DENTAL MATERIALS 23 (2007) 1211–1220

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Two-dimensional gradient platforms for rapid assessment of dental polymers: A chemical, mechanical and biological evaluation☆

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ARTICLE INFO

Article history:
Received 26 September 2006
Accepted 14 November 2006

Keywords:
Dental restorative materials
Combinatorial
Degree of conversion
NIR
FTIR-RM
Mechanical properties
Nanoindentation
Viability
Cytotoxicity

ABSTRACT

Objectives. The increased usage of composite dental restorations underscores the need for continued improvements in material properties. Well-controlled sample fabrication and reproducible methods to quantify and compare material properties will accelerate material design and optimization. Our objectives were to fabricate samples encompassing a range of processing parameters and develop techniques that systematically quantify multiple properties of these samples, thus reducing sample-to-sample variation while concurrently testing numerous processing conditions.

Methods. Gradient samples were prepared to evaluate the effects of composition and irradiation time. Comonomer ratio of 2,2-bis[*p*-2′-hydroxy-3′-methacryloxypropoxy]-phenyl]propane (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) was varied discretely, and irradiation time was varied continuously across each composition. Degree of conversion was measured using infrared spectroscopy, mechanical properties were evaluated using nanoindentation, and cell viability and density were quantified using fluorescence microscopy.

Results. Higher BisGMA contents increased elastic moduli while higher TEGDMA contents increased conversions. Cell response depended only on irradiation time and not composition, with conversions of at least 52% and 60% required for unaffected viability and cell density, respectively. A single composition–irradiation combination to achieve all of the 'best' properties (highest conversion, highest elastic modulus, lack of cytotoxicity) was not identified, illustrating the necessity of testing all combinations for multiple relevant properties.

Significance. Simultaneously screening composition and conversion increased the experimental throughput and allowed for the quantification of chemical, mechanical, and biological properties in a controlled, reproducible fashion. This 2D gradient approach is useful for optimizing compositions and processing parameters to achieve the desired combination of properties.

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doi:10.1016/j.dental.2006.11.020

1. Introduction

Polymeric composites are widely used as dental restorations, yet a number of challenges remain. In a recent review on the current status and challenges of restorative dental composites, the lack of measurement techniques for intra- and inter-laboratory data comparison was repeatedly noted [1]. This is important because data relating chemistry, physical behavior, and clinical performance must be thoroughly evaluated prior to clinical applications. Two main factors contribute to the difficulties in comparing and correlating data: (1) a lack of standard test methods, and (2) variation in the physical test specimens. These difficulties are due in part to the complexity of dental composites. The matrix alone is typically comprised of a binary or ternary resin mixture in which the material properties can be easily adjusted by compositional changes and a large number of processing parameters. In the absence of standard test methods, one can still improve the data comparability if a series of uniform samples is attainable. The variation in test specimens arises from the difficulties in preparing identical samples for various measurement methods that often require different sample geometries and curing protocols. This is particularly prevalent for photopolymerized dimethacrylate polymers because the resulting properties can vary greatly depending on the sample preparation procedure. Preparing a series of systematically varied samples for multiple materials characterization can be a challenging task given the sensitivity of the polymerization process. To overcome those measurement issues, it has been recommended that "test suites" be developed to facilitate data correlation and inter-laboratory comparison [1].

Properly designed test suites should accelerate current efforts in "rational" material design to achieve new or improved materials by relating various material properties and identifying optimum synthetic strategies. For example, the degree of conversion (DC) is one of the benchmark properties often used to relate various material properties and predict clinical performance. A thorough test of multiple material properties is labor intensive, and hence most research is focused on identifying the "best" properties of a few discrete samples rather than using systematic approaches to evaluate an entire composition range. A test platform to characterize multiple material properties would be extremely valuable. It is therefore our goal to develop fabrication methods for producing systemically varied samples and corresponding characterization techniques for quantifying several material properties on these samples.

In developing our test suites, combinatorial approaches allow for systematic evaluation of various material parameters. High throughput and combinatorial methods have become increasingly popular in materials discovery, characterization, and optimization [2]. Combinatorial methods have several advantages over traditional techniques, including faster data acquisition, more thorough examination of experimental variables, equal processing conditions for a given specimen, and lower experimental error. The use of two-dimensional combinatorial arrays would allow multiple material properties to be characterized as a function of two predefined variables. Appropriately designed combinatorial methods greatly reduce uncertainties associated with sample differences as well as increase the experimental throughput. A thorough mapping of properties would rapidly identify compositions or processing conditions leading to the most desirable properties overall. It would be useful to optimize the chemical structure and composition using combinatorial approaches prior to in depth analyses. In addition to rapid screening of material properties, combinatorial platforms accommodating multiple characterization techniques permit direct data comparison of the material properties. Given the numerous attractive properties of high throughput and combinatorial methods, it is no surprise that they have been used to study the degree of conversion [3], reaction kinetics [4], and reactivity ratios [5] of free radical copolymerizations. In addition, mechanical properties have been characterized using nanoindentation [3,6–8].

Combinatorial methods have also been used to evaluate the cell response [9]. Appropriate cell response for a given application is essential for clinical use of new and existing materials. Materials with poor biocompatibility can lead to hypersensitivity, inflammation, pulp tissue necrosis, and a loss of pulp vitality, often resulting in restoration failure and replacement. A simple and straightforward screening process to assess the toxicity of the material and the corresponding cell response would be useful prior to in depth *in vitro* or *in vivo* characterization.

