycling included 55 cycles of denaturation (30 s, 95 °C), annealing (1.5 min, 58 °C) and extension (2 min, 72 °C). The final products were analyzed using a melt curve from 50 to 95 °C and also by electrophoresis in a 2% agarose gel with ethidium bromide staining. Copy number was calculated by matching the threshold cycle for each sample to its corresponding standard curve and normalizing to the 18 S internal reference.

2.11. Statistical analysis

AFM and water contact angle measurements from two separate, aged gradient substrates were evaluated using an unpaired, two-tailed Student’s t-test with \(p < 0.05\) to indicate statistical significance. All other assays were performed on at least three separate aged gradient substrates, either whole or sectioned, as described above. Viability, flow cytometry and RT-PCR data were analyzed using one-way analysis of variance (ANOVA) and a comparison-wise Fisher’s least-significant difference (LSD) test to indicate statistical significance, with a confidence interval of 95%. The standard deviations computed from multiple measurements were used as an estimate for the standard uncertainty associated with each measurement technique.

3. Results

3.1. Gradient substrate fabrication and characterization

Conversion gradient substrates were fabricated by exploiting the non-uniform illumination of the Triad light source. The visible-light curing unit consists of a halogen
light bulb shining down on a sample stage, so the resulting stage illumination varies as a function of radial position with the highest intensity at the center of the stage. Thus, the final conversion profile was dependent upon the cure time and the sample position.

NIR spectra taken before and after curing revealed a reduction in the 6164 cm\(^{-1}\) peak area after curing, while the internal reference peak area at 4623 cm\(^{-1}\) was unchanged (Fig. 2). Conversion profiles for the gradient substrates were calculated for cure times of 40, 60 and 100 s (Fig. 3). The substrates selected for cell studies were cured for a total of 40 s, resulting in a conversion ranging from 43.0 ± 1.7% at one edge to 61.2 ± 0.7% at the opposite edge.

Surface characteristics were evaluated on aged, whole gradient substrates to determine if hydrophilicity and surface roughness were changing as a function of conversion and possibly affecting the cell response. Surface analysis of aged substrates revealed no significant differences (\(p > 0.05\)) as a function of conversion. Water contact angle measurements varied from 60.0° ± 2.3° to 61.4° ± 1.1° for the advancing measurements and from 40.2° ± 0.7° to 44.0° ± 3.1° for the receding measurements, low to high conversion. AFM measurements of r.m.s. roughness ranged from 8.3 ± 7.4 nm at 45% conversion to 6.1 ± 6.7 nm at 60% conversion.

3.2. Gradient substrate aging and extract analysis

The presence of leachable components that diffused into the PBS during the aging process was determined qualitatively using UV absorption. The overall absorption of the leachables decreased as a function of time, but leachables were still detectable on day 7. To better understand the source of the leachable components, gradient substrates were sectioned and then aged for 7 days. The amount of leachables depended upon the conversion, with 45% conversion producing the most leachables and 60% conversion producing the lowest concentrations of leachables, as anticipated.

Spectra for the individual components (Fig. 4a) contained the following absorption peaks: BisGMA (228, 277, 284 nm), TEGDMA (227 nm), CQ (225 nm) and 4EDMAB (228, 311 nm). As the concentrations were...
decreased, the peaks at 225–228 nm shifted to the left. Spectra for the extracts (Fig. 4b) contained absorption peaks at 225 nm (corresponding to BisGMA, TEGDMA, CQ and 4EDMAB), 270 nm (corresponding to BisGMA) and 315 nm (corresponding to 4EDMAB). The 225 nm peak also shifted to the left as the leachables were diluted. Thus, the leachable spectra were found to overlap with all four of the individual components. The absorption spectra for TEGDMA and CQ were very similar, so additional studies would be needed to calculate the quantity of each component present in the extracts.

Leachables from gradient substrates aged in RPMI growth medium were evaluated using a cell viability assay. Whole gradient leachables from days 1 and 3 were cytotoxic and resulted in decreased cell viability, whereas leachables from days 5 to 7 did not reduce viability as compared to controls on TCPS (Fig. 5). When leachables from separate sections were studied, cell viability increased as the conversion level of the section increased (data not shown).

3.3. Calcein/ethidium staining for viability analysis

Cell viability was selected as the initial screening assay to assess overall cell response to the substrates due to the assay’s simplicity and ease of completion. Two dyes were used to determine viability. Cells with intracellular esterase activity and intact cell membranes converted calcein AM to green fluorescent calcein (indicative of live cells). In cells with compromised membranes, ethidium homodimer-1 entered and bound to nucleic acids to fluoresce red (indicative of dead cells). Viability images (Fig. 6a) were quantified to reveal that viability increased with increasing methacrylate conversion (Fig. 6b). Cell viability on TCPS controls was similar to viability at 55% and 60% conversion, whereas 45% and 50% conversion had significantly reduced viability. Moreover, cell images revealed few adherent cells at locations with a conversion less than 50%.

