©Operative Dentistry, 2022, **47-4,** 437-448

Quality of Cure in Depth of Commercially Available Bulk-fill Composites: A Layer-by-layer Mechanical and Biological Evaluation

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Clinical Relevance

Of the nine bulk-fill materials studied, only four conform to the "bulk" designation, ie, they show no significant difference up to a depth of 4 mm for all properties considered. Among these four, very large differences could be observed.

SUMMARY

Despite their popularity, the use of bulk-fill composites remains controversial, both in terms of their properties and their in-depth development. The objectives of the present work were (1) to provide a more comprehensive evaluation of the quality of cure in depth of commercially available bulk-fill composites by combining various key mechanical and biological characterization methods, (2) to evaluate the inter-material differences when optimally cured, and (3) to evaluate the efficiency of an antioxidant—N-acetyl-cysteine (NAC)—to restrain the adverse effects of the leached components on cell viability.

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http://doi.org/10.2341/21-084-L

Nine bulk-fill composites (including flowable and high-viscosity materials) were investigated and compared to two conventional resin-based composites, one flowable and one high-viscosity restorative material. The materials were injected or packed into Teflon molds of various configurations, up to 6 mm material thickness. They were then light-cured from the top for 20 seconds with Bluephase G2 (Ivoclar Vivadent, irradiance = 1050 mW/cm^2). The following physico-mechanical properties were measured for the upper (0-2 mm), intermediate (2-4 mm), and lower (4-6 mm) layers: degree of conversion using Raman Spectrometry (DC, in %), microhardness using a Vickers micro-indenter before (VHN dry) and after 24 hours of storage in ethanol (VHN EtOH), and flexural strength (in MPa) and flexural modulus (in GPa) using a three-point bend test. Each composite layer and an uncured layer were also stored for one week in a standard cell growth medium to generate conditioned media. Human dental pulp cells were then cultured for 24 hours with the latter and cell viability was measured using an MTS assay. A similar experiment was repeated with conditioned media produced in contact with uncured composites, with and without the addition of 4 mM NAC. The data were subjected to a Shapiro-Wilk test, then one-way ANOVA or Kruskal-Wallis test, followed either by Tukey's test (inter-material comparison) or by Dunnett's or Dunn's test (comparison between layers relative to the upper one). The level of statistical significance was set at 0.05.

Some materials (EverX, X-traF, VenusBF, X-traB) did not show any significant differences (p>0.05) for any of the properties considered between the intermediate layers compared to the upper one (considered as reference). Others displayed significant differences, at least for some properties, highlighting the value of combining various key mechanical and biological characterization methods when investigating the quality of cure in depth. Significant inter-material differences (p<0.05) were observed when comparing the properties of their upper layer, considered as "optimally" polymerized. Hence, one needs to consider the absolute property values, not only their relative evolution concerning layer thickness. Finally, the use of NAC appeared as beneficial to reduce the risk of harmful effects to dental pulp cells, especially in case of excessive thickness use, and may therefore be of potential interest as an additive to composites in the future.

INTRODUCTION

The indications regarding the use of resin-based composites (RBCs) have considerably evolved over time since their introduction to the market. They were initially used for small restorations, but are now routinely used for larger and larger restorations. However, an increase in the failure rate has also been reported as the number of restored surfaces increases,[1-4] making the mechanical and biological performance of the materials all the more important in large cavities. Due to the need to place the composite restorations in layers,[5,6] the restoration of large cavities with RBCs is very time-consuming for both clinicians and patients. To reduce the procedure duration, two different strategies were developed: (1) changing the photoinitiator to significantly reduce the curing time (below 5 seconds) while maintaining, or in some cases improving, mechanical and biological key material properties[7-10]; and (2) to increase the depth of cure by modifying certain material characteristics, thereby giving birth to a "new" RBC category, namely bulk-fill composites. Most notably, increased light transmission through the composite was obtained by changes in material composition, mainly a reduction in filler content, an adjustment of filler size relative to the light wavelength, and an adaptation of the refractive index between the inorganic and organic fractions.[11]

Bulk-fill composites are marketed as a solution to restore large tissue losses in layers of 4-mm thickness or sometimes more (Table 1). Very few randomized clinical studies comparing the success of bulk-fill composite restorations to conventional ones are currently available, and those available have relatively short follow-up and include restorations of limited size.[12,13] The randomized clinical trial with the longest follow-up period[14] compared a bulk-fill strategy (4-mm bulk-fill RBC covered with 2-mm conventional RBC) to a classic incremental filling, in class I and II cavities. Over the six-year evaluation period, no significant difference could be observed between both groups. Despite being promising, these results need to be verified for other materials, given the large differences in physico-mechanical properties reported within the bulk-fill RBC category.[15] Moreover, the performance of bulk-fill restorations in larger cavities remains unknown and is therefore subject to caution.