In the current study, we have developed a test suite for the characterization of dental polymers by combining multiple measurement techniques. This test suite includes DC by near infrared spectroscopy (NIR) and Fourier transform infrared spectroscopy-reflectance mode (FTIR-RM), mechanical characterization by nanoindentation, and bioassays modified specifically for combinatorial studies. All these properties are essential for the clinical performance of dental composites. Combinatorial approaches were selected to examine the relationships between chemical composition, conversion, mechanical properties, and cytotoxicity of two-component dimethacrylate polymers. Two-dimensional gradient samples varying in resin composition and irradiation time along orthogonal axes were prepared and characterized. The resin mixtures were comprised of bisphenol-A dimethacrylate (BisGMA) base monomer blended with the diluent monomer triethylene glycol dimethacrylate (TEGDMA) at different compositions.

2. Materials and methods

2.1. *Materials*[2]

BisGMA and TEGDMA were obtained from Esstech Inc. Camphorquinone (CQ) and ethyl 4-*N*,*N*-dimethylaminobenzoate (4E) were purchased from Aldrich Corp. Methacryloxypropy-

[2] Certain commercial materials and equipment are identified in this work for adequate definition of the experimental procedures. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology that the material and the equipment identified is necessarily the best available for the purpose.

ltrimethoxysilane (MPTMS) and *n*-octadecyltrimethoxysilane (OTMS) were purchased from Gelest, Inc. Cell culture reagents were purchased from Invitrogen Corp. All reagents were used as received.

2.2. Fabrication of gradient samples

BisGMA and TEGDMA were mixed to obtain the following compositions: 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, and 20:80 mass ratios. The resin mixtures were activated for blue light photopolymerization with 0.2% CQ and 0.8% 4E (by mass fraction) and stored in the dark until use.

The gradient specimen consisted of a discrete gradient in monomer composition along one axis with an orthogonal gradient in methacrylate conversion, which was varied in a continuous fashion. A sandwich mold was prepared using two surface treated microscope glass slides and a poly(dimethylsiloxane) (PDMS) spacer (thickness ≈1.5 mm) with 5–7 channels (3 mm × 65 mm) stamped out (Fig. 1). One glass slide was surface treated with MPTMS to enhance adhesion between the dimethacrylate polymer and glass slide while the other glass slide was surface treated with OTMS to allow easy separation of the polymer and glass slide. Resin mixtures of different compositions were syringed into the separate channels, thus keeping the monomer composition discrete. The assembly was placed 10 cm beneath a light source (Dentsply Triad 2000 replacement Tungsten halogen light bulb 250 W, 120 V) and positioned with one edge of the 20:80 composition directly under the center of the light source. The sample for mechanical testing was irradiated for 20 s on each side, then placed under a shield such that only 15 mm of each composition was uncovered and further exposed for 1 min per side. This photopolymerization process generated conversion gradients on a single glass slide (gradient sample shown in Fig. 1). Samples for the cell studies were irradiated for 15 s per side positioned with one edge of the 20:80 composition directly under the center of the light source. The samples were then partially shielded and further exposed for 30 s on each side.

All measurements were carried out at least 24 h after light exposure to ensure that the conversion no longer changed with post-cure time. Storage under ambient light conditions over 4 weeks did not notably change the DC on the gradient sample. A notch was made across the composition gradient at the high conversion end and was defined as the zero position for subsequent measurements. Conversion measurements and mechanical testing data were collected and reported over 50 mm at 5 mm intervals beginning at the zero position for each composition. Bioassay data was collected every 10 mm starting with the zero position.

2.3. Viscosity of dental resin mixtures

Steady-shear measurements of the resin mixtures were performed using a Rheometric Scientific ARES instrument in the cone-and-plate configuration (diameter = 50 mm, cone angle = 0.04 rad). All samples exhibited the Newtonian behavior (constant viscosity) over the entire shear rate studied (1–100 s^{-1}). Duplicate measurements showed excellent reproducibility with a relative standard uncertainty of 1%.

2.4. FTIR spectroscopy

Transmission NIR spectroscopy was performed using a Nicolet Magna 550 FTIR spectrometer (Madison, WI) configured with a white light source, a CaF_2 beam splitter, and an InSb detector. The NIR spectra in the region of 7000–4000 cm^{-1} were acquired from 64 co-added scans at 6 cm^{-1} resolution. The gradient sample was clamped to a card with a wide opening for the NIR beam and was shifted vertically or horizontally so that the appropriate location on the sample could be analyzed. The relative uncertainty associated with the NIR measurements is 5%.

The FTIR-RM analyses were performed using a Nicolet Magna-IR 550 FTIR spectrophotometer interfaced with a Nic-Plan IR microscope operated in reflectance mode. The microscope was equipped with a video camera, a liquid nitrogen cooled mercury cadmium telluride (MCT) detector and a computer controlled *x*–*y* translation stage. A total of 64 scans were collected from 650 cm^{-1} to 4000 cm^{-1} at 8 cm^{-1} resolution with a beam spot size of 300 μm × 300 μm. Each sample was manually focused before data collection. The reflectance spectra were proportioned against a background of a gold-coated disk and transformed to absorbance spectra using the Kramers–Kronig transform algorithm [10] for dispersion correction.

2.5. Nanoindentation

Nanoindentation measurements were performed using a MTS Nanoinstruments NanoXP instrument (Oak Ridge, TN) equipped with a 10 μm radius, 90° diamond cone indenter. The continuous stiffness method, using 45 Hz, 5 nm dynamic

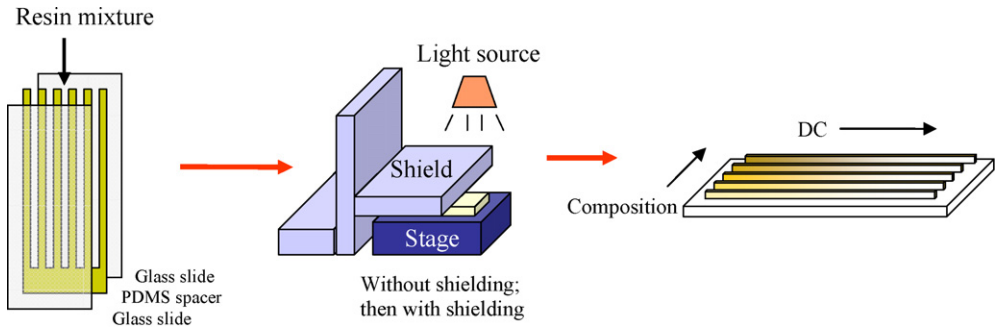

Fig. 1 – Preparation of 2D gradient samples varying in resin composition and DC.

