

I. Foreword

Dental restorative materials play a central role in contemporary oral healthcare, at the interface between clinical performance and biological safety. Over the past decades, resin-based composites have become the dominant restorative materials worldwide, driven by aesthetic demands, adhesive dentistry, and minimally invasive treatment strategies. While their mechanical performance and clinical longevity have been extensively investigated, their biological interactions with oral tissues, particularly the dental pulp, remain an area of ongoing scientific debate.

The present thesis addresses this issue by examining resin-based composites not as inert restorative materials, but as dynamic systems capable of interacting with their biological environment. Incomplete polymerization, residual monomer release, and degradation processes may lead to cellular stress in pulp and oral tissues, even in the absence of overt tissue necrosis. Understanding these interactions requires an integrated perspective that combines material science, cellular biology, and clinically relevant considerations.

Accordingly, this thesis is structured around a multidimensional approach. The first part provides a comprehensive review of resin-based composite chemistry, polymerization mechanisms, elution phenomena, and the biological effects of substances released from these materials. This section is intentionally written to be accessible to both dental and non-dental readers, establishing a shared conceptual framework for the experimental work that follows.

The experimental chapters then pursue two complementary objectives. First, the physico-mechanical performance and biological effects of commercially available resin-based composites are evaluated, with particular attention to curing efficiency in depth and its consequences for both material properties and cellular responses. Second, strategies aimed at improving composite biocompatibility are explored

through the incorporation of protective additives, with a focus on antioxidants such as N-acetylcysteine. Rather than proposing such additives as a means to compensate for inadequate material formulations, this work examines their potential to modulate sublethal biological effects when incorporated into well-designed composite systems.

Finally, the discussion integrates these findings in light of current regulatory frameworks, experimental limitations, and clinical reality. Particular emphasis is placed on distinguishing material-related biological effects from other contributors to pulpal pathology, such as inflammation and operative factors, and on identifying realistic perspectives for the development of safer and more biologically compatible restorative materials.

Through this integrated analysis, the present thesis aims to contribute to a more balanced understanding of resin-based composites, one that acknowledges both their undeniable clinical benefits and the biological challenges that accompany their long-term use.

II. List of Abbreviations

Abbreviation Full term

BPA	Bisphenol A
BisGMA	Bisphenol A-glycidyl methacrylate
CQ	Camphorquinone
DC	Degree of Conversion
DMAEMA	N,N-Dimethylaminoethyl methacrylate
DPC	Direct Pulp Capping
EPR	Electron Paramagnetic Resonance
GI	Glass Ionomer
GSH	Glutathione
hDPCs	Human Dental Pulp Cells
HEMA	2-Hydroxyethyl methacrylate
ISO	International Organization for Standardization
LC/MS	Liquid Chromatography–Mass Spectrometry
LDH	Lactate Dehydrogenase
MDR	Medical Device Regulation

List of Abbreviations

Abbreviation	Full term
--------------	-----------

MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay
------------	--

NAC	N-Acetylcysteine
------------	------------------

RBCs	Resin-Based Composites
-------------	------------------------

ROS	Reactive Oxygen Species
------------	-------------------------

TC₅₀	Toxic Concentration inducing a 50% reduction in cell viability
------------------------	--

TEGDMA	Triethylene glycol dimethacrylate
---------------	-----------------------------------

TPO	2,4,6-Trimethylbenzoyl diphenylphosphine oxide
------------	--

UDMA	Urethane dimethacrylate
-------------	-------------------------

III. Table of contents

I. Foreword	1
II. List of Abbreviations	3
III. Table of contents	5
IV. Introduction	11
References	17
V. Thesis Structure	19
VI. Resin-Based Composite	21
1. Composition	22
1.1. Resin matrix	22
1.2. Fillers	25
1.3. Initiators	28
2. Photopolymerization Reaction	30
2.1. Degree of conversion	34
2.2. Degree of crosslinking	35
3. Mechanical Properties	36
4. Elution of Compounds from Cured Resin-Based Composites	37
4.1. Types of eluted substances	38
4.2. Elution rate	39
4.3. Parameters influencing elution	40
5. Conclusion	41
6. References	43
VII. Biological Effects of Substances Released by Resin-Based Composites	46

1. Pathways of Absorption	47
2. Types of Effects	48
2.1. Systemic effects	48
2.1.1. Toxicokinetics and systemic toxicity of resin monomers	48
2.1.2. Bisphenol A as an impurity or by-product	50
2.1.3. Overall appraisal	50
2.2. Allergic reactions	50
2.2.1. Types of hypersensitivity	51
2.2.2. Allergenic components	51
2.2.3. Prevalence	51
2.2.4. Clinical manifestations	52
2.2.5. Summary	53
2.3. Local effects	54
2.3.1. Toxicity	55
2.3.2. Molecular mechanisms of toxicity	57
2.3.2.a. Molecular mechanisms of TEGDMA toxicity	57
2.3.2.a.i. Depletion of GSH and generation of oxidative stress	58
2.3.2.a.ii. Mitochondrial dysfunction	61
2.3.2.a.iii. Cellular stress responses	62
2.3.2.a.iv. Genotoxicity	67
2.3.2.a.v. Outcomes: apoptosis versus necrosis	73
2.3.2.b. Molecular mechanisms of BisGMA toxicity	76
2.3.2.b.i. Lipophilicity and reactivity	77
2.3.2.b.ii. Mechanisms of GSH depletion	77
2.3.2.b.iii. Cystine uptake inhibition	78
2.3.2.b.iv. Mitochondrial effects	78
2.3.2.b.v. Adaptive responses to oxidative stress	79
2.3.2.b.vi. DNA damage and cell cycle arrest	79
2.3.2.b.vii. Cell death	80

Table of contents

3. Summary	81
4. References	83
VIII. Objectives	91
IX. Methods used in this thesis	93
1. Three-Point Bending (Flexural Strength and Modulus)	93
2. Vickers Microhardness	94
3. Electron Paramagnetic Resonance (EPR) Spectroscopy for Trapped Free Radicals	95
4. Degree of Conversion by Micro-Raman Spectroscopy	95
5. Chemical Analyses for Monomer and Photoinitiator Elution	96
6. Biological Properties	97
6.1. Cell metabolic activity – MTS Assay	97
6.2. Reactive Oxygen Species Quantification	98
7. References	100
X. Quality of Cure in Depth of Commercially Available Bulk-fill Composites: A Layer-by-layer Mechanical and Biological Evaluation	101
1. Introduction	104
2. Materials and Methods	106
3. Results	111
4. Discussion	115
5. Conclusions	119
6. References	120

XI.	<i>Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites</i>	125
1.	Introduction	127
2.	Materials and methods	129
2.1.	Experimental composites preparation	129
2.2.	Sample preparation	129
2.3.	Material properties	131
2.4.	Chemical analyses	132
2.5.	Biological properties	133
2.6.	Metabolic activity	134
2.7.	ROS production	134
2.8.	Statistical analysis	134
3.	Results	136
3.1.	Material properties	136
3.2.	Chemical analyses	138
3.2.1.	NAC Elution	138
3.2.2.	Elution of residual monomers and photoinitiators	138
3.3.	Biological Properties	139
3.3.1.	Metabolic activity	139
3.3.2.	ROS production	140
4.	Discussion	142
5.	Conclusion	149
	References	150
6.		150
XII.	<i>Discussion and Perspectives</i>	155
1.	Material Properties and Intrinsic Modifications	156

Table of contents

1.1.	Degree of conversion _____	156
1.2.	Filler phase _____	157
1.3.	Alternative monomers _____	158
2.	Additives and Protective Strategies _____	160
2.1.	Antioxidants incorporated into resins _____	160
2.2.	External antioxidant treatments _____	161
3.	Clinical Mitigation: Residual Dentin and Operative Conditions _	162
3.1.	Residual dentin thickness _____	162
3.2.	Use of liners _____	163
3.3.	Curing protocols _____	164
4.	Perspectives _____	164
5.	References _____	168

IV. Introduction

The World Health Organization recognizes untreated dental caries in permanent teeth as the most prevalent health condition globally, reflecting the extraordinary scale of oral disease as a public health burden. This is confirmed by the Global Burden of Disease Study (1), which estimated that untreated caries of permanent teeth affected 2.3 billion people, while untreated caries of deciduous teeth affected 532 million children. Altogether, oral disorders were estimated to affect 3.47 billion individuals, nearly half of the global population, and accounted for 8.8 million years lived with disability.

Caries arises from the interaction of bacterial biofilms, fermentable sugars, and host factors, leading to acid production and progressive demineralization of enamel and dentin. If not treated, lesions advance into the dentin–pulp complex, resulting in pulpitis, pulp necrosis, and eventual tooth loss. These pathological consequences explain why caries, although preventable in principle, continue to have a major impact on health and quality of life. Against this backdrop, restorative treatment is a cornerstone of modern dentistry.

For over a century, dental amalgam was the principal restorative material, valued for its durability, ease of placement, and low cost. However, its metallic appearance limited esthetics, and its mercury content raised concerns. While evidence of direct patient toxicity remains limited, environmental risks became decisive. The Minamata Convention on Mercury, signed by more than 120 countries, mandated a phase-down of amalgam, primarily for ecological reasons. This global policy shift accelerated the transition to resin-based composites (RBCs).

RBCs now dominate restorative dentistry. Their adhesive bonding to enamel and dentin enables minimally invasive preparations, and their ability to mimic natural tooth color meets modern esthetic demands. Advances in RBCs chemistry, filler technology, and

photoinitiator systems have expanded their indications from anterior to posterior restorations, making them the material of choice worldwide.