One particularly important aspect regarding the performance of RBCs placed in thick layers is their quality of cure in depth. Several methods have been proposed in

Table 1: *List of Tested Materials*

Materials	Abbreviation	Manufacturer	Composite Type	Shade	Batch	Maximum Layer Thickness Recommended by the Manufacturer in Instructions for Use
Grandio	Grandio	Voco (Cuxhaven, Germany)	Hybrid paste conventional composite	A3	1408240	2 mm
Grandio Flow	GrandioF	Voco (Cuxhaven, Germany)	Hybrid flowable conventional composite	A3	1208317	2 mm
Sonic Fill	SonicF	Kerr (Orange, CA, USA)	Bulk-fill paste composite with sonic hand-piece	A3	5139879	5 mm
Tetric Evo Ceram Bulk Fill	TECBF	Ivoclar Vivadent (Schaan, Liechtenstein)	Bulk-fill paste composite	Bulk IVA	540860	4 mm
Venus Bulk Fill	VenusBF	Heraeus Kulzer (Hana, Germany)	Bulk-fill flowable composite	Universal	10105	4 mm
Filtek Bulk Fill	FiltekBF	3M-ESPE (St Paul, MN, USA)	Bulk-fill flowable composite	A3	536127	4 mm
X-tra fil	X-traF	Voco (Cuxhaven, Germany)	Bulk-fill paste composite	Universal	1343523	4 mm
X-tra base	X-traB	Voco (Cuxhaven, Germany)	Bulk-fill flowable composite	Universal	1345335	4 mm
Surefil SDR Flow	SDR	Dentsply (Konstanz, Germany	Bulk-fill flowable composite	Universal	1407000667	4 mm
Ever-X posterior	EverX	GC Europe (Leuven, Belgium)	Bulk-fill paste composite with glass microfibers	/	1309091	4 mm
Fill-up!	Fill-up	Coltene Whaledent (Alstätten, Switzerland)	Dual-cure bulk-fill flowable composite	Universal	F33233	Arbitrary thickness owing to its dual curing properties

the past to evaluate the "depth of cure",[16-18] corresponding to the depth at which the RBC is considered "adequately" cured. It was underlined that the depth of cure values vary greatly depending on the method considered, which may result in an overestimation of the true value.[19] Therefore, a combination of various methods is more likely to provide a more complete assessment of the quality of cure in depth.[16] In this sense, while physico-mechanical properties are often studied in this context, biological aspects are more often omitted or studied separately. Therefore, they need to be integrated into the laboratory evaluation of the performance of thick RBC restorations. Notably, RBCs can release various active compounds such as monomers or photoinitiators,[20]

which can diffuse through the adhesive layer and dentin[21] and ultimately reach pulp cells. Numerous undesirable biological responses have been described following contact of the released substances with the cells,[22] responses that have been so far mostly attributed to an oxidative stress through the generation of reactive oxygen species.[23]

Hence, the objectives of the present work were (1) to provide a more comprehensive evaluation of the quality of cure in depth of commercially available bulk-fill composites by combining various key mechanical and biological characterization methods, (2) to evaluate the inter-material differences when appropriately cured, and (3) to evaluate the efficiency of an antioxidant—N-acetyl-cysteine (NAC)—to restrain the adverse effects of the leached components on cell viability when the composite was unpolymerized, to simulate an inappropriate use.

METHODS AND MATERIALS

Nine bulk-fill RBCs (including flowable and high-viscosity materials) were investigated and compared to two conventional RBCs, one flowable and one high-viscosity restorative material (Table 1).

Mechanical Evaluation

For the evaluation of flexural properties (modulus and strength) in depth, the composites were placed into three rectangular white Teflon molds of 2 mm thickness, 2 mm width, and 25 mm length (Figure 1a). The three Teflon molds were aligned and assembled by two screws, each composite layer being isolated from the others by polyester films to allow ulterior individual processing of each separate layer. The uppermost surface was covered with a polyester film to prevent the formation of an oxygen inhibition layer. Photopolymerization of the three layers was initiated by three successive and non-overlapping irradiations of 20 seconds on the upper side, with the light tip in close contact with the polyester film (inner tip diameter = 9 mm). All light-curing procedures were performed with the Bluephase G2 light-curing unit (Ivoclar Vivadent, Schaan, Liechtenstein) set to "high-power" mode (irradiance = 1050 mW/cm^2 as measured before each experiment by Bluephase Meter - Ivoclar Vivadent, Schaan, Liechtenstein).