oscillations, was used to determine the elastic modulus continuously throughout the loading portion of the experiment [3]. The reported modulus values are the average of the moduli obtained over a depth range from 1000 nm to 4000 nm. All indentation experiments were conducted using a strain rate of $0.05\,s^{-1}$. The relative uncertainty associated with the nanoindentation measurements is 3%.

2.6. Cell culture

Cell experiments were performed using the murine RAW 264.7 macrophage-like cell line (passages 8–12) from the American Type Culture Collection (ATCC TIB-71, Manassas, VA). Cells were maintained in humidified incubators (5% by volume CO_2, 37 °C) and cultured in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% (volume fraction) heat-inactivated fetal bovine serum.

2.7. Cell seeding

Gradient samples were sterilized with ethylene oxide gas (Anprolene Sterilization System, Andersen Products, Inc., Haw River, NC) and degassed for at least 3 days at room temperature. The samples were then washed twice with phosphate buffered saline (PBS) and aged in PBS for 7 days at 37 °C to remove initial toxic leachables. The PBS solution was changed on days 1, 3, 5, and 7. After the PBS was removed on day 7, samples were washed with PBS and seeded with RAW 264.7 macrophages. An initial suspension of cells in 5 mL growth medium (1.2×10^6 cells/mL) was added to the top of the glass slide to cover the sample. The samples were undisturbed for 15 min to allow for initial cell attachment, and 60 mL growth medium containing an additional 6×10^6 cells (1×10^5 cells/mL) were added to fill the dish (150 mm) and completely immerse the samples. After 5 min, samples were transferred to the incubator. Negative controls consisted of cells seeded in six-well tissue culture polystyrene (TCPS) plates (2 mL per well of 1.5×10^5 cells/mL).

2.8. Quantitative viability assay

After 24 h, the samples were evaluated for viability using calcein acetoxymethyl ester (calcein AM) and ethidium homodimer-1 (EthD-1). Live cells with intact cell membranes and normal intracellular esterase activity converted the non-fluorescent, cell-permeable calcein AM to green fluorescent calcein that was retained by the intact cell membranes. EthD-1 entered cells with damaged membranes and was therefore excluded from live cells with intact membranes. Once inside the cell, it bound to nucleic acids and fluoresced red. Cells were simultaneously stained with Hoechst 33342 (H33342) to label all cell nuclei. The growth medium was removed, and fresh growth medium containing 2 μmol/L calcein AM, 2 μmol/L EthD-1, and 10 μmol/L H33342 was added to cover the cells. The dishes were incubated at 37 °C for 10 min prior to imaging.

Cells were visualized on a Leica DMA upright microscope with epifluorescence capabilities (Leica Microsystems AG, Wetzlar, Germany). Images were captured using a digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). The samples were imaged every 10 mm starting at the marked zero position for a total of six locations per composition. At each location, three separate fields of view were imaged.

Cell viability was quantified using macros written in Image-Pro Plus. Dead cells were determined by counting the number of nuclei stained with EthD-1. Live cells were calculated as the H33342 stained cells minus any cells stained with both H33342 and EthD-1. Viability was calculated as the live cells divided by the total number of cells (live plus dead). Data from the three fields of view were combined to produce one viability measurement for each position on each of the seven compositions. Cell density was calculated as the total number of cells divided by the total surface area included in the three fields. Eight separate gradient samples were analyzed, and results were binned with respect to conversion. Each bin encompassed a 4% conversion range, and overall the bins covered a 40% conversion range from 36% to 76%.

Viability and cell density data were analyzed statistically using analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test with a 95% confidence interval to indicate significant differences. The relative uncertainties associated with these measurements are 15% and 30% for viability and cell density, respectively.

3. Results and discussion

Two-dimensional gradient samples with orthogonal variation in two parameters (i.e., comonomer composition and DC) were fabricated and characterized. The same sample specimen can be used to generate data on chemical, mechanical, and biological properties for several compositions and a range of conversion values. The comonomer composition was purposefully varied in a discrete fashion since this provides several advantages from sample fabrication to data collection and analysis. Dental resins typically consist of a base monomer that is significantly more viscous than the diluent monomer(s), so co-syringing the monomers to form a continuous composition gradient is impractical due to insufficient mixing. As shown in Table 1, the viscosity increased by over two orders of magnitude over the composition range of

Table 1 – Viscosity of the BisGMA:TEGDMA monomers

	BisGMA content (%)						
	20	30	40	50	60	70	80
η_0 ($\times 10^{-3}$ Pa s)	22	39	81	186	513	1413	7304
η_0 standard deviation ($\times 10^{-3}$)	0.39	0.36	0.59	1.84	4.48	11.9	67.4

20–80% BisGMA (mass fraction). Combining fluids of a high viscosity mismatch without proper mixing would result in laminar flow. Keeping the compositions separate also allowed for the exact comonomer composition to be known, thus eliminating uncertainties that would arise from the complex data analysis necessary for determining both the monomer composition and DC simultaneously. In addition, each composition strip was designed to be wide enough for quantitative FTIR spectroscopy, to allow for multiple nanoindentation data collection, and to accommodate cell seeding and subsequent analyses. Thus, these samples achieved the purpose of characterizing multiple material properties on a single test specimen.