Yet despite their success, RBCs are not biologically inert. Their polymerization chemistry imposes intrinsic limitations that continue to drive scientific and clinical concern. Polymerization by free-radical reaction is never complete: the degree of conversion (DC) seldom exceeds 70%, leaving residual monomers and photoinitiator fragments entrapped in the polymer network (2). Over time, residual compounds can leach into the oral environment. More than 30 substances have been identified in composite eluates, among which bisphenol A-glycidyl dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGDMA) are particularly significant (3). These two monomers are among the most widely used in RBCs formulations and carry particular toxicological relevance: TEGDMA as a low-molecular-weight diluent monomer and BisGMA as the high-molecular-weight base monomer. Their release undermines mechanical stability and raises biological concerns. Laboratory studies consistently show that methacrylate monomers impair pulp cell viability, deplete glutathione, and induce oxidative stress (4).

While systemic effects are generally minimal, the local effects on pulp and oral tissues are clinically relevant. Monomers can diffuse through dentinal tubules, reaching pulp cells and eliciting stress, notably through the generation of oxidative stress, even in the absence of outright necrosis(5). Such toxic effects may compromise pulpal homeostasis and predispose teeth to pulpitis and early endodontic failure.

Clinical evidence aligns with this concern. Meta-analyses of thousands of composite restorations report acceptable survival overall, but with a striking pattern: failures within the first year are dominated by endodontic complications, while later failures are due to fractures, and later on become dominated by secondary caries (Fig. 1) (6).

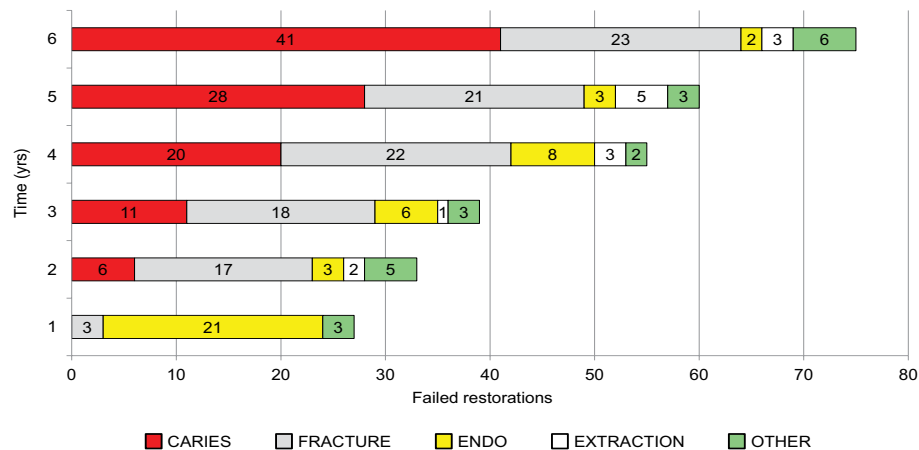


Figure 1: Number of failed restorations with type of failure during the first six-years observation time. Reproduced from Opdam et al. 2014 (6).

It is important to emphasize that pulpal pathology is multifactorial. Inflammation related to residual caries, bacterial by-products, operative trauma, and host-related factors all contribute to pulpal responses. Material-related toxicity should therefore be considered as one component within a broader biological and clinical context, rather than as an isolated or exclusive cause of pulpal injury.

Yet assessing pulp health before or after restoration remains difficult. Conventional tests (thermal or electric pulp testing) measure neural response rather than true vitality and often misrepresent actual histological status. Radiographs reveal late-stage changes but not early inflammation. As a result, early pulp injury often progresses silently until irreversible damages (tissue necrosis) demands endodontic treatment.

These biological and clinical challenges underline the importance of robust regulatory oversight with regard to the materials used for dental restoration. In the European Union, medical devices are regulated under the Medical Device Regulation (MDR, Regulation (EU) 2017/745), which classifies devices into four categories according to patient risk:

Class I: low-risk devices such as dental mirrors and hand instruments.

Class IIa: medium-risk devices such as RBCs and impression materials.

Class IIb: higher-risk devices, including most implantable and long-term surgically invasive devices.

Class III: the highest-risk devices, such as heart valves and resorbable bone substitutes.

In principle, this tiered system ensures that the degree of scrutiny reflects the level of clinical risk. In practice, RBCs are classified as Class IIa devices. By contrast, implantable and long-term surgically invasive devices are generally classified as Class IIb. However, Annex VIII, Rule 8 of Regulation explicitly provides an exception: *“All implantable devices and long-term surgically invasive devices are classified as Class IIb unless they are intended to be placed in the teeth, in which case they are classified as Class IIa”*. This downgrading is paradoxical: RBCs are invasive, long-term materials placed in close proximity to pulp through permeable dentin, yet they are regulated as moderate-risk devices.

The consequence is significant. Class IIb devices undergo stricter conformity assessment, including more comprehensive pre-clinical testing, stronger clinical evidence, and closer oversight by notified bodies (independent third parties that verify compliance with EU safety and performance standards). Class IIa devices follow a lighter pathway, with fewer mandatory endpoints and reduced regulatory scrutiny. As a result, RBCs are not subject to the same biological testing or clinical evaluation requirements as other long-term invasive devices, despite their ability to release residual compounds and exert toxic effects on pulp tissues.

Biological safety under MDR is demonstrated through a risk-based evaluation plan, typically aligned with ISO 10993-1. This includes endpoints such as cytotoxicity (ISO 10993-5), sensitization and irritation (ISO 10993-10). Additional endpoints such as genotoxicity (ISO 10993-3), and systemic toxicity (ISO 10993-11) are only recommended

but not mandatory. In practice, regulatory testing still focuses mainly on acute cytotoxicity. Sub-toxic and long-term effects, which are especially relevant for RBCs, are often overlooked. Residual compounds may not cause immediate cell death in pulp tissues, but they can gradually impair cellular function, effects that remain undetected by standard cytotoxicity assays.

Technical performance is separately assessed according to ISO 4049, which specifies requirements for flexural strength, water sorption, and solubility, along with standardized test methods for other parameters such as depth of cure and radiopacity. While the DC is an important property for RBCs, it is not explicitly regulated by ISO 4049 but is often evaluated in scientific studies. These tests are conducted under highly standardized laboratory conditions: specimens are prepared in defined geometries, cured under optimal light intensity, and stored in controlled environments. While this ensures reproducibility and comparability across materials, it does not capture the variability of clinical reality, where curing lights may be angled obliquely, cavity geometries restrict light penetration, and contamination from saliva or blood may interfere. Consequently, polymerization achieved *in vivo* may be less effective than indicated by ISO 4049 testing, raising not only mechanical concerns but also biological risks through increased residual compounds release.

The combined result is a regulatory paradox: RBCs are classified in a lower-risk category, not subject to the same rigorous testing as other long-term invasive devices, and assessed under idealized performance conditions. Yet sublethal pulp effects and early endodontic failures remain insufficiently captured, leaving a critical gap between regulatory approval and clinical reality.

Taken together, the global burden of caries, the ecological phase-down of amalgam, the widespread use but intrinsic limitations of RBCs, the evidence of early pulp-related failures, the challenges of pulp diagnosis, and the limitations of MDR/ISO frameworks converge on a single problem: **how to design RBCs that balance mechanical performance with biological safety?** This challenge defines the rationale for the present thesis.

Introduction

To address this challenge, the present thesis pursues a dual approach. First, it evaluates the physico-mechanical performance and biological effects of current commercial RBCs, aiming to clarify the balance between durability and safety. Second, it explores the development of novel composite formulations designed to minimize biological risks without compromising essential physico-mechanical properties including the use of additives such as antioxidants.

Taken together, these considerations highlight the need for an integrated evaluation of resin-based composites that simultaneously addresses physico-mechanical performance, curing efficiency, and biological responses. Approaches focusing on only one of these dimensions risk overlooking clinically relevant interactions between material properties and pulp biology.

References

1. James SL, al. e. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2017;392(10159):1789-858.
2. Leprince JG, Palin WM, Hadis MA, Devaux J, Leloup G. Progress in dimethacrylate-based dental composite technology and curing efficiency. *Dent Mater*. 2013;29(2):139-56.
3. Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J Biomed Mater Res*. 1998;41(3):474-80.
4. Juranova J. Illuminating the cellular and molecular mechanism of the potential toxicity of methacrylate monomers used in biomaterials. *Drug Chem Toxicol*. 2020;43(3):266-78.
5. Hampe T, Wiessner A, Frauendorf H, Alhussein M, Karlovsky P, Burgers R, et al. Monomer Release from Dental Resins: The Current Status on Study Setup, Detection and Quantification for In Vitro Testing. *Polymers (Basel)*. 2022;14(9).
6. Opdam NJ, van de Sande FH, Bronkhorst E, Cenci MS, Bottenberg P, Pallesen U, et al. Longevity of posterior composite restorations: a systematic review and meta-analysis. *J Dent Res*. 2014;93(10):943-9.

V. Thesis Structure

The thesis is organized as follows:

- **Chapter VI – Resin-Based Composites**
Reviews the composition, polymerization chemistry, and clinical use of RBCs.
- **Chapter VII – Biological Effects of Substances Released by Resin-Based Composites**
Summarizes substances eluted from composites and their biological effects.
- **Chapter VIII – Objectives**
States the research aims guiding this thesis.
- **Chapter IX – Methods Used in this Thesis**
Describes the experimental approaches and analytical techniques.
- **Chapter X – Quality of Cure in Depth of Commercially Available Bulk-Fill Composites: A Layer-by-Layer Mechanical and Biological Evaluation**
Presents experimental evaluations of depth of cure, mechanical performance, and biological effects in commercially available bulk-fill composites.
- **Chapter XI – Effect of N-Acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites**
Explores the incorporation of N-acetylcysteine into composites and its consequences for polymerization, material properties, and cytotoxicity.

- **Chapter XII – Discussion and Perspectives**

Integrates findings across chapters, evaluates strategies to mitigate biological risks, and outlines perspectives for future research.

VI. Resin-Based Composite

RBCs are among the most widely used restorative materials in modern dentistry, offering excellent esthetics, adhesion, and versatility. Despite their clinical success, these materials are inherently imperfect because the degree of polymerization is never complete under intraoral conditions. As a result, a fraction of unreacted monomers and other low-molecular-weight components remain trapped within the cured network. Over time, these residual species, along with degradation products, can diffuse out of the material into the surrounding oral environment.