The three 2-mm-thick layers—0-2 mm (upper layer), 2-4 mm (intermediate layer), and 4-6 mm (lower layer)—were removed from the molds and polished using SiC paper grit 1000 then placed in distilled water in the dark for one week at 37°C. They were then submitted to a three-point bend test in a universal testing machine (LRX Plus, Lloyd Instruments, Largo, FL, USA) at a crosshead speed rate of 0.75 mm/min until a fracture occurred (n=5). Flexural modulus (based on the tangent to the initial slope) and strength were calculated based on ISO 4049:2000.

For the measurement of microhardness and degree of conversion, the composites were injected into a rectangular white Teflon mold of 5 x 5 mm aperture and 10-mm depth (Figure 1b), covered by a polyester film, and light-cured from the aperture in a single

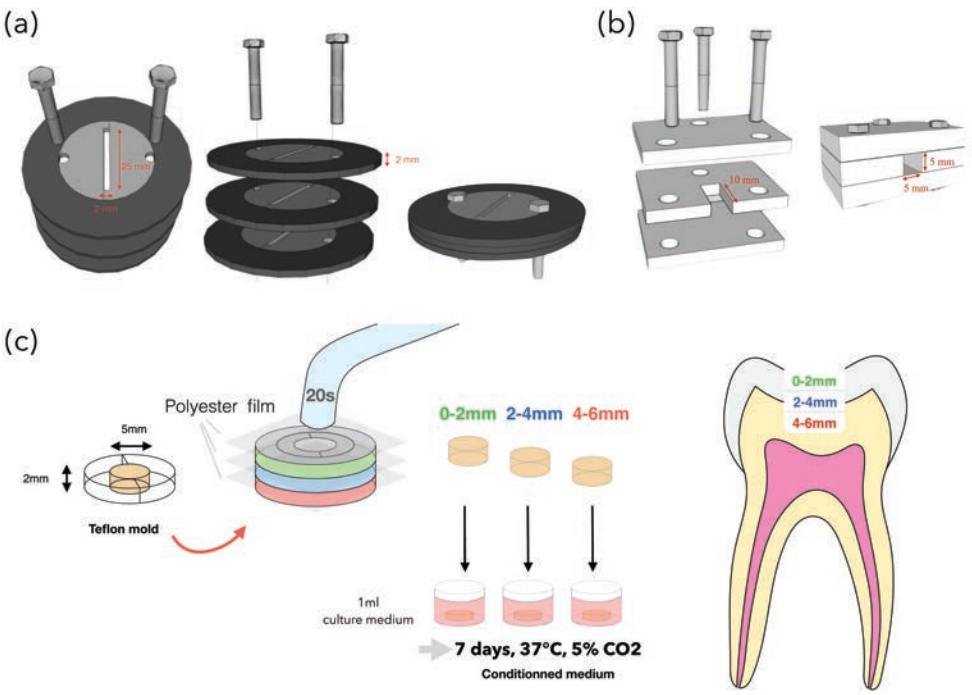

Figure 1. *Experimental setups for layer-by-layer polymerization: (a) molds for samples for 3-point-bending; (b) molds for samples for DC and VHN measurements; and (c) measurement of cell toxicity. (c) presents how each layer of the composite cylinder was then used to produce the conditioned medium.*

20-second irradiation with the same parameters described above. The degree of conversion (DC, in %; n=3) was measured on the side of the sample at 0-, 2-, 4-, and 6-mm depth using a Raman spectrometer (DXR Raman microscope, Thermo Scientific, Madison, WI, USA). Briefly, the samples were excited at 780 nm by a frequency-stabilized single-mode diode laser through a microscope objective (50×) and spectra were obtained in the region 1600 cm^{-1}, with the following conditions: microhole: 50; irradiation time: 60 seconds; number of accumulations: 5; and grating: 400 lines/mm. The DC was then calculated based on the decrease in intensity of the peak corresponding to the methacrylate C=C groups at 1640 cm^{-1} compared to the uncured sample; the aromatic peak at 1610 cm^{-1} was used as the internal standard.[24] On the same samples, Vickers microhardness was measured in dry conditions (VHN dry; n=3) along the side of the sample at a similar depth (0, 2, 4, and 6 mm) using a Durimet microhardness tester (Leitz, Wetzlar, Germany) and applying a 200 g load for 30 seconds. The samples were then immersed in pure ethanol for 24 hours, before re-measuring the microhardness (VHN EtOH; n=3).[15] To enable comparison with flexural properties, an average value was calculated between 0 and 2 mm (upper layer), 2 and 4 mm (intermediate layer), and 4 and 6 mm (lower layer).