The DC of a given monomer composition depends on the time and intensity of light exposure. Different monomer compositions also have different polymerization kinetics. Since the complexity of the photopolymerization process does not allow precise control of the conversion gradient under the current set-up, the DC was carefully measured at each position. The DC was determined using FTIR spectroscopy both in the mid-IR and near-IR region. Since the sample was thick (≈1.5 mm), NIR was carried out in transmission mode;

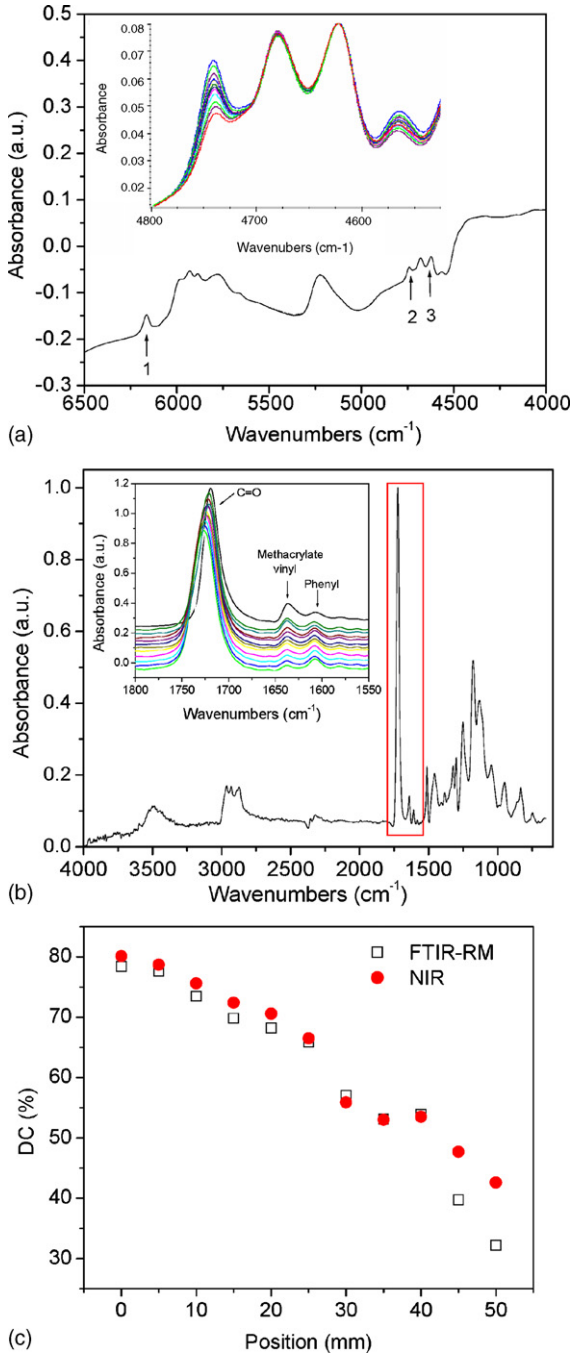

Fig. 2 – (a) A NIR spectrum of a typical composition, peaks 1 and 2 correspond to methacrylate vinyl and peak 3 corresponds to the phenyl stretch. The region containing peaks 2 and 3 is enlarged and overlapped for NIR spectra collected along the conversion gradient. (b) FTIR-RM spectrum of a typical BisGMA:TEGDMA polymer. The inset shows the methacrylate vinyl peak and the reference phenyl peak. (c) Conversion determined using NIR and FTIR-RM as a function of gradient position.

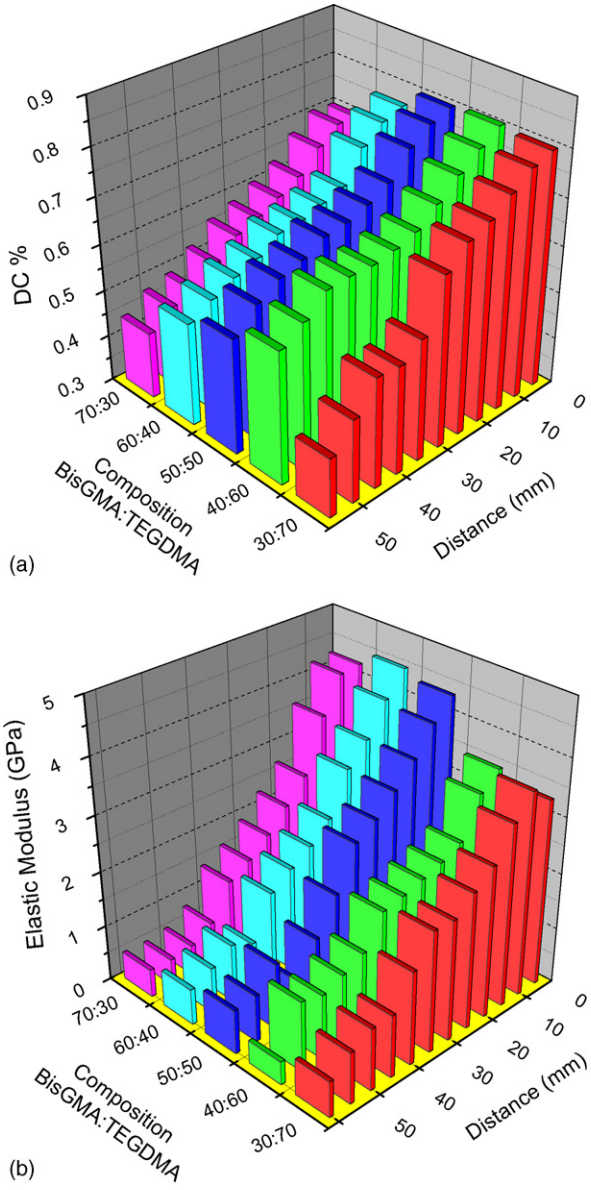

Fig. 3 – DC (a) and elastic modulus (b) for a gradient specimen varying in irradiation and composition.

however, FTIR spectroscopy in the mid-IR range had to be performed in the reflectance mode. As such, results obtained from NIR correspond to DC averaged through the entire sample depth whereas FTIR-RM results represent DC calculated from the top few microns of the sample surface.