3.4. Annexin V staining to assess apoptosis

Evaluation of apoptosis in non-adherent cells provided information on overall cell attachment and cell health. Enumeration of non-adherent cells via flow cytometry revealed that over 15% of the initially seeded cells on whole gradient substrates were non-adherent at 24 h, whereas less than 8% were floating in TCPS control wells (Fig. 7a), indicating that cells were less likely to either initially attach or to remain adherent on the substrates. To determine if this was a function of the conversion, substrates were cut and cultured as individual sections. Sections encompassing conversion levels of 55% and 60% were not significantly different from TCPS. However, sections with 45% and 50% conversion were each significantly different from all other conversions, with the lowest conversion of 45% having the highest percentage (49%) of floating cells.

Within that floating cell population, the viable, early apoptotic, late apoptotic and dead cell fractions were measured.
(Fig. 7b), and statistical differences were calculated using ANOVA and Fisher’s LSD tests. As compared to the TCPS controls, whole and sectioned gradient substrates had statistically fewer viable cells (except for the 50% section), fewer early apoptotic cells and more dead cells, indicating an overall increase in cell death on the gradient substrates. In comparing the substrate sections, the 60% section had reduced cell death as compared to 45%, 55% and whole gradients, and the 45% section had more cell death than the 50% and 60% sections.

Apoptosis in adherent cells on whole gradient substrates was evaluated using fluorescence microscopy. LPS was used as a positive control to stimulate apoptosis at 24 h. Apoptosis was reduced as the conversion increased (Fig. 8). As with the viability staining, areas of 45% conversion had very few cells adherent after 24 h of culture.

3.5. Quantitative real-time RT-PCR to measure gene expression

Quantitative real-time RT-PCR revealed no significant changes due to the methacrylate conversion in the expression levels of IL-1β and TNF-α after 24 h of culture (Fig. 9). Overall, as compared to cells on TCPS, cells cultured on the whole substrates had significantly elevated TNF-α gene expression but unchanged IL-1β expression. TNF-α expression at 45%, 50% and 55% conversion was upregulated as compared to TCPS controls. IL-1β
LPS significantly elevated IL-1β expression positions on the gradient substrate. The endotoxin decrease shrinkage may come at the cost of a lower final photopolymerized dental materials today, but efforts to other material properties. For instance, post-polymerization conversion possible. However, the conversion does affect one might think all materials should aim for the highest conversions have less cytotoxicity and improved cell viability, so of the oral cavity. Our results suggest that higher conversion. Therefore, understanding the effects of the conversion level on other aspects, such as the biological response, is important for and relevant to dental research.

Culturing cells on a gradient surface poses significant technical challenges, especially if a large variation in cellular response, such as viability, is present. If a significant portion of the material is highly toxic, cells on the entire sample may be adversely affected. This was evident with the gradient substrates when the lowest conversion was below 35%, in which case cell death was significant even at the higher conversions of 60% (data not shown). Since the cells share a common growth medium, this uniformly increased cell death may have been due to soluble factors from one area diffusing and affecting cells on other areas. Possible factors include toxic levels of extractables leaching from significantly undercured portions of the substrate, intracellular signaling via secretion of cytokines and other signaling molecules, and cellular debris from dead and dying cells. On the other hand, if the conversion range was high (55–65%), no variation in viability was seen. A 40 s cure time was chosen to produce gradient substrates with a minimum conversion of 43.0 ± 1.7%. The resulting conversion profile on these substrates induced a range of cell responses, an important aspect for the development and assessment of methods to analyze cell response.

Substrates were aged prior to cell seeding in order to remove any globally toxic effects. Results seen after the aging process should be due to either the polymer network itself or the leachables present in the local microenvironment. Leachable studies were conducted to determine an appropriate duration for substrate aging prior to culturing the cells. When substrates were aged for 1 day prior to cell seeding, few cells were viable after 24 h of culture on the substrates. One plausible explanation is that toxic levels of extractables leaching from the substrate, particularly from undercured portions. UV spectroscopy was chosen as a simple technique to monitor the extent of overall leachables present in the extracts. The primary peaks of interest were 225 and 270 nm for the absorption from TEGDMA/CQ and BisGMA, respectively. Evaluation of these wavelengths was an efficient method to monitor leachables and could easily be used for simple leachable studies in the future. UV absorption confirmed significant levels of leachables from the gradient substrates for at least 7 days. Other studies have also shown that molecules with high aqueous solubility leach from dental polymers [7,11]. Although leachables were still detectable on day 7, the concurrent studies of leachable toxicity demonstrated that cell viability was unaffected by extracts from day 5 and later. Therefore, all substrates were aged for 7 days prior to cell seeding since the leachable concentration after 7 days of aging was no longer significantly cytotoxic.