Cell Culture

The human dental pulp cells (hDPCs) used in the present work were obtained from the pulps of four wisdom teeth extracted for orthodontic reasons (female patient, 18-years-old) after informed consent. hDPCs were isolated by the outgrowth method.[25] Briefly, after surface disinfection, the teeth were split to recover the pulp tissue, which was then rinsed in a growth medium (see above), minced into small pieces, and placed in 6-well plates. The growth medium was changed every 2-3 days. After 10 days, the cells were harvested using Acutase (Life Technologies, Gent, Belgium), centrifuged, and replated in a new flask. For the next passages and experiments, the cells were harvested upon reaching 80% confluence, and either frozen or plated at 2.5 x 10^5 cells/T75 flask for subsequent use. For all experiments performed in the present work, the cells were used at passage 6.

Biological Evaluation

For the study of hDPC viability, a conditioned medium was produced by incubating composite disks into a cell culture medium (Dulbecco's Modified Eagle Medium—supplemented with 10% bovine serum, L-Glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, Thermo Fischer-Scientific, Waltham, MA, USA). The composites were packed into three superimposed and aligned white Teflon molds (5-mm diameter, 2-mm thickness) separated by polyester films to allow for individual processing of each separate layer (Figure 1c). Like for the other measurements, the curing light tip was placed in close contact with the polyester film covering the uppermost surface. Each of the three 2-mm-thick disks—0-2 mm (upper layer), 2-4 mm (intermediate layer), 4-6 mm (lower layer)—was then placed in 1 mL of culture medium in a 24-well plate and incubated for 7 days at 5% CO_2, 95% humidity (Figure 1c). After one week, conditioned media were collected and incubated with hDPCs to evaluate their impact on cell viability. Positive controls were obtained by incubating an identical volume of uncured materials. For the flowable materials, a volume of 40 mm^3 corresponding to the volume of the mold was injected directly into the well.

To evaluate the effect of the components released by the materials on cell viability, hDPCs were seeded onto 96-well plates at 10^4 cells per well ($3.03*10^4$ cell/cm^2). The culture was maintained with 5% CO_2, at 37°C for 24 hours to allow cell adhesion. After that, the cell culture medium was removed and replaced by the conditioned medium for 24 hours at 5% CO_2, 37°C, standard culture medium was used as control. Cell viability was assessed using an MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) as per supplier instructions. Briefly, the culture medium was removed from the wells, which were washed three times with PBS. MTS solution (100 µL) was then added to each well for 30 minutes at 5% CO_2, 37°C. The absorbance of each well was then determined using a microplate reader (SpectraMax M2, Molecular devices) at a wavelength of 490 nm.

In a second series of experiments, an antioxidant (N-acetyl-cysteine—NAC) was added to the conditioned media obtained with the uncured materials, to determine whether the cytotoxic effect of the released compounds could be attenuated or annihilated. NAC was prepared as 1 mol/L stock solution in HBSS solution and pH was adjusted to 7.2. In preliminary experiments using various concentrations (0 - 2 - 4 - 6 - 8 - 10 mM), cell viability was affected above 6 mM after 24 hours of incubation (data not shown). The final concentration of NAC added to the medium was therefore 4 mM (n=4 wells per condition).

Statistical Analyses

Statistical analyses were performed using JMP software (SAS Institute, Cary, NC, USA). The normality of the

distributions was verified using a Shapiro-Wilk test, after logarithmic transformation of the data if necessary. For inter-materials comparisons (upper layer), one-way ANOVA was performed followed by Tukey's test for multiple comparisons. For the effect of the layer (intra-material comparisons), one-way ANOVA was performed in case of normal distribution of the data, followed by Dunnett's test for comparison with the layer considered as reference, ie, the upper layer (0-2 mm). When normality was rejected, a Kruskal-Wallis test was performed, followed by Dunn's test for comparison with the reference layer (0-2 mm). The level of statistical significance was set at 0.05.

RESULTS

Mechanical Evaluation

The evolution in depth (upper, intermediate, and lower layers) of the various material properties appeared to be clearly material-related. Indeed, while some materials (EverX, X-traF, VenusBF, X-traB) did not show any significant differences ($p>0.05$) for any of the properties considered between the lower or intermediate layers compared to the upper one (considered as reference), others displayed significant differences, at least for some properties. For the vast majority of the materials and properties, no significant differences could be observed between the upper and the intermediate layer, with no clear trend appearing when comparing bulk-fill or conventional materials. On the contrary, significant differences were frequently detected when considering the lower layer, and this was systematically for the two conventional materials (Grandio and GrandioF) for all properties (Figure 2).