A NIR spectrum collected from a typical composition is shown in Fig. 2a. Two characteristic absorption bands (4743 cm^{-1} and 6165 cm^{-1}) represent the methacrylate = C—H stretch and can be used to monitor the DC. Both peak height and area decreased as the amount of irradiation increased, indicative of increased methacrylate conversion. The specific region of interest between 4525 cm^{-1} and 4800 cm^{-1} is enlarged and overlapped for spectra collected along the conversion gradient (Fig. 2a inset). The overlapped spectra reveal that while the absorption band at 4743 cm^{-1} changed, the aromatic C—H absorption at 4623 cm^{-1} remained essentially constant. Similar NIR spectra were observed for the all comonomer compositions.

For the DC quantification using NIR spectroscopy, measuring the methacrylate peak height at 4743 cm^{-1} and normalizing it against the aromatic peak height at 4623 cm^{-1} provided the most reliable and reproducible results. An alternative method for calculating the DC is to directly monitor the methacrylate absorption and then correct it for any changes in sample thickness [11], but this method did not produce satisfactory results with the current system. Conversion calculations based on the 6165 cm^{-1} band normalized against the 4623 cm^{-1} band were less reproducible presumably due to baseline shifts over the large wavenumber difference.

We also used FTIR-RM to characterize the DC on the gradient specimen. FTIR-RM, a method for characterizing surface chemical composition both qualitatively and quantitatively, has been used previously to map the curing of epoxy films [12], polymer blends [8,13], human crown dentin [14], and calcified deposits on polyurethane implants [15], but has not been used to study the conversion of dimethacrylates. A typical FTIR-RM absorbance spectrum is illustrated in Fig. 2b and is comparable to that obtained from more commonly used transmission FTIR. The region between 1550 cm^{-1} and 1800 cm^{-1}, which includes the carbonyl, methacrylate vinyl, and phenyl stretches, is expanded in the inset of Fig. 2b. The expected trend was observed in which the methacrylate vinyl peak decreased with increased irradiation. Using the phenyl peak height as the internal standard, the DC was calculated as the reduction in the methacrylate peak height, as typically performed.

FTIR-RM is used less often because it is more complex to perform than transmission FTIR and also requires the Kramers–Kronig transformation procedure. In order for the data to be valid, the sample refractive index and surface roughness should be identical. The sample roughness on BisGMA:TEGDMA conversion gradients was comparable from one end to the other. Changes in methacrylate refractive index were relatively small upon polymerization [16], especially within the conversion range examined under the current

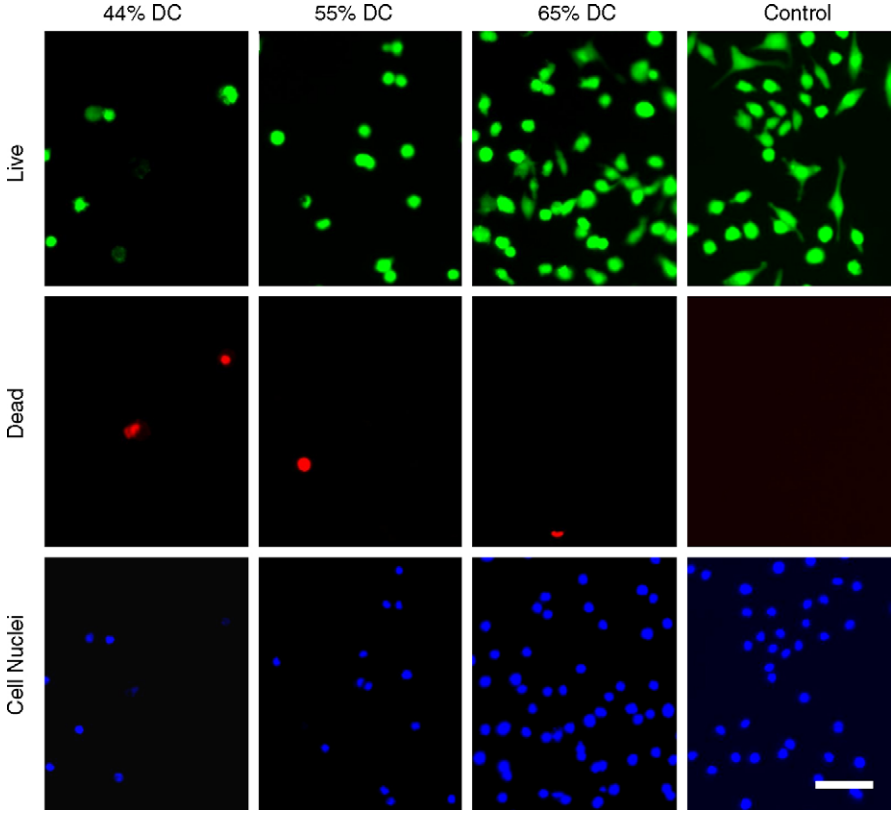

Fig. 4 – Viability stain for cells cultured on a typical composition. Images are shown for three DC levels and for control cells cultured on tissue culture polystyrene (TCPS). Live cells were detected with calcein AM, dead cells were labeled with EthD-1, and cell nuclei were stained with H33342. Scale bar = 50 μm.

study. We estimate that errors resulting from the refractive index changes were small and did not significantly affect the DC calculation. In addition, the sample thickness varied with respect to position, so manual focusing prior to each data point collection was necessary. Although FTIR-RM is more complex, the results demonstrated that FTIR-RM could successfully determine the DC. Moreover, the DC obtained using FTIR-RM and NIR for a typical composition agreed well at the DC levels greater than 50%, with a greater discrepancy observed at low DCs (Fig. 2c). As noted earlier, NIR provides an average DC for the entire depth of the specimen whereas FTIR-RM provides the conversion for only the top few microns of the sample surface. These results suggest that the surface and bulk conversions are comparable over most of the conversion range; therefore, results obtained from NIR spectroscopy are appropriate measures of DC.