The elution of such compounds is of considerable scientific and clinical interest for two reasons. First, elution contributes to the long-term chemical and mechanical stability of the composite, influencing properties such as hardness, wear resistance, and color stability. Second, leached substances may interact with oral tissues, saliva, and biofilms, raising questions of biological safety and biocompatibility. More than 30 organic and inorganic species have been identified in eluates, including unreacted monomers, photoinitiators, co-initiators, filler-derived ions, and hydrolytic degradation products. Their release is time-dependent and influenced by numerous factors, such as resin chemistry, filler composition, curing conditions, and the chemical nature of the storage medium.

Understanding the mechanisms and kinetics of elution is therefore critical for interpreting material performance and anticipating biological outcomes. To this end, this chapter reviews the composition and polymerization of RBCs, identifies the compounds released, describes their elution profiles, and outlines the key parameters that govern their release.

1. Composition

Ever since the introduction of light-curable RBCs in the 1970s, these mixtures of organic and inorganic phases have continuously evolved to meet the increasing requirements of material design and dental practitioners. However, fundamentally, the chemistry of composite phases has not significantly changed, with material design that commonly involves particle dispersion within a resin matrix. Such matrix is typically based on (di)methacrylate chemistry and an initiator system to initiate polymerization upon light activation. The lack of any substantial shift in the use of conventional manufacturing approaches is, in part, testament to the relative success of RBCs as restorative dental filling material.

1.1. Resin matrix

The resin phase of dental composites is a blend of reactive monomers engineered to balance three competing needs: (i) rapid polymerization in an oxygen-rich clinical environment, (ii) workable paste rheology in a highly filled system with intrinsically limited molecular mobility, and (iii) low shrinkage with sufficient rigidity in the cured network. In practice, high-molecular-weight, rigid monomers are combined with low-viscosity diluents. The former reduce volumetric shrinkage and raise modulus; the latter lower paste viscosity to accommodate high filler loads and improve handling and permit incorporation of a high proportion of filler particles (1).

Historically, resin systems have relied on monomers displaying methacrylate groups to provide the reactive sites for rapid free-radical polymerization (Fig.6.1). Methacrylates cure quickly at room temperature, even in the oxygen-rich oral environment, and produce a strong three-dimensional polymer network (2). This chemical design principle has remained the foundation of composite technology for over half a century.

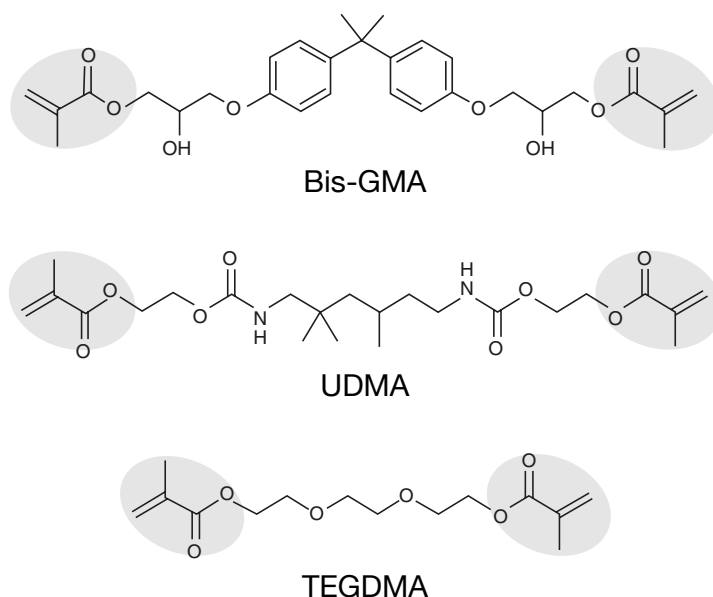


Figure 6.1 : Molecular structure of typical dimethacrylate monomers used in resin-based dental composites. The methacrylate group is highlighted in gray.

The best-known example is BisGMA, introduced by Bowen in 1962 (3). BisGMA contains an aromatic backbone that restricts molecular mobility and has been associated with increased elastic modulus and reduced polymerization shrinkage in cured composites(4). These properties contribute to wear resistance, marginal stability, and restoration longevity. However, BisGMA is extremely viscous ($\eta \approx 1200 \text{ Pa}\cdot\text{s}$) (5), which limits flow and makes it difficult to incorporate large amounts of filler (2).

To overcome this, BisGMA is blended with a low-viscosity comonomer. The most common is TEGDMA, which has a viscosity of only about $0.06 \text{ Pa}\cdot\text{s}$ (4). TEGDMA reduces the thickness of the resin blend, allowing high filler loading and better adaptation to cavity walls. It also increases monomer mobility during curing, which improves the DC and cross-linking. The disadvantage is that TEGDMA

raises polymerization shrinkage and increases water sorption, which can lead to marginal stress, postoperative sensitivity, and gradual softening of the resin (1).

Another important monomer is urethane dimethacrylate (UDMA). UDMA is less viscous than BisGMA, cures more completely, and produces a polymer network that is tougher and more fracture-resistant (1). Its urethane linkages provide flexibility, while still giving cured composites good mechanical properties. Nevertheless, UDMA exhibits higher polymerization shrinkage than BisGMA, shows susceptibility to water sorption and hydrolytic degradation, which can compromise the long-term stability of dental composites.

Monomer	Molecular weight (g/mol)	logP (octanol/water)	Polarity	Electrical charge (physiological pH)	Water solubility
TEGDMA	286.3	1.4–1.6	Moderate	Neutral	Moderate
UDMA	470,5*	3.0–3.5	Low–moderate	Neutral	Low
BisGMA	512.6	3.4–3.9	Low	Neutral	Very low

Table 6.1 : Physicochemical properties of major dimethacrylate monomers used in resin-based dental composites.

The table summarizes key physicochemical descriptors, including molecular weight, lipophilicity (logP, octanol/water), polarity, electrical charge at physiological pH, and water solubility, which are relevant to monomer behavior within polymer networks and aqueous environments. Reported values correspond to ranges commonly found in the literature and may vary slightly depending on experimental conditions and molecular substitutions.

Though by combining BisGMA (for rigidity), UDMA (for toughness), and TEGDMA (for viscosity control), manufacturers create resin matrices that are workable for clinicians, cure efficiently under light, and remain durable in the challenging oral environment.

1.2. Fillers

To enhance the mechanical properties of RBCs, inorganic fillers are incorporated into the resin matrix. Among the most widely used are amorphous silica, barium or strontium aluminosilicate glass, which not only reinforce the material but also impart radiopacity, making restorations easily visible on radiographs, and zirconia–silica nanoclusters designed to optimize strength and wear resistance. The overall performance of an RBC is closely tied to its filler volume content, with higher loading generally translating into improved mechanical properties (Fig.6.2) (1, 2).

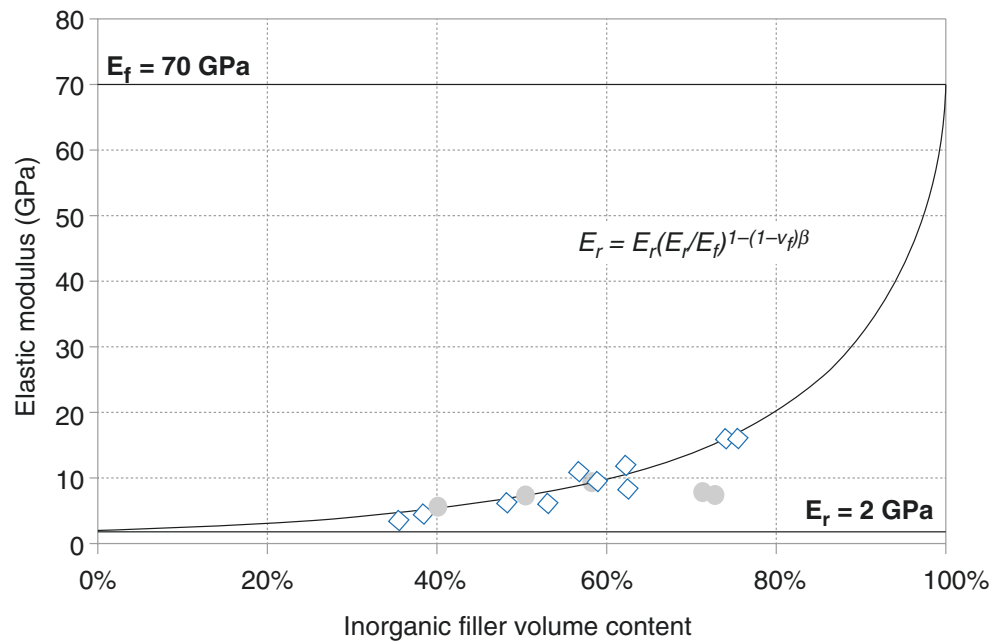


Figure 6.2 : Theoretical relationship (plain line) between the elastic modulus and filler volume content. The elastic moduli of resin (E_r) and fillers (E_f) were chosen as 2 and 70GPa respectively. Reproduced from Randolph et al. (6)

Over time, the evolution of RBCs has closely mirrored progress in filler technology, with each innovation in particle type, size, and distribution contributing to the superior

esthetics and clinical reliability of modern composites. In the 1960s, macrofilled composites containing large glass or quartz particles (5–50 μm) offered good strength but produced rough, stain-prone surfaces as particles were lost during wear. The 1970s brought microfilled composites based on colloidal silica ($\sim 40\text{ nm}$), which delivered excellent polishability and enamel-like gloss but suffered from low filler loading and poor strength, restricting their use to anterior restorations. Hybrid composites emerged in the 1980s and 1990s, combining small silica with larger glass fillers to achieve higher filler fractions (70–80 wt%) and balancing strength with polishability. In the 2000s, nanofilled composites incorporated silica and zirconia–silica nanoparticles (20–75 nm) and nanoclusters, achieving excellent esthetics and polish retention, though with somewhat reduced toughness compared with hybrids. The latest development, nanohybrids, integrates nanoparticles with submicron glass fillers, uniting the mechanical reliability of hybrids with the gloss of nanofills. These materials now dominate as universal composites, applicable in both anterior and posterior regions (Fig. 6.3) (7).