Surface features are another group of material properties known to affect cell behavior. It is possible that variations in surface features due to the changing conversion may affect the cell viability and response. However, AFM studies of aged substrates revealed no significant differences

Fig. 9. Gene expression of inflammatory cytokines IL-1β and TNF-α measured using real-time RT-PCR. TCPS-polymer data represent the cells in the well with the polymer but attached to the TCPS. Data points represent the average of triplicate readings from three individual substrates, and error bars represent one standard deviation and are the estimate of standard uncertainty. *Significantly higher gene expression as compared to the TCPS control (95% confidence interval).
in surface roughness due to the conversion level, and no significant differences were detected in water contact angle across the aged substrates. This was expected given our chemistry and preparation techniques. The aging process may affect the surface architecture due to the leaching of unincorporated material, but our results suggest that any changes on the surface did not result in significant differences across the gradient substrate. Therefore, it is unlikely that surface features contributed to the variation seen in cell response.

Cells seeded on the aged conversion gradient substrates showed a range of cell responses as a function of conversion. No global toxicity was seen, and cells at higher conversion levels of 55% and 60% had viability and apoptosis similar to the TCPS negative controls. Thus the reduced viability and increased apoptosis on areas of lower conversion were indeed due to the local conversion level of the polymer. The conversion affects both the polymer network and the leachables, so effects on the cells may have been due to pendant groups on the polymer surface or to locally released leachables such as unreacted monomer, initiator or reaction byproducts. The gradient substrates were not disturbed during the 24 h culture period, so toxic levels of leachables could be present at the low conversion end. The leachable concentration would decrease as a function of the distance from the low conversion end, eliminating the toxic effects at significant distances. This is supported by results showing that cells in the same well as the polymer but adherent to TCPS also exhibited decreased viability and increased apoptosis similar to the TCPS negative controls. Cells on other areas of the TCPS away from the low conversion end had viability similar to that of negative controls. Moreover, cell attachment was significantly reduced on the substrate at conversions less than 50%, but many cells were attached on the TCPS in the vicinity of the low cure portion of the substrate (data not shown). Cells on the low cure end of the substrate (data not shown). Cells on the low cure end of the substrate (data not shown). Cells on the low cure end of the substrate (data not shown). Cells on the low cure end of the substrate (data not shown).

The floating cell analysis, which indicated an increase in both the number and the apoptosis/death of non-adherent cells at lower conversions, also supports a combined toxicity from the leachables and the polymer network. The increased number of floating cells suggests that the cells did not adhere well to the surface, and the apoptosis and cell death suggest the presence of toxic leachables in the growth medium. It is therefore likely that in addition to effects from the undercured polymer network, leachables from the low conversion end of the substrate remained in the local area to negatively affect the cells. Future studies could isolate effects from the polymer network by completely removing the leachables, perhaps using thin films as the test material to reduce the diffusion distance and accelerate release of any unincorporated materials. The polymers and leachables could then be tested separately.

The final bioassay evaluated gene expression of inflammatory cytokines. Analysis of inflammatory cytokines complements the viability and apoptosis assays and provides a more comprehensive assessment of the macrophage-specific response. In the control samples, basal levels of TNF-α in LPS-stimulated macrophages were expected at 24 h, since TNF-α expression has been shown to be upregulated by LPS at earlier time points and then return to normal levels within 24 h [38,39]. Although no significant differences were seen as a function of conversion, some samples did not have detectable IL-1β expression at 45% conversion (these samples were not included in the statistical analysis). The corresponding reduction in viability and increase in apoptosis and cell death seen at 45% conversion indicated that cells on this portion of the substrate were significantly affected by the incomplete conversion. This compromised state may have been interfering with normal cell functions, including production of basal levels of IL-1β. Further, this may indicate a reduced ability to respond to bacterial challenges, as demonstrated for solutions containing BisGMA, TEGDMA or leachables from dental adhesives containing BisGMA [5,14,15].

5. Conclusions

Conversion gradient substrates were used to evaluate the effects of methacrylate conversion on cell response, with both local leachables and the undercured polymer network potentially affecting the cell response. Based on the bioassay results, it is recommended that equimolar BisGMA/TEGDMA mixtures be cured to over 50% conversion. Conversions of 45% and 50% demonstrated acute toxicity at 24 h, but conversions greater than 50% resulted in lower concentrations of leachables, improved cell adhesion/retention, decreased numbers of floating cells, increased cell viability and decreased apoptosis. Interestingly, conversion level did not influence the inflammatory cytokine response to these materials. Overall, this in vitro screening platform was able to distinguish the cell response relative to incremental changes in a given parameter, namely conversion, on single test specimens. This methodology was developed using unfilled BisGMA/TEGDMA polymers and a murine macrophage cell line, but its utility extends to the initial biological screening of novel dental polymers/filled composites and any adherent cell type. This will establish the biologically acceptable baseline from which physical and mechanical properties can then be optimized for a given application.

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References