The DC calculated using NIR spectroscopy and the corresponding elastic modulus determined by nanoindentation for all compositions and all irradiation levels are illustrated in Fig. 3a. For these substrates, one end of the sample was irradiated for a longer time to drive the DC as high as possible, which we refer to as the ultimate conversion. For the BisGMA:TEGDMA system, all compositions had an ultimate conversion ranging between 70% and 79%. For compositions with high TEGDMA content (≥50%), the ultimate conversion was relatively constant (≈79%). The ultimate conversion was lower at higher BisGMA contents. In addition, the conversion range was consistently higher for the 50:50 and 40:60 compositions, presumably due to higher polymerization rates at these compositions. These trends are consistent with previous findings [17,18]. The corresponding mechanical properties (i.e., elastic modulus, Fig. 3b) demonstrate that compositions with a BisGMA content greater than or equal to 50% (by mass) exhibited higher moduli even though they had a lower conversion. The elastic modulus decreased for the lower BisGMA compositions. The chemical structure of the monomers and their mass ratio strongly affect the conversion, reaction kinetics, and properties. The rigid, more viscous BisGMA is known to reduce the DC while increasing the mechanical properties due to the stiffness of its backbone. The flexible, lower molecular mass TEGDMA is considered a reactive diluent. However, comonomer compositions containing higher TEGDMA fractions also have a higher number of methacrylates per volume. At a given reaction conversion, it is reasonable to conclude that the cross-link density is higher for a system containing more TEGDMA. In the current system, compositions with a higher BisGMA content had superior mechanical properties even though they had lower DC and lower cross-link densities, indicating that the stiffness of the BisGMA base monomer dominated the mechanical response. In our previous study on an ethylated BisGMA (EBPADMA):TEGDMA system, we found that the ability for TEGDMA to increase the cross-link density dominates the mechanical response of the resultant networks [3].

Bioassays were performed on the gradient specimens to complement the chemical and mechanical analysis. While many biocompatibility assays focus on the toxic effects of dental monomers or leachables [19–21], the current study focuses on the polymer toxicity. Each dimethacrylate monomer has a functionality of 4. At lower DC values, a significant amount of monomer is unreacted and unattached to the network, and therefore could leach out over time. It is also pos-

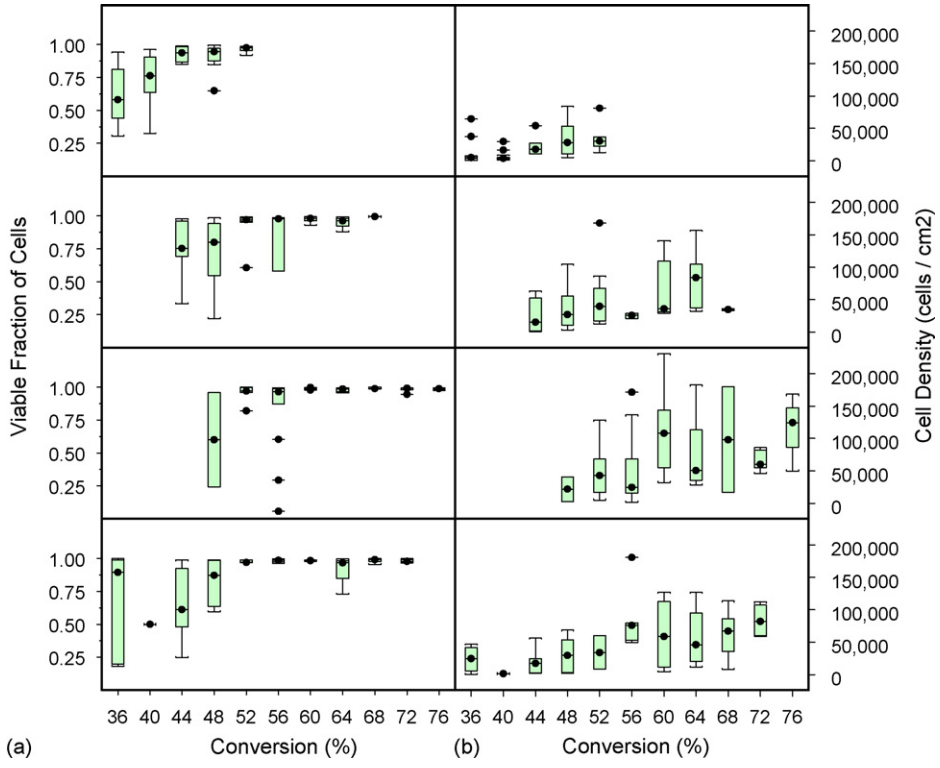

Fig. 5 – Quantification of viable cell fraction (a) and cell density (b) as a function of DC and composition. Results are shown for four of the seven BisGMA:TEGDMA compositions, (top to bottom, 80:20, 60:40, 40:60, 20:80).

sible for some monomers that only one dimethacrylate reacts and becomes coupled to the polymer network, leaving an unreacted methacrylate. These attached, unreacted methacrylates can potentially have long-term toxic effects. All gradient specimens were leached for 7 days to remove a majority of the leachable components. Our previous work established that 7 days leaching was sufficient to reduce the leachables to levels that no longer affected cell viability [22].