Significant differences ($p<0.05$) in absolute values were observed between the materials for all properties (upper layers), particularly for microhardness and flexural modulus (Table 2). Specifically, with regard to hDPC viability, and in the experimental conditions used, the conditioned media from each composite cured under optimal conditions (upper layer) led to significant inter-material differences in hDPC viability ($p<0.05$; Table 2), with values ranging from 54% (FiltekBF) to 98.7% (GrandioF).

Biological Evaluation

Relative hDPC viability was not significantly different ($p>0.05$) between intermediate and upper layers for all materials, except for GrandioF for which a slight increase was observed. On the contrary, the lower composite layer led to a reduction of hDPC viability compared to the upper layer, in a significant manner ($p<0.05$) for half of the materials investigated.

A significant reduction in relative cell viability was observed for cells grown in a conditioned medium prepared with uncured composites compared to the lower composite layer ($p<0.05$ for all materials except SonicF, TECBF, and VenusBF). Average relative values for uncured composites ranged from 0 to 60.3% compared to the standard growth medium (Figure 3). The addition of 4 mM of NAC to the conditioned media resulted in a significant increase in relative cell viability for all materials ($p<0.05$), with values ranging from 29.4-102.3%, depending on the material considered. The addition of 4mM NAC was sufficient to lead to a full recovery of cell viability for some materials (SonicFill, X-traB), while the viability remained quite low for others (X-traF, SDR). Fill-up! could not be considered for this experiment due to its additional chemical cure.

DISCUSSION

To our knowledge, this is the first study performing a layer-by-layer evaluation of such a large array of commercially-available bulk-fill composites that combined a mechanical and biological characterization.

With regard to the first goal of this work, it appeared that the quality of cure in depth of commercially available composites is both material- and property-dependent. This can account for the inconsistency reported in the literature regarding the determination of the depth of cure of bulk-fill composites since all properties do not necessarily evolve similarly with depth.[26] Our results also underline the interest to combine various methods allowing the characterization of key mechanical and biological properties to provide a more representative picture of the performances of a material or group of materials with regards to their quality of cure in depth. For example, the conversion of bulk-fill composites has in the past been characterized mostly based on DC or microhardness measurements,[27-31] which does not reflect sufficiently their performance when laid in thick layers. This reinforces the need to combine complementary methods to characterize the depth of cure demonstrated in the past, notably to reduce the risk of overestimating the maximum material layer thickness.[19,32]

Not only is the maximum recommended thickness complicated to evaluate accurately by scientists, but also difficult to control accurately in practice during the layering process. Therefore, clinicians may use excessive composite thicknesses, which underlines the need to investigate beyond the recommended maximum thickness. In that sense, the lower (4-6 mm) and uncured layers were considered in the present work as, respectively, off-label use and a worst-case scenario.

As mentioned above, bulk-fill composites are usually considered as materials allowing polymerization in