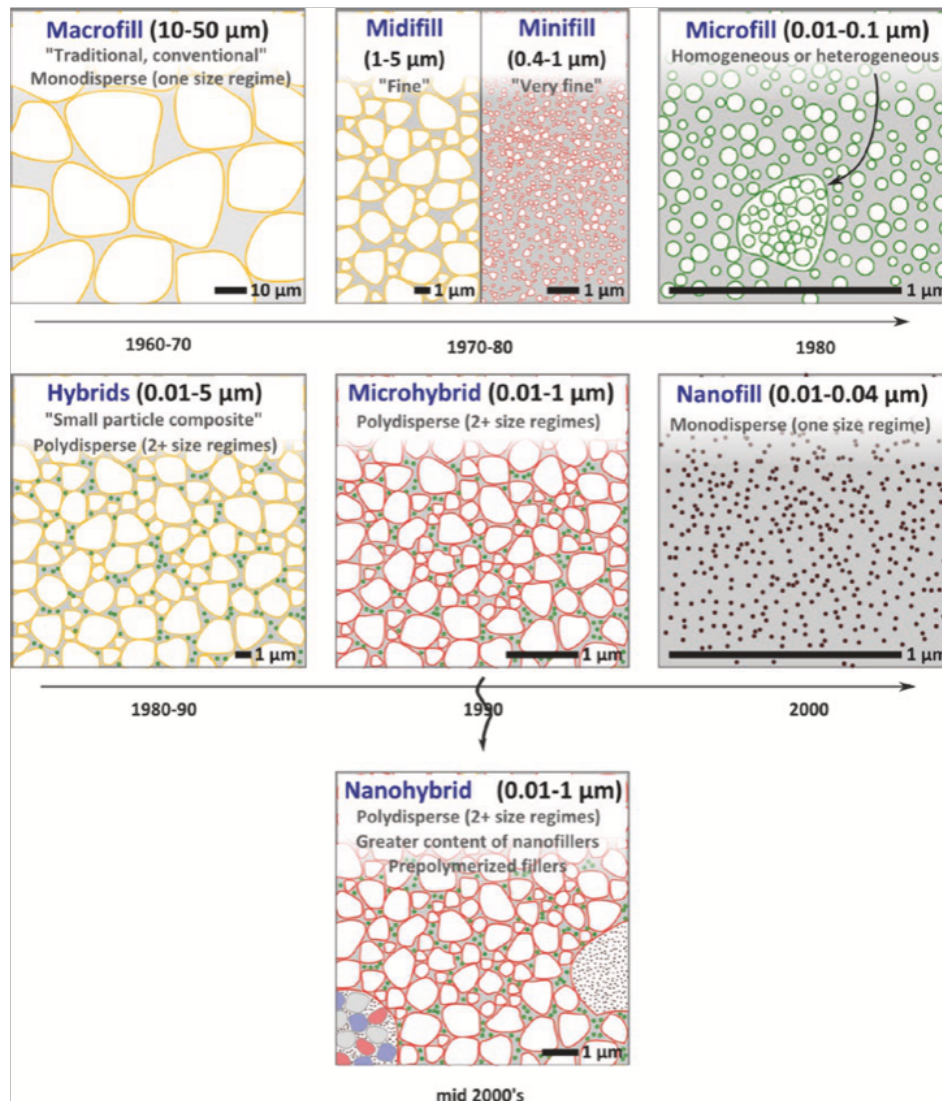


Figure 6.3 : Evolution of particle size and classification of dental composites. Modern materials are labeled nanohybrid. Reproduced from Randolph et al. (6)

Based on the rule of mixtures, the conventional strategy in composite design has been to maximize filler content in order to enhance mechanical performance. In modern resin formulations, most notably nanohybrids RBCs, fillers typically account for 50–80% by weight (30–70% by volume) (1, 8). Achieving such high loading levels is made

possible through careful control of particle size distribution, which allows dense packing and uniform dispersion within the resin matrix.

Clinically, advances in filler technology have transformed composites from materials once suited primarily for anterior esthetic use into versatile systems capable of withstanding high occlusal stresses in posterior teeth, while also maintaining long-term polish and esthetic integration.

1.3. Initiators

The in situ photopolymerization of RBCs requires a photoinitiator system capable of producing efficient polymerization throughout a clinically relevant increment of material (≈ 2 mm) within practical irradiation times (≈ 20 s). Although initiators represent only about 0.1–1.0 wt% of the resin, they largely determine the DC of RBCs (1, 2).

Historically, and still typical of most commercial formulations, this role has been fulfilled by the Type II system camphorquinone (CQ) with a tertiary amine co-initiator such as N,N-dimethylaminoethyl methacrylate (DMAEMA) (Fig.6.4). CQ is a yellow diketone that absorbs in the 400–500 nm range, with a maximum around 470 nm (9). Upon photon absorption, CQ reaches an excited state and reacts with the amine, generating free radicals that initiate polymerization (1). This system is efficient and reliable, but it has well-known limitations: CQ imparts a yellow hue that can affect lighter shades, amines oxidize over time causing discoloration, and the Type II mechanism generates only one initiating radical per absorbed photon, which reduces initiation efficiency (1).

To overcome these drawbacks, Type I photoinitiators such as acylphosphine oxides have been introduced, the most notable example being 2,4,6-trimethylbenzoyl diphenylphosphine oxide (TPO) (Fig. 6.4).

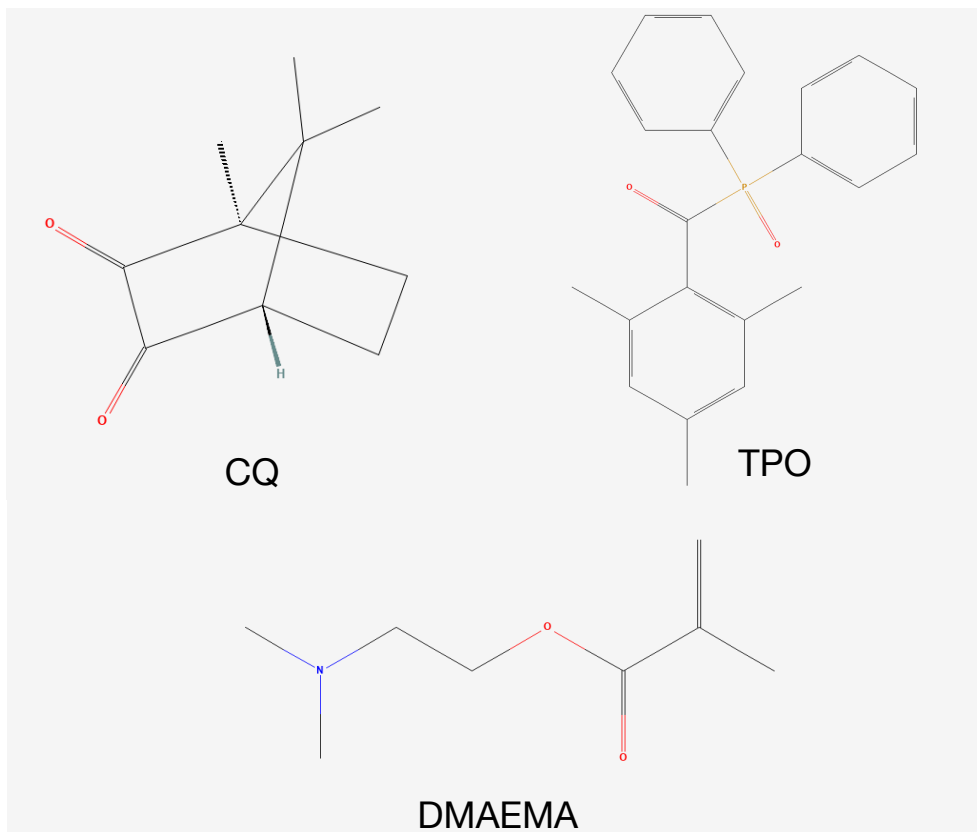


Figure 6.4 : Initiators and co-initiators generally used in dental composites for photopolymerization. Camphorquinone (CQ), 2,4,6-trimethylbenzoyl diphenylphosphine oxide (TPO) and N,N-dimethylaminoethyl methacrylate (DMAEMA).

These molecules absorb at shorter wavelengths, with maxima around 400 nm (Fig.6.5). A key advantage of TPO is its higher absorption yield efficiency which describes how many photons can be absorbed per initiator molecule at a given wavelength (Fig.6.5) (10). Compared with CQ, TPO therefore utilizes incident light far more effectively and, importantly, can achieve similar or greater curing performance at concentrations up to ten times lower (10, 11).