Cell viability and cell density after 24 h of culture were used to assess the acute cytotoxicity of the polymers. These two measures provide a quick screen of the cell response and are easily adapted for gradient substrates. Macrophage-like cells cultured directly on the gradient samples were fluorescently labeled to determine the cell response to these materials. As with the typical live/dead staining procedure, calcein AM and EthD-1 were used to label live and dead cells, respectively (Fig. 4). The quantification of cell viability was automated to increase the throughput of the bioassay. Dead cells were quantified by counting nuclei labeled with EthD-1, but the calcein stain was not sufficient to count the live cells due to artificially lower numbers of live cells when cell–cell contact was high. Instead, a third dye was utilized to provide an accurate, reproducible count of the live cells. H33342, which passes through cell membranes and binds to nuclei acids to produce blue fluorescence, was used to label all cell nuclei. Live cells were thus counted as the number of nuclei stained with H33342 and not co-stained with the dead cell stain. Co-stained cells were removed from the count by subtracting the dead cell image from the H33342 image. This method was verified manually and found to be accurate and reproducible. Even though the H33342 should provide a total cell count by staining all the nuclei, some dead cells stained brightly with EthD-1 and very dimly with H33342 while other dead cells stained brightly with both dyes. This variation in dead cell staining was likely due either to competition between the two dyes for the same nucleic acids or to differences in the degree of cell membrane disruption. Since the H33342 staining by itself did not provide an accurate count of total cells, the image subtraction method was utilized.

The automated image analysis was then used to quantify cell viability and density as a function of DC and composition (Fig. 5). Both cell viability and density provide important information regarding the material toxicity. Statistical analysis revealed that there were no significant differences in viability and density with respect to composition, meaning that for a given conversion level, there were no differences among the compositions. Therefore, all compositions were grouped together to evaluate cell viability and density as a function of DC (Fig. 6). A clear cutoff conversion was evident for both measures of cell response. Conversions below 52% resulted in significantly decreased viability. Interestingly, a higher conversion was required for improved cell density. Only conversions greater than 60% had unaffected cell densities at 24 h. Therefore, for this BisGMA:TEGDMA system, a minimum DC of 52% is required for normal cell viability while a minimum DC of 60% is needed for normal cell attachment and retention for all the comonomer mass ratios tested.

The difference in these minimally acceptable conversions may be the result of cell detachment. The average cell viability

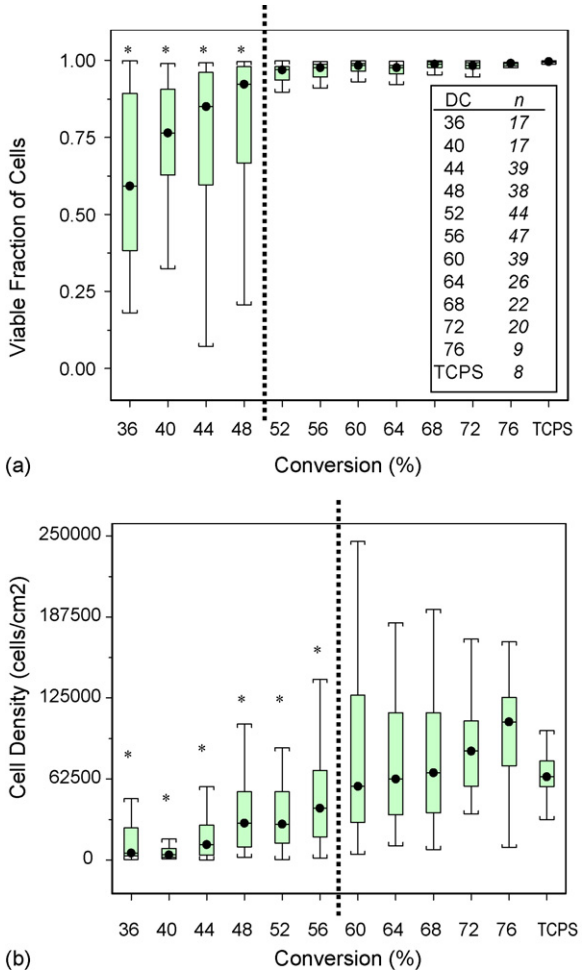

Fig. 6 – Standard box plots of viable cell fraction (a) and cell density (b) as a function of conversion for data pooled from all compositions. The TCPS control is also included. Sample size (*n*) is given for each conversion bin. Outliers have been omitted for clarity. Vertical dashed lines indicate the cutoff conversions. Conversions to the left of the line (*) are statistically different ($P < 0.05$) from all conversions to the right of the line, and conversions to the right of the line are statistically the same ($P > 0.05$).

does not fall below 60% even at the lowest DC of 36%. When the reduced cell attachment is taken into consideration together with the reduced viability at lower conversions, it is plausible that cells were either unable to attach initially due to surface toxicity or that cells detached from the surface after dying. If cells died and then detached either during the culture period or as a result of the solution changes during the staining procedure, the viable fraction would be artificially inflated due to the loss of dead cells. It is therefore possible that a conversion higher than 52% is required for optimal cell viability. Future studies could probe this using time-lapse microscopy to monitor cell attachment and detachment, or flow cytometry to evaluate non-adherent cells. It was not feasible to analyze the floating cells with respect to conversion in this study since the entire gradient sample shared a common growth medium. Therefore, effects on cell density are especially important, as

they may be a more sensitive screen of cytotoxicity than cell viability.

Although there were no statistical differences in cell response with respect to composition, this does not suggest that comonomer composition does not matter. Other material properties must also be considered. As shown in Fig. 3, increased BisGMA content reduced the conversion attained while increasing the mechanical properties. Even though the higher BisGMA content improved the elastic modulus, the reaction kinetics simply did not allow it to attain the high conversions seen in systems with more TEGDMA. Since its ultimate conversion is lower, it is therefore more likely to have a toxic effect if slightly, unintentionally undercured in a clinical setting. While higher BisGMA content might seem preferable for its superior mechanical properties, the biological response suggests that this should be carefully considered. These results clearly illustrate the importance of evaluating multiple properties in order to optimize a material for a given application. Our 2D gradient samples and corresponding test suites provide one such method to do so in a controlled, reproducible fashion.