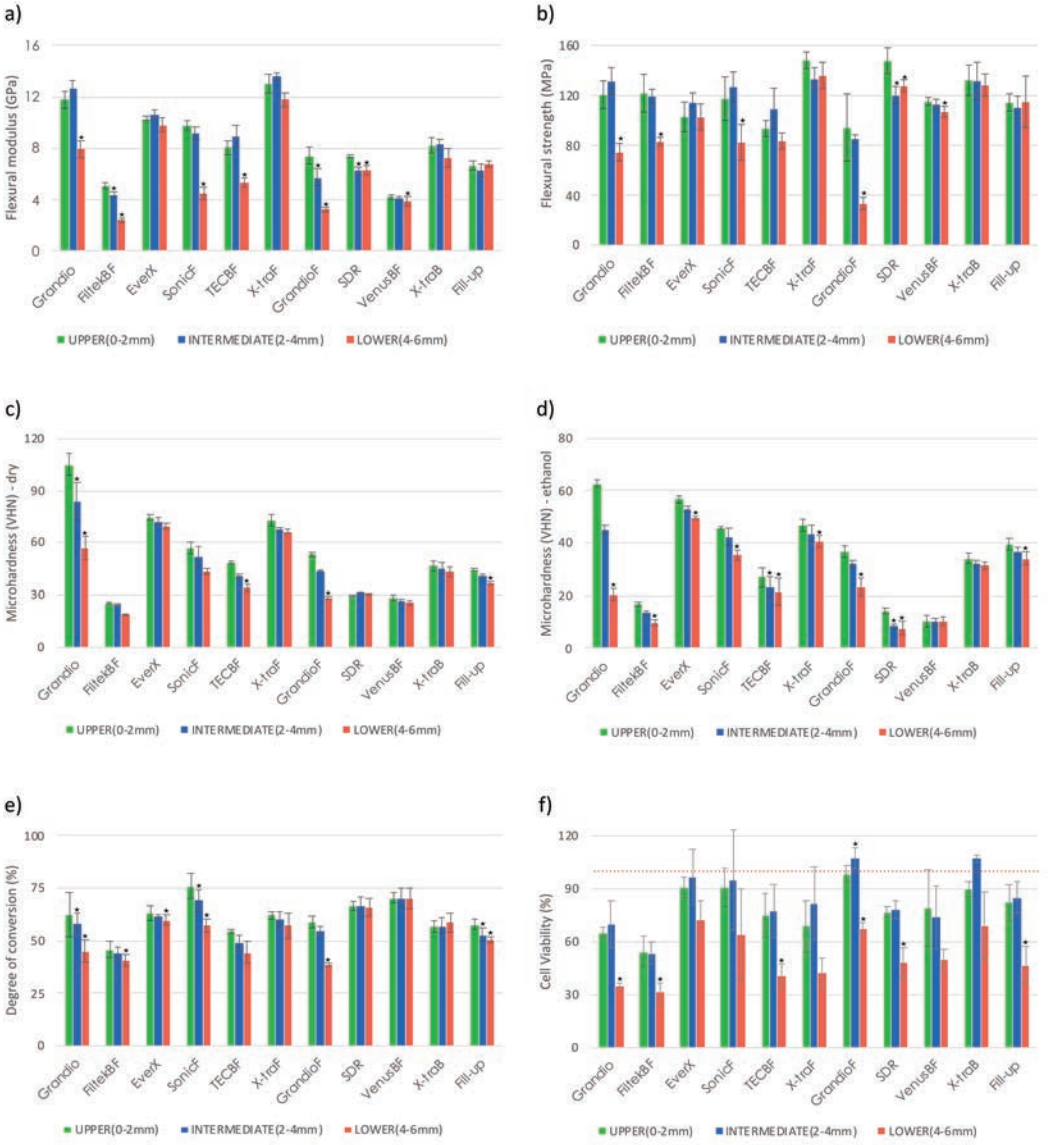

Figure 2. *Mechanical and biological properties for each material according to the three layers (upper, intermediate, and lower): (a) flexural modulus; (b) flexural strength; (c) microhardness after dry storage; (d) microhardness after 24 hours of ethanol storage; (e) degree of conversion; and (f) human dental pulp cell viability when grown in conditioned media, 100% viability (horizontal dotted line) corresponding to the values measured for the cells cultivated in regular growth medium; stars above the histograms indicate a significant difference (p<0.05) between the intermediate or lower layer and the upper layer considered as reference.*

4-mm thick layers. Within the presently considered materials, only four materials (EverX, X-traF, VenusBF, X-traB) presented no significant reduction between the upper and intermediate layers (≤ 4 mm) for all tested properties, which supports the recommendation made in previous work to be cautious with the bulk fill "label." [29] In fact, this labeling is further challenged by the fact that significantly increasing the curing time (100 seconds or more) may lead to comparable levels of conversion at 4-mm depth.[11]

It must be mentioned, however, that both in classic and recent work, the mold material was shown to have a significant impact on the depth of cure.[33,34] The choice of white Teflon in the present investigation was made to be closer to the remaining dental tissue in terms of light transport. However, a lower polymerization quality in depth would be expected when using metal molds, which better simulate the situation of a metal matrix band. Therefore, the quality of cure measured here in the depth of the material should not be extrapolated to all clinical situations, particularly not to those where a metal matrix is used. Also, it should be noted that the use of various types of molds represents a limitation preventing a direct correlation between the various

Table 2: *Inter-Material Properties Comparison for the Upper (0-2 mm) Layer (Means and Standard Deviations); Similar Letters within Columns Connect Materials Which are not Statistically Different* (p>*0.05) for the Considered Property*