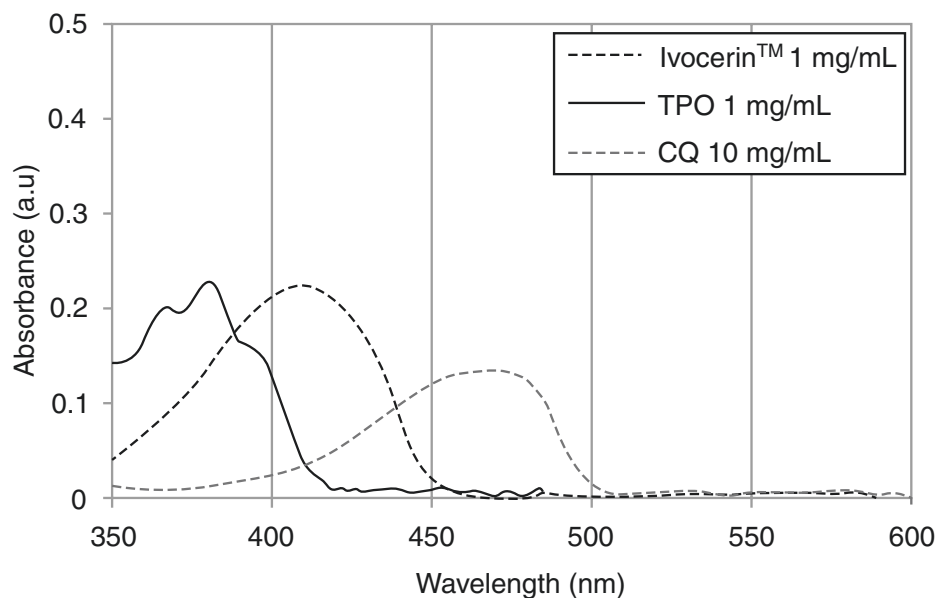


Figure 6.5 : Absorption spectra of 2,4,6-trimethylbenzoyl diphenylphosphine oxide (TPO), camphorquinone (CQ) and Ivocerin® (a bis-acylphosphine oxide derivative commercialized by Ivoclar Vivadent®) in the range 350-600 nm. Reproduced from Randolph et al. (6).

Beyond efficiency, TPO also exhibits a distinct mechanistic benefit: upon excitation, each TPO molecule generates two active radicals (12), whereas CQ/amine systems typically yield only one. This higher radical yield translates into faster polymerization, higher degrees of conversion, and shorter irradiation times (10). However, violet light (380–425 nm), required to activate TPO effectively, penetrates less deeply than blue light, which may reduce curing depth (13).

2. Photopolymerization Reaction

The photopolymerization process of dimethacrylate-based composites is triggered by free radicals, which are generated when a light-sensitive photoinitiator is irradiated. These radicals attack and open the carbon–carbon double bonds of methacrylate

groups (C=C), thereby initiating a chain reaction that transforms the monomer mixture into a cross-linked polymer network. This process is classically described in three stages: initiation, propagation, and termination (Fig. 6.8).

During initiation, the photoinitiator absorbs photons of an appropriate wavelength and produces reactive radical species.

In CQ/amine systems, absorption of blue light around 470 nm excites CQ, which then undergoes electron or hydrogen transfer from a tertiary amine, generating free radicals capable of attacking the C=C bonds of methacrylate monomers (Fig. 6.6) (2). The resulting amine-derived radical initiates polymerization by addition to the carbon-carbon double bond of the methacrylate monomer, whereas the camphorquinone-derived radical does not initiate polymerization and instead undergoes recombination to form pinacol, returns to its ground state, or is converted into a secondary alcohol. (14)

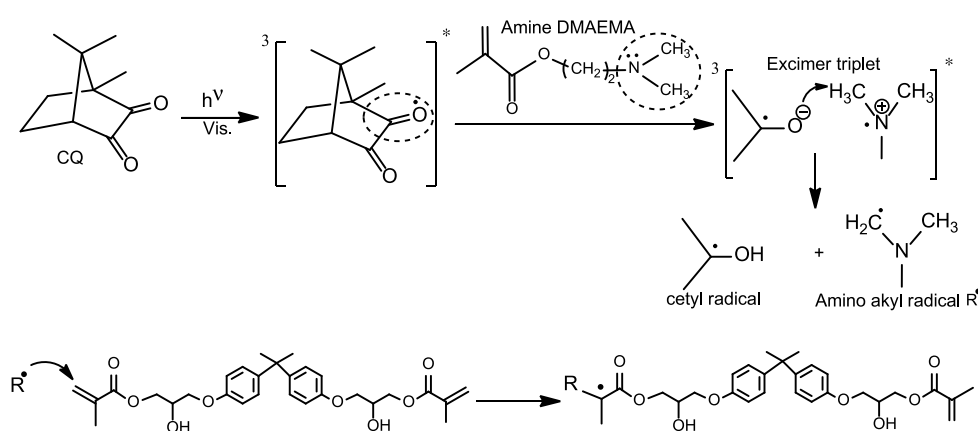


Figure 6.6 : Mechanism of CQ decomposition and formation of the first radical monomer. Upon visible-light irradiation, camphorquinone (CQ) is excited to its triplet state and interacts with the tertiary amine co-initiator DMAEMA to form an excimer. Hydrogen transfer from the α -carbon of the amine leads to the generation of an initiating amine radical ($R^\bullet$) and a camphorquinone (cetyl) radical. (14).

By contrast, Type I initiators such as TPO absorb violet light near 400 nm, and the excited TPO molecule undergoes homolytic cleavage to yield two radical fragments. This direct bond scission eliminates the need for an amine co-initiator (Fig.6.7). *Both radicals are highly reactive and directly initiate free-radical polymerization by addition to carbon-carbon double bonds of methacrylate monomers(11).*

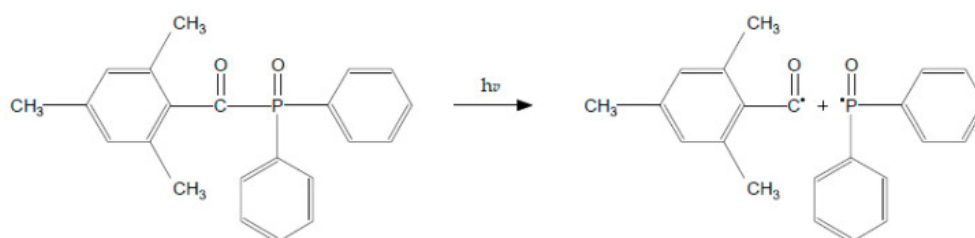


Figure 6.7 : Alpha-cleavage of photoinitiation mechanism of TPO. Upon violet light irradiation, TPO undergoes photochemical α -cleavage of the carbon-phosphorus bond, yielding two primary radicals: a trimethylbenzoyl radical and a diphenylphosphinoyl radical (11).

The propagation step consists of the sequential addition of monomer units to the growing radical chain. Each addition converts a methacrylate double bond into a single bond and transfers the radical site to the chain end, enabling rapid growth and network formation (Fig.6.8).

Termination occurs when radical activity is quenched, either by radical combination, disproportionation, or by interaction with oxygen. In the latter case, oxygen reacts with active radicals to form stable peroxy radicals, creating an oxygen-inhibited layer at the composite surface(Fig.6.8).

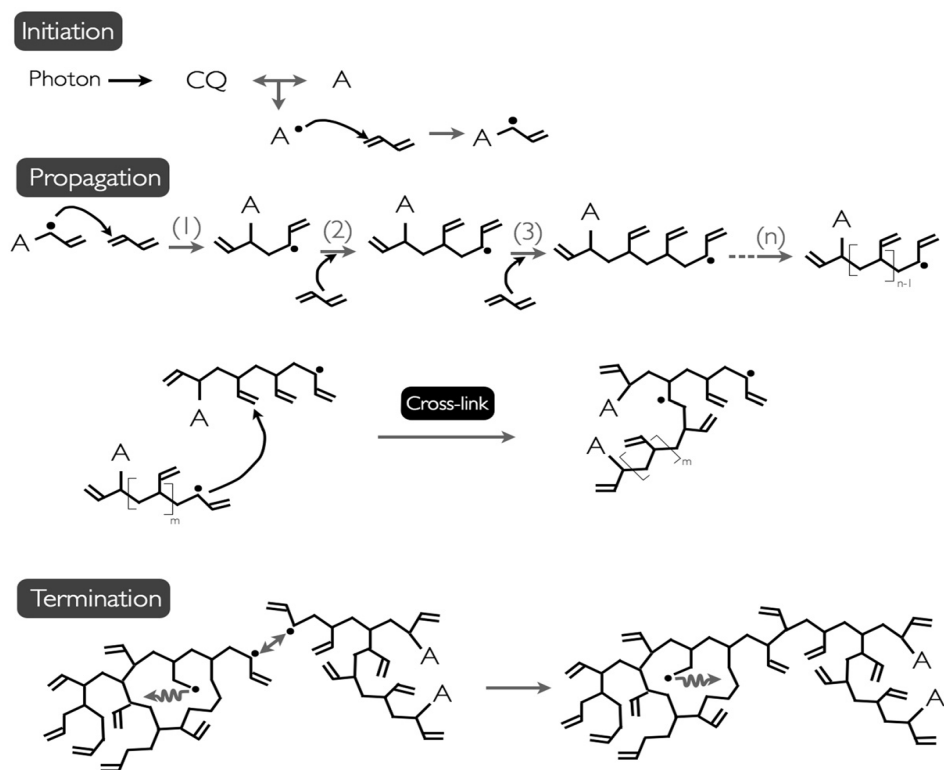


Figure 6.8 : Schematic representation of the 3 steps of photopolymerization reaction – CQ: camphorquinone, A: tertiary amine, [•] indicates a radical species. (1), (2), (3) and (n) represent the theoretical steps of linear monomer addition. n and m: large amount of monomer units. A trapped radical is illustrated in the termination section and segmental mobility is represented by a twisting gray arrow. A double gray arrow indicates radicals about to react together (bimolecular termination) – during initiation, a free radical is generated through the activation of a photosensitive molecule or photoinitiator by a photon of specific wavelength. The newly formed free radical quickly reacts with a nearby monomer, opening its C=C bond. Then follows a chain reaction, the propagation, by which the polymer grows by repetition of this C=C opening by the radical and addition of a large number of monomer units one after the other. The polymer can either grow linearly by reaction with monomers (reactions 1, 2, 3, n), leaving unreacted or “pendant” double bonds, or grow tri-dimensionally by reacting with another polymer chain, creating cross-links. The addition of supplementary units continues until free radicals react with each other to form a stable covalent link through bimolecular termination reaction. Reproduced from Leprince et al. (15)

2.1. Degree of conversion

Conventionally, the extent of polymerization in RBCs is quantified by the DC, which is defined as the fraction of initial carbon-carbon double bonds (C=C) that are consumed during polymerization. DC is calculated from the reduction in the number of C=C bonds upon curing, according to the relationship :

$$\text{DC (\%)} = [(\text{C}=\text{C})_{\text{initial}} - (\text{C}=\text{C})_{\text{residual}}] / (\text{C}=\text{C})_{\text{initial}} \times 100$$

In practice, DC values vary widely among commercial RBCs, typically ranging from 35% to 77% (16). Importantly, these values do not mean that 23–65% of monomer molecules remain unreacted, because each dimethacrylate contains two methacrylate groups; one group may have reacted while the other remains as a pendant group covalently attached to the polymer backbone (Fig. 6.8).