4. Conclusions

The present study demonstrated that multiple properties can be measured on a single test specimen, and the results exhibited the similar trends and agreed well with those obtained from traditional one-at-a-time testing methods. Our methods of screening chemistry and processing parameters at the same time are advantageous over traditional methods, as they increase experimental throughput, reduce sample-to-sample variation, and reproducibly quantify multiple properties. This approach therefore addresses the measurement issues related to a lack of test methods and a variation in test specimens, both of which can undermine inter-laboratory data comparison. In addition, the current results reaffirm that a single optimum composition does not exist and highlight the need to thoroughly test all important properties for each combination of material and processing parameters. That being the case, the sample fabrication techniques and test methods in this study could easily be used to evaluate novel dental monomers and fillers to optimize the final combination of chemical, mechanical, and biological properties. This should facilitate future experimental design and accelerate the identification of promising candidate materials for clinical applications.

Acknowledgements

The dental resins were kindly donated by Esstech Inc. We would also like to thank Drs. Joy Dunkers, Forrest A. Landis, Joseph Antonucci, and Naomi Eidelman, as well as Mr. Edward Perry for their technical assistance.

REFERENCES

[1] Sakaguchi R, Review of the current status and challenges for dental posterior restorative composites: clinical, chemistry, and physical behavior considerations. In Summary of discussion form the Portland Composites Symposium (POCOS), June 17–19, 2004. Portland, Oregon: Oregon Health & Science University, Dental Mater 2005;21:3–6.

[2] Amis EJ. Combinatorial materials science—reaching beyond discovery. Nat Mater 2004;3:83–5.

[3] Lin-Gibson S, Landis FA, Drzal PL. Combinatorial investigation of the structure-properties characterization of photopolymerized dimethacrylate networks. Biomaterials 2006;27:1711–7.

[4] Johnson PM, Reynolds TB, Stansbury JW, Bowman CN. High throughput kinetic analysis of photopolymer conversion using composition and exposure time gradients. Polymer 2005;46:3300–6.

[5] Shaikh S, Puskas JE, Kaszas G. A new high-throughput approach to measure copolymerization reactivity ratios using real-time FTIR monitoring. J Polym Sci Part A: Polym Chem 2004;42:4084–100.

[6] Tweedie CA, Anderson DG, Langer R, Van Vliet KJ. Combinatorial material mechanics: high-throughput polymer synthesis and nanomechanical screening. Adv Mater 2005;17:2599.

[7] Warren OL, Wyrobek TJ. Nanomechanical property screening of combinatorial thin-film libraries by nanoindentation. Measure Sci Technol 2005;16: 100–10.

[8] Simon CG, Eidelman N, Deng Y, Washburn NR. High-throughput method for determining modulus of polymer blends. Macromol Rapid Commun 2004;25: 2003–7.

[9] Simon CG, Eidelman N, Kennedy SB, Sehgal A, Khatri CA, Washburn NR. Combinatorial screening of cell proliferation on poly(D,L-lactic acid)/poly(D,L-lactic acid) blends. Biomaterials 2005;26:6906–15.

[10] Chalmers JM, Everall NJ, Ellison S. Specular reflectance: a convenient tool for polymer characterisation by FTIR-microscopy? Micron 1996;27:315–28.

[11] Stansbury JW, Dickens SH. Determination of double bond conversion in dental resins by near infrared spectroscopy. Dental Mater 2001;17:71–9.

[12] Eidelman N, Raghavan D, Forster AM, Amis EJ, Karim A. Combinatorial approach to characterizing epoxy curing. Macromol Rapid Commun 2004;25:259–63.

[13] Eidelman N, Simon CG. Characterization of combinatorial polymer blend composition gradients by FTIR microspectroscopy. J Res Natl Inst Standards Technol 2004;109:219–31.

[14] Tesch W, Eidelman N, Roschger P, Goldenberg F, Klaushofer K, Fratzl P. Graded microstructure and mechanical properties of human crown dentin. Calcified Tissue Int 2001;69: 147–57.

[15] Eidelman N, Fowler BO, Joshi R, Levy RJ. Characterization of calcified deposits on polyurethane implants by FTIR microscopy. J Dental Res 1996;75:574.

[16] Fujita K, Nishiyama N, Nemoto K, Okada T, Ikemi T. Effect of base monomer's refractive index on curing depth and polymerization conversion of photo-cured resin composites. Dental Mater J 2005;24:403–8.

[17] Feilzer AJ, Dauvillier BS. Effect of TEGDMA/BisGMA ratio on stress developmentand viscoelastic properties of experimental two-paste composites. J Dental Res 2003;82:824–8.

[18] Asmussen E, Peutzfeldt A. Influence of UEDMA, BisGMA and TEGDMA on selected mechanical properties of experimental resin composites. Dental Mater 1998;14:51–6.

[19] Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. Dental Mater 2004;20:12–20.

[20] Schweikl H, Hiller KA, Bolay C, Kreissl M, Kreismann W, Nusser A, et al. Cytotoxic and mutagenic effects of dental composite materials. Biomaterials 2005;26:1713–9.
[21] Geurtsen W. Biocompatibility of resin-modified filling materials. Crit Rev Oral Biol Med 2000;11:333–55.
[22] Lin NJ, Bailey LO, Becker ML, Washburn NR, Henderson LA. Macrophage response to methacrylate conversion using a gradient approach. Acta Biomater 2007;3:163–73.