Material	Flexural Modulus (GPa)			Flexural Strength (MPa)			DC (%)			Microhardness (VHN) Dry			Microhardness (VHN) EtOH			Cell Viability (%)		
	M	SD		M	SD		M	SD		M	SD		M	SD		M	SD	
Fill-up	6.7	0.4	D	114.3	6.9	CDE	57.9	2.3	DE	44.5	2.2	D	39.5	0.8	BC	82.3	10.4	AB
EverX	10.3	0.2	B	102.8	11.9	DE	63.1	1.3	CD	74.6	3.2	B	56.7	1.6	A	90.7	5.6	AB
FiltekBF	5.2	0.2	E	121.7	15.0	ABCD	45.7	0.7	F	25.2	3.9	E	16.9	0.2	E	54.5	8.5	C
Grandio	11.8	0.6	A	120.6	11.0	ABCDE	62.5	1.4	CD	105.1	10.7	A	62.6	6.3	A	65.0	3.6	BC
GrandioF	7.4	0.7	CD	94.2	26.9	DE	58.9	2.1	DE	53.1	2.9	CD	36.7	1.5	C	98.7	4.8	A
SDR	7.4	0.1	CD	147.8	10.4	A	66.9	1.0	BC	29.5	2.2	E	14.3	0.5	E	77.0	3.1	ABC
SonicF	9.8	0.4	B	117.4	17.5	BCDE	76.1	0.7	A	56.8	6.0	C	45.6	3.7	B	90.6	10.8	AB
TECBF	8.1	0.5	C	93.4	6.5	E	54.4	3.8	E	48.5	1.1	CD	27.0	0.7	D	74.8	12.3	ABC
VenusBF	4.3	0.2	E	115.1	3.2	CDE	70.4	2.1	AB	28.1	2.2	E	10.3	1.6	E	79.2	21.9	ABC
X-traB	8.2	0.6	C	132.2	12.0	ABC	57.0	2.1	DE	46.5	2.7	CD	34.1	3.1	CD	89.6	4.2	AB
X-traF	13.1	0.7	A	148.1	6.6	AB	62.1	2.3	D	72.8	1.5	B	47.0	3.6	B	68.7	14.5	BC

Abbreviations: M, mean; SD, standard deviation.

properties measured. Future work should consider designing experiments allowing the measurements of key physical, mechanical, and biological properties in depth on the same samples.

Not only does the evolution of a given property in depth need to be considered about the upper layer, but also the actual absolute value. To illustrate that, among the four materials strictly complying with "bulk-fill" criteria, very large differences could be observed, notably between X-traF and VenusBF. Hence, not only is the effect of depth to consider when choosing a material, but also and especially inter-materials differences.

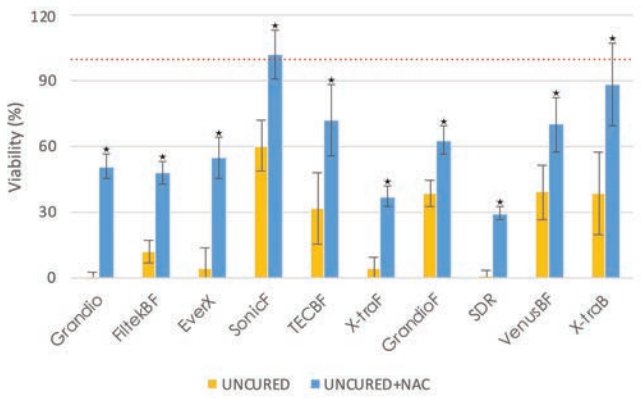

Figure 3. *Relative cell viability of cells grown in conditioned medium prepared with uncured composites, with and without the addition of 4 mM of NAC; 100% viability (horizontal dotted line) corresponds to the values measured for the cells cultivated in regular growth medium. *Identifies a statistical difference between uncured and uncured+NAC.*

This is in line with previous work based mostly on physico-mechanical aspects,[15,26] complemented here by the effect of composite conditioned media on hDPC viability, with similar conclusions.

It is well known that the conversion of dimethacrylate-based resins and composites is never complete,[35] and that substances can be released that have harmful effects on human pulp cells.[36] The cytotoxicity of the composite leachates depends both on the nature and the amount of the released molecules.[37] Monomers and compounds of the photoinitiator system represent most of the released molecules,[38] each molecule being associated with a different toxicity level. For example, the EC50 (concentration of substance necessary to kill 50% of a cell population) measured on human gingival fibroblasts varied from 0.087 mM for BisGMA to 11.53 mM for HEMA and 3.460 mM for TEGDMA.[37] Hence, a first possible explanation of the inter-material differences observed here regarding hDPC viability is logically the specific monomer composition of the composites. The ones containing the compounds with the lowest EC50 are most likely to lead to a more important drop in cell viability. Moreover, synergistic interactions between monomers are also possibly at play, since they were shown to potentiate cytotoxicity.[39] However, an additional parameter is essential regarding the cytotoxicity of a molecule, ie, its solubility in an aqueous medium. To induce cell damage, they must indeed be soluble in the aqueous growth medium. For example, the solubility of BisGMA in water is 9.5 ug/mm^3 as

compared to 27.5 ug/mm^3 for TEGDMA.[40] It was very elegantly demonstrated by Meng and others that no Bis-GMA could be detected in water storage after one week, while the monomer was detected in large amounts one week later once the solvent was switched to ethanol.[41] Consequently, the measurement of EC50 of hydrophobic monomers such as Bis-GMA required their prior dissolution into DMSO and subsequent dilution in an aqueous cell growth medium.[37] Since the growth medium without DMSO was used in the present work (aqueous media, 7 days extraction) to maximize clinical relevance, it is very likely that some hydrophobic compounds could not leach out of the composite due to their lack of hydrophilicity.