The kinetics of polymerization explain this limitation. After irradiation, radical generation rapidly initiates chain growth and cross-linking, leading to a sharp increase in viscosity. The system undergoes its first transition, from a viscous liquid to an elastic gel, at the gel point. At this stage, mobility restriction affects radicals bound to growing chains, whereas small monomers can still diffuse relatively freely. Bimolecular termination becomes inefficient, while initiation continues to create new growth centers. This imbalance causes a rise in radical concentration and a sharp acceleration of polymerization rate (autoacceleration) (17). As the network continues to densify, mobility is increasingly restricted even for monomers, and the system undergoes autodeceleration. The second transition, vitrification, marks the conversion of the material from a rubbery to a glassy state, at which point diffusion is so limited that further extensive polymerization is prevented (18). This explains why DC never reaches 100%, even under optimal irradiation conditions.

The DC in dental composites is critical because it influences most material properties that determine clinical success. As reviewed by Leprince et al. (15), DC is positively correlated with mechanical characteristics such as microhardness, elastic modulus,

and flexural strength (19-24), and negatively correlated with monomer elution. For these reasons, DC is one of the most frequently measured indicators of polymerization efficiency.

Nevertheless, the DC only measures the average number of double bonds reacted and does not capture the composite's structural heterogeneity or crosslinking density, which are also shaped by polymerization kinetics and curing conditions. Consequently, while DC is a useful indicator, it alone cannot fully describe the composite's mechanical behavior. To this end, complementary parameters such as the degree of crosslinking density and mechanical performance testing should be considered to provide a more comprehensive framework for evaluating and comparing dental composites.

2.2. Degree of crosslinking

Besides the DC, the degree of crosslinking is another critical determinant of the mechanical properties (1, 4) and structural stability of dental composites. Materials with lower crosslink density are more prone to swelling and degradation in solvents (25). Crosslinking arises from the polymerization of multifunctional dimethacrylate monomers such as TEGDMA or BisGMA (2). The extent of crosslinking influences the final network structure: lightly crosslinked polymers exhibit higher elasticity and recovery, as desired in rubbers, whereas highly crosslinked networks provide rigidity and resistance to deformation (4).

At present, there is no direct method available to quantify the degree of crosslinking in these materials. However, it can be assessed indirectly. The ethanol softening test is widely used: a smaller reduction in hardness after ethanol storage indicates higher crosslinking (26).

3. Mechanical Properties

The study of the mechanical properties of dental composites is of great clinical relevance, as these materials must maintain their structural integrity in the oral cavity for a long period of time, ideally for the entire life of the patient. The clinical success of RBCs is inseparably linked to their ability to withstand mechanical challenges encountered in the oral environment. Fracture remains the most common reason for restoration failure, particularly in posterior regions subjected to high occlusal loading. Laboratory testing of mechanical properties provides critical insight into the behavior of composites under simulated conditions, thereby guiding material development, regulatory evaluation, and clinical selection. However, not all properties bear equal predictive value, and the choice of test methodology can substantially influence both reported outcomes and their translational relevance.

The Academy of Dental Materials (Ilie et al. 2017) has identified a hierarchy of mechanical properties that are most strongly supported by empirical evidence and best reflect clinical failure modes. These include strength, elastic modulus, fracture toughness, fatigue, hardness, and wear resistance, which together form a core test panel reflecting the multifactorial nature of clinical failure. Secondary properties, such as edge strength and toothbrush wear, provide supplementary but less central insights. While each test carries methodological limitations and variable correlation with clinical performance, their collective interpretation allows for a comprehensive evaluation of material behavior.

Although the detailed testing of mechanical properties is not the primary focus of this thesis, their evaluation remains essential for understanding the clinical relevance of polymerization parameters. Clinical evidence confirms that the reasons for failure evolve over time. In the first year after placement, failures are predominantly endodontic, often related to pulpal complications from the underlying pathology or restorative procedure. From the second year onward, fracture becomes the most frequent cause and remains a stable, long-term concern. With increasing service time,

however, caries-related failures accumulate and eventually surpass fracture as the leading reason for replacement (Fig. 1) (Opdam et al. 2014). Importantly, early endodontic and fracture failures are mainly material, or technique-related, whereas caries-associated failures are largely patient-dependent, reflecting caries susceptibility and oral hygiene.

For both researchers and clinicians, this evidence underscores that an integrated, multi-property assessment framework, one that considers not only material performance but also biological concerns such as the elution of residual compounds, provides the most robust pathway to developing, selecting, and deploying RBCs capable of meeting the rigorous demands of long-term intraoral function.

4. Elution of Compounds from Cured Resin-Based Composites

Since the DC of RBCs is never complete under clinical circumstances, a fraction of unreacted monomers inevitably remains in the set material. These molecules, together with unbound components such as initiators can leach into the oral environment.

More than 30 different organic compounds have been identified in eluates from polymerized composites. Among the most frequently detected are the major dimethacrylate monomers BisGMA and TEGDMA, along with various photoinitiators (27).

Release of substances is a critical issue in dental materials research, not only because it affects long-term material stability but also because of potential biological consequences, which will be reviewed in detail in the next chapter. Importantly, it has been shown that essentially any organic component present in a RBCs has the potential to be leached, albeit in varying quantities depending on the chemical environment (28).

Extraction tends to be more pronounced in organic-rich solvents such as ethanol, which simulate worst-case clinical conditions, but even in water or artificial saliva, elution of residual species can be detected, sometimes at very low concentrations, such as the photoinitiators. (29).

This section therefore explores the chemical nature of eluates, their release kinetics, and the material and environmental factors that govern their elution.

4.1. Types of eluted substances

The organic matrix is the primary source of eluates (28). Residual monomers show distinct elution behaviors depending on their molecular structure. Low-molecular-weight, polar monomers such as TEGDMA are consistently reported to elute at higher concentrations and earlier time points compared with bulkier dimethacrylate monomers like BisGMA migrate more slowly but may still be detected over extended periods (30, 31). UDMA generally exhibits intermediate behavior, reflecting its balance of size and polarity.

Initiators and co-initiators, such as CQ and DMAEMA may be detected in eluates if they are not fully consumed during curing (32). Concerning TPO, however, its elution remains theoretical, as no studies to date have detected its release from adequately cured RBCs.

Fillers represent another source of eluates. The inorganic particles themselves are generally insoluble, but ions can leach out from radiopaque fillers such as barium, strontium, or zinc aluminosilicate glasses (33, 34).

4.2. Elution rate

The elution of substances from RBCs follows a characteristic time-dependent pattern, but the kinetics differ among monomers and initiators.

For monomers, the release is generally biphasic. A rapid burst release occurs within the first hours to days after curing, dominated by small, mobile monomers such as TEGDMA and HEMA (28, 30). These molecules are easily extracted into aqueous solutions and even more efficiently into ethanol–water mixtures (29, 31). Larger species like BisGMA or UDMA elute more slowly and at lower concentrations, especially in organic-rich media (30, 31). Several studies have confirmed that while a substantial proportion of releasable monomers is extracted in the first 24–72 h, release may continue for weeks to months or even a year, reflecting slow diffusion from the bulk matrix (31).

Long-term elution kinetics of initiators and co-initiators remain poorly characterized. To date, only one notable study by Meng et al. (35) monitored eluates over 14 days in both deionized water and ethanol–water mixtures. They reported that CQ elution was extremely low, with detectable levels only in specimens containing high initiator loadings and stored in 75% ethanol. In water, virtually no CQ was released. This result is somewhat surprising, given that the Nernst partition coefficient of CQ is about six times higher than that of BisGMA (36), which would predict a greater affinity for organic media.

Elution from RBCs is time-dependent and compound-specific. Monomers show biphasic release: small, mobile species such as TEGDMA and HEMA leach rapidly within the first hours to days, while larger monomers like BisGMA and UDMA elute more slowly and at lower levels, with release persisting over longer periods. By contrast, initiators and co-initiators contribute minimally to eluates and remain less well characterized.

4.3. Parameters influencing elution

As mentioned above, several factors can influence the release of monomers from dental composites, including the material properties, curing parameters, and the storage medium. The elution profile is ultimately determined by the interplay between the composite's chemistry and the external environment.

Monomers type : low-molecular-weight, hydrophilic monomers such as TEGDMA diffuse rapidly, whereas bulkier, hydrophobic species like BisGMA and UDMA are more restricted but can still leach over time (30, 31).

Degree of conversion and cross-link density : a low DC or insufficient crosslinking leaves more free monomers mobile within the matrix. By contrast, higher conversion and crosslink density reduce free volume and molecular mobility, limiting the diffusion of monomers and initiators (4, 20).

Filler content: highly filled composites contain a smaller resin fraction and therefore release fewer monomers in absolute terms (1, 29).

Storage medium: the nature of the extraction medium strongly influences these release patterns: aqueous media such as water or artificial saliva primarily extract more hydrophilic monomers, while ethanol–water solutions or other organic-rich solvents penetrate the hydrophobic polymer network and mobilize larger, less soluble species (28, 29, 31). It was elegantly demonstrated by Meng and colleagues (35) that no BisGMA could be detected in water storage after one week, whereas the same monomer appeared in large amounts one week later once the storage medium was switched to ethanol (Fig. 6.9). This finding underscores that the solvent environment not only affects the total concentration of released species but also dictates which components can be detected in eluates.

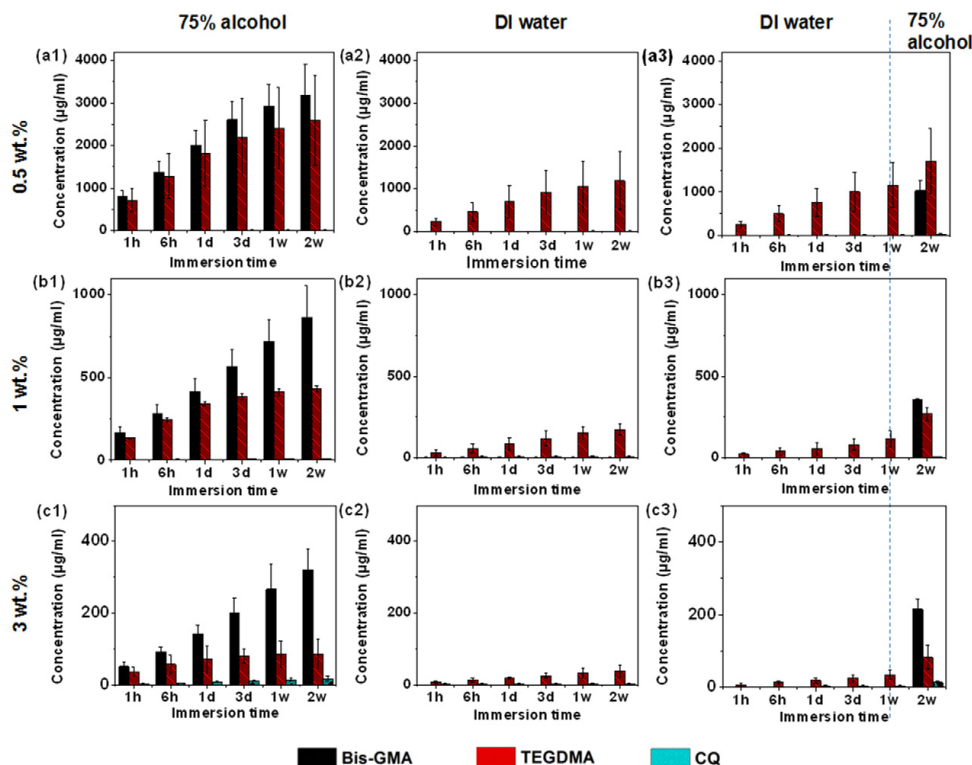


Figure 6.9 : Concentrations of dissolvable ingredients from cured composite resins containing different amounts of CQ/DMAEMA by soaking them in different media for 14 days and being determined by LC/MS. Left column: soaking in 75% ethanol solution; middle column: soaking in DI water; right column: soaking in DI water for 7 days followed by soaking in 75% ethanol solution for another 7 days. Reproduced from Meng et al. (35)

5. Conclusion

The elution of substances from RBCs is a dynamic, multi-phase process shaped by both material composition and environmental conditions. Monomers, particularly small hydrophilic species such as TEGDMA, dominate the early release phase through rapid diffusion, while larger monomers like BisGMA and UDMA elute more slowly at

lower levels. Initiators and co-initiators are released in trace amounts, though their long-term kinetics remain insufficiently studied.

Factors such as DC, cross-link density, filler content, curing conditions, and the storage medium all play decisive roles in determining the extent and pattern of release. Together, these insights emphasize that elution is not merely a material property but the result of a complex interplay between intrinsic formulation and external environment.

While this chapter has focused on the chemical aspects and kinetics of elution, the broader clinical relevance lies in the biological effects of these leached substances. The next chapter therefore examines the potential health implications of eluates from dental composites, addressing cytotoxicity, genotoxicity, and other biological responses that inform the biocompatibility of these widely used restorative materials.

6. References

1. Ferracane JL. Resin composite--state of the art. *Dent Mater.* 2011;27(1):29-38.
2. Peutzfeldt A. Resin composites in dentistry: the monomer systems. *Eur J Oral Sci.* 1997;105(2):97-116.
3. Bowen RL, inventor. Dental filling material comprising vinyl silane treated fused silica and a binder consisting of the reaction product of bisphenol and glycidyl methacrylate. 1962.
4. Stansbury JW. Dimethacrylate network formation and polymer property evolution as determined by the selection of monomers and curing conditions. *Dent Mater.* 2012;28(1):13-22.
5. Davy KW, Kalachandra S, Pandain MS, Braden M. Relationship between composite matrix molecular structure and properties. *Biomaterials.* 1998;19(22):2007-14.
6. V.Miletic, editor. *Dental Composite Materials for Direct Restorations* 2018.
7. Ferracane JL. A Historical Perspective on Dental Composite Restorative Materials. *J Funct Biomater.* 2024;15(7).
8. Ilie N, Hickel R. Resin composite restorative materials. *Aust Dent J.* 2011;56 Suppl 1:59-66.
9. Stansbury JW. Curing dental resins and composites by photopolymerization. *J Esthet Dent.* 2000;12(6):300-8.
10. Neumann MG, Schmitt CC, Ferreira GC, Correa IC. The initiating radical yields and the efficiency of polymerization for various dental photoinitiators excited by different light curing units. *Dent Mater.* 2006;22(6):576-84.
11. Kowalska A, Sokolowski J, Bociong K. The Photoinitiators Used in Resin Based Dental Composite-A Review and Future Perspectives. *Polymers (Basel).* 2021;13(3).
12. Jockuschlgor S, Koptuyug V, McGarry PF, Sluggett GW, Turro NJ, Watkins DM. A Steady-State and Picosecond Pump-Probe Investigation of the Photophysics of an Acyl and a Bis(acyl)phosphine Oxide. *Journal of the American Chemical Society.* 1997.
13. Ilie N, Hickel R. Can CQ be completely replaced by alternative initiators in dental adhesives? *Dent Mater J.* 2008;27(2):221-8.
14. Bouzidi A, Bayou S, Khier N, Dehamchia M. Photoinitiated polymerization of a dental formulation, part 2: kinetic studies. *Polymer Bulletin.* 2023;81(5):4221-35.
15. Leprince JG, Palin WM, Hadis MA, Devaux J, Leloup G. Progress in dimethacrylate-based dental composite technology and curing efficiency. *Dent Mater.* 2013;29(2):139-56.
16. Schmalz G. Resin-based composites. In: G S, D A-B, editors. *Biocompatibility of Dental Materials*: Springer-Verlag; 2009. p. p. 99–137.
17. Anseth KS, Newman SM, Bowman CN. Polymeric dental composites: properties and reaction behavior of multimethacrylate dental restorations. *Advances in Polymer Science.* 1995.
18. Lovell LG, Stansbury JW, Syrpes DC, Bowman CN. Effects of Composition and Reactivity on the Reaction Kinetics of Dimethacrylate:Dimethacrylate Copolymerizations. *Macromolecules.* 1999.

19. Lovell LG, Newman SM, Donaldson MM, Bowman CN. The effect of light intensity on double bond conversion and flexural strength of a model, unfilled dental resin. *Dent Mater.* 2003;19(6):458-65.
20. Petronijevic Sarcev B, Balos S, Markovic D, Sarcev I, Vukcevic M, Labus Zlatanovic D, et al. Effect of the Degree of Conversion on Mechanical Properties and Monomer Elution from Self-, Dual- and Light-Cured Core Composites. *Materials (Basel).* 2021;14(19).
21. Leprince JG, Leveque P, Nysten B, Gallez B, Devaux J, Leloup G. New insight into the "depth of cure" of dimethacrylate-based dental composites. *Dent Mater.* 2012;28(5):512-20.
22. Li J, Li H, Fok AS, Watts DC. Multiple correlations of material parameters of light-cured dental composites. *Dent Mater.* 2009;25(7):829-36.
23. Ferracane JL. Correlation between hardness and degree of conversion during the setting reaction of unfilled dental restorative resins. *Dent Mater.* 1985;1(1):11-4.
24. Ferracane JL, Greener EH. The effect of resin formulation on the degree of conversion and mechanical properties of dental restorative resins. *J Biomed Mater Res.* 1986;20(1):121-31.
25. Sideridou I, Tserki V, Papanastasiou G. Effect of chemical structure on degree of conversion in light-cured dimethacrylate-based dental resins. *Biomaterials.* 2002;23(8):1819-29.
26. Asmussen E, Peutzfeldt A. Influence of selected components on crosslink density in polymer structures. *Eur J Oral Sci.* 2001;109(4):282-5.
27. Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J Biomed Mater Res.* 1998;41(3):474-80.
28. Ferracane JL. Elution of leachable components from composites. *J Oral Rehabil.* 1994;21(4):441-52.
29. Ortengren U, Wellendorf H, Karlsson S, Ruyter IE. Water sorption and solubility of dental composites and identification of monomers released in an aqueous environment. *J Oral Rehabil.* 2001;28(12):1106-15.
30. Sideridou ID, Achilias DS. Elution study of unreacted Bis-GMA, TEGDMA, UDMA, and Bis-EMA from light-cured dental resins and resin composites using HPLC. *J Biomed Mater Res B Appl Biomater.* 2005;74(1):617-26.
31. Polydorou O, Konig A, Hellwig E, Kummerer K. Long-term release of monomers from modern dental-composite materials. *Eur J Oral Sci.* 2009;117(1):68-75.
32. De Angelis F, Sarteur N, Buonvivere M, Vadini M, Steffl M, D'Arcangelo C. Meta-analytical analysis on components released from resin-based dental materials. *Clin Oral Investig.* 2022;26(10):6015-41.
33. Zhou M, Drummond JL, Hanley L. Barium and strontium leaching from aged glass particle/resin matrix dental composites. *Dent Mater.* 2005;21(2):145-55.
34. Tiskaya M, Salihi A, Shahid S, Hill R. Are inert glasses really inert? *Dent Mater.* 2022;38(11):1742-8.
35. Meng J, Yang H, Cao M, Li L, Cai Q. Correlating cytotoxicity to elution behaviors of composite resins in term of curing kinetic. *Mater Sci Eng C Mater Biol Appl.* 2017;78:413-9.

36. Durner J, Spahl W, Zaspel J, Schweikl H, Hickel R, Reichl FX. Eluted substances from unpolymerized and polymerized dental restorative materials and their Nernst partition coefficient. *Dent Mater*. 2010;26(1):91-9.

VII. Biological Effects of Substances Released by Resin-Based Composites

Although RBCs offer clear advantages in restorative dentistry, concerns remain regarding their biological safety. Central to these concerns is the release of residual compounds from incomplete polymerization, particularly residual monomers and photoinitiators residues, which may be released over time (1).

RBCs are primarily composed of methacrylate monomers, including BisGMA, UDMA and TEGDMA. Although 2-hydroxyethyl methacrylate (HEMA) is not a major component of RBCs, it is widely used in dental adhesives that are invariably applied in conjunction with composites. Consequently, exposure to HEMA is clinically inseparable from the use of RBCs. Moreover, HEMA is a small, highly hydrophilic monomer with high diffusivity, which allows it to readily penetrate dentin and soft tissues (Tab.2).

Monomer	Molecular weight (g/mol)	logP (octanol/water)	Polarity	Electrical charge (physiological pH)	Water solubility
HEMA	130.1	-0.4 to -0.1	High	Neutral	Very high
Bisphenol A (BPA)	228.3	3.2–3.6	Low	Neutral	Very low

Table 2: Physicochemical properties of selected low-molecular-weight resin-related compounds relevant to dental materials. The table summarizes key physicochemical descriptors, including molecular weight, lipophilicity (logP, octanol/water), polarity, electrical charge at physiological pH, and water solubility, for HEMA, a hydrophilic monomer commonly used in dental adhesives, and Bisphenol A (BPA), a resin-related compound that may be present as an impurity or degradation by-product. Reported values correspond to ranges commonly found in the literature and may vary depending on experimental conditions.

For these reasons, HEMA has been frequently implicated in studies of both systemic and local effects. Including HEMA in this review therefore provides a more complete assessment of the biological risks associated with contemporary composite restorations, which depend not only on the composite itself but also on the adhesive systems required for their placement.

This chapter first maps the exposure pathways through which leached substances can reach human tissues. It then reviews evidence from experimental and clinical studies on how these substances are absorbed, distributed, metabolized, and eliminated in the body. Particular attention is given to potential health risks associated with specific monomers, including the controversial compound bisphenol A (BPA). Although BPA is not intentionally added to composites, it can be present in trace amounts due to incomplete synthesis of BisGMA. The discussion then addresses allergic and sensitization risks before examining the molecular mechanisms of cytotoxicity, with dedicated sections on the widely used matrix monomer BisGMA and the co-monomer TEGDMA.

1. Pathways of Absorption

Leached substances from RBCs can reach human tissues through several distinct routes of exposure.

For patients, there are three principal routes of exposure. Ingestion is the most direct pathway, as leached monomers mix with saliva and are swallowed into the gastrointestinal tract (1). A second route is transmucosal absorption, particularly when restorations come into direct contact with oral soft tissues. Finally, diffusion through dentinal tubuli may occur, especially when the remaining dentin thickness is reduced (2).

For dental personnel, dermal exposure represents a more significant route. Contact with uncured RBCs can allow methacrylates to penetrate the skin, occasionally causing allergic contact dermatitis despite gloves use (3). Inhalation is another concern: during polishing, composite dust containing resin-coated filler particles and entrapped monomers can become airborne. Prolonged occupational exposure to such dust has been associated with respiratory irritation and, in rare cases, with chronic lung diseases, including pneumoconiosis in dental professionals (4).

2. Types of Effects

2.1. Systemic effects

Systemic effects of RBCs depend not only on the amount of substances leached but also on their toxicokinetics once in the body. Residual compounds can be released from restorations and may reach systemic circulation (5), where their absorption, distribution, metabolism, and elimination have been investigated in both experimental and clinical studies.

2.1.1. *Toxicokinetics and systemic toxicity of resin monomers*

Once absorbed, methacrylate monomers are distributed systemically but are generally cleared rapidly. Toxicokinetic studies in rodents show that low-molecular-weight monomers such as HEMA and TEGDMA are metabolized and exhaled as carbon dioxide within 24 hours, with negligible tissue accumulation (6). Tissue concentrations measured after 24 hours were several orders of magnitude below toxic thresholds, consistent with rapid clearance. Larger and more hydrophobic monomers, such as BisGMA and UDMA, persist somewhat longer but undergo enzymatic hydrolysis by plasma esterases, producing more polar metabolites such as bis-hydroxypropoxyphenyl-propane, which are more easily excreted. Although these

bulkier monomers exhibit higher cytotoxicity in vitro, in vivo data indicate that they are also efficiently metabolized and eliminated, generally within one to two days (7).

Animal toxicology studies support these kinetic findings, indicating that dental resin monomers have low systemic toxicity at clinically relevant doses. Acute high-dose exposures can produce transient effects such as local irritation or cytotoxicity but typically do not result in lasting organ damage. In a 90-day rat study with TEGDMA composites, no behavioral or clinical abnormalities and no histopathology were observed in 30 organs (8). Other feeding studies likewise reported tissue levels far below toxic thresholds. For instance, Reichl et al. (6) showed that even high oral doses of HEMA or TEGDMA in rodents did not yield organ concentrations sufficient to elicit known toxic effects.

Human clinical studies have provided similar results. No clinical syndrome has been definitively linked to composite restorations. A comprehensive Norwegian systematic review of pediatric and adult trials (9) found no differences in renal function, neurocognitive development, or growth trajectories over 5–7 years of follow-up, aside from minor findings such as tooth sensitivity. Some smaller investigations have suggested subtle biomarker changes, for example, altered B-cell and monocyte activation markers (10) or slight increases in oxidative stress following composite placement (11). However, these effects were modest, inconsistent across studies, and not associated with clinical illness. Importantly, biomonitoring studies consistently show that systemic exposure is both short-lived and minimal: monomers from RBCs and adhesive systems can be detected in saliva shortly after treatment but typically fall below detection limits within one week (12), and when measurable, concentrations in saliva or blood remain far below levels associated with systemic pathology. Biomonitoring consistently shows that residual monomer levels in saliva or blood remain too low to cause systemic pathology (13, 14).

2.1.2. *Bisphenol A as an impurity or by-product*

Although not intentionally added to composites, BPA may be present as an impurity in BisGMA-based composites (15). BPA is an endocrine-disrupting chemical associated with adverse effects on human health, including reproductive, developmental, and metabolic disorders (16). Clinical studies consistently report that BPA is detectable in saliva or urine after placement of resin sealants and composites, but typically only as a transient spike within the first 24 hours (17-21). Importantly, BPA levels from dental sources remain well below toxicological thresholds and contribute only minimally to total BPA exposure, which is dominated by dietary and environmental sources (22). Accordingly, BPA release from composites is not considered a significant health concern, though it represents a minor contributor to overall exposure.

2.1.3. *Overall appraisal*

Taken together, the evidence indicates that although RBCs can release potentially harmful monomers, systemic exposure in patients is low, short-lived, and well below established toxic thresholds. Animal toxicokinetics and human biomonitoring consistently demonstrate rapid metabolism and elimination of absorbed compounds. Clinical studies, including large randomized trials and systematic reviews, have not demonstrated clinically significant systemic toxicity attributable to dental composites. However, the limitations of clinical follow-up may mask subtle or long-term effects. Thus, while composites are considered safe in everyday practice, continued vigilance through biomonitoring and long-term epidemiological studies remains essential.

2.2. Allergic reactions

Although systemic toxicity from RBCs appears to be negligible at clinically relevant exposure levels, their components can act as sensitizers, triggering allergic reactions in susceptible individuals. Hypersensitivity to dental materials has been documented since the early 20th century, with clinical manifestations ranging from localized irritation to systemic symptoms (23).

2.2.1. *Types of hypersensitivity*

Both type I (immediate, IgE-mediated) and type IV (delayed, T-cell-mediated) hypersensitivity reactions have been reported in association with RBCs.

Type I reactions to RBCs are rare. Most immediate hypersensitivity in dentistry are related to latex or medications, but isolated reports have linked RBCs or sealants to systemic events (23).

Type IV reactions are the most frequent, presenting as allergic contact dermatitis on the hands of dental personnel, or as intraoral lesions in patients. Clinical presentations include stomatitis, lichenoid lesions, or cheilitis (23).

2.2.2. *Allergenic components*

RBCs and adhesives contain multiple potential allergens. Methacrylate monomers, particularly HEMA and TEGDMA, are among the most potent sensitizers (24, 25), whereas high-molecular-weight monomers such as BisGMA and UDMA are less frequently implicated (24, 26). Additives such as amine accelerators or initiators, may occasionally provoke reactions, though far less commonly than the monomers themselves (27). In contrast, inorganic fillers (silica, glass) are generally immunologically inert.

2.2.3. *Prevalence*

Occupational allergy is the best-documented context for methacrylate sensitization. Repeated exposure to uncured RBCs or adhesives during placement puts dentists, hygienists, assistants, and technicians at particular risk. Epidemiological studies consistently report a higher prevalence of methacrylate allergy among dental personnel compared with patients.

One of the most comprehensive studies was conducted by Goon et al. (24) who patch-tested 1,632 individuals (1,322 patients and 310 staff members). They found methacrylate sensitization in 5.8% (18/310) of dental personnel versus 2.3% (30/1,322) of patients. Across both groups, the most frequent allergen was HEMA. Among the methacrylates commonly present in composites, including TEGDMA, BisGMA, and UDMA, the prevalence was considerably lower: these compounds accounted for allergic reactions in only 0.6% of patients but in 3.2% of dental personnel. This disparity underscores the increased risk associated with