Last, although most of the cytotoxicity of resin-based composites has been attributed to monomers via oxidative stress,[23] the involvement of other components than the monomers cannot be ruled out. It has notably been shown that photoinitiators are released from cured composites, in various quantities depending on the extraction medium,[20] and that they can contribute to various extents to the dimethacrylate-based material cytotoxicity.[42,43] The presence of unconverted photoinitiator molecules was shown to result from insufficient light exposure,[44] which may be the case beyond 4-mm depth and explain the reduced cell viability of the lower layer.

It remains impossible to determine the relative part played by the aforementioned variables on cell viability due to the commercial nature of the materials. Despite being clinically relevant to investigate the materials used by practitioners, this remains a limitation of any work on commercial materials because their precise composition is rarely disclosed.[45] A full quantification of the monomers released would be useful to discuss certain hypotheses, but this is beyond the scope of the present work, and would in any case be much more accurate if the experiment were repeated with experimental materials of known composition.[8]

Finally, one needs to consider the layer effect (upper, intermediate, and lower) on hDPC viability. Both monomer-polymer conversion and cross-linking density of the polymer network were shown to affect the way unconverted monomers leach out of the material,[7] thereby affecting cell viability.[46] Hence, the first and most obvious explanation for the reduction in cell viability beyond 4-mm thickness would be the reduction in monomer conversion, which is known to be inversely correlated with monomer elution.[47] This is globally in line with the present data, and in agreement with previous work, including one bulk-fill and three conventional composites, and reporting no differences in viability until 4-mm depth.[48]

However, it can be noticed that the variations in DC between layers are generally smaller than those observed for hDPC viability. This can be related to the rather complex relationship existing between polymer conversion and cross-linking density. A rather large polymer network heterogeneity has been reported for di-methacrylate systems, which can include highly and loosely crosslinked domains.[35] Consequently, even at a similar DC, a different local cross-linking density can be measured.[19,32] This might affect both the proportion of the various co-monomers bound to the polymer network and their ability to diffuse out of the material.

Concerning the addition of 4 mM NAC, it was shown efficient to restrain the adverse effects of the released components on cell viability. NAC is an antioxidant, and the improvement of cell viability observed when it was added to the conditioned media is consistent with the observation of other articles.[49,50] Nevertheless, under the present experimental conditions, the addition of NAC at maximum non-cytotoxic concentration did not allow a full recovery of hDPC viability except for SonicF. This could first be explained by the fact that the maximum concentration of NAC remains too low (4 mM) to overcome all the generated reactive oxygen species. The second explanation is that monomers could exert their cytotoxic effect through another pathway. It has indeed been described that the increased levels of reactive oxygen species similar to those induced by 2-hydroxyethyl methacrylate were not associated with an increase in cell death or cell growth inhibition, unlike when the monomer was present.[51] This tends to indicate that monomer-induced cell damage may not be caused exclusively by the increase in reactive oxygen species.

CONCLUSIONS

Within the limitations of this work, and about the objectives, it can be concluded that:

1. There is an interest to combine various key mechanical and biological characterization methods to provide a more comprehensive picture of the performances of a group of materials. This concerns both their quality of cure in depth, which is material and property-specific, and their properties when "optimally" cured.
2. Not only does the evolution of a given property in depth need to be considered about the upper layer, but also the actual absolute value since significant inter-material differences were observed.
3. The use of NAC appears beneficial for reducing the risk of the harmful effect on dental pulp cells, especially in case of excessive thickness use, and

may be of potential interest as an additive to composites in the future.

Acknowledgments

The authors would like to thank the manufacturers for supplying us with the commercial materials investigated. JG Leprince thanks Cliniques universitaires St-Luc (Brussels, Belgium) for his FRC fellowship.

Regulatory Statement

This study was conducted in accordance with all the provisions of the human subjects oversight committee guidelines and policies of UCLouvain, Brussels, Belgium. The approval code was #2013/16DEC/550.

Conflict of Interest

The authors of this article certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

(Accepted 29 September 2021)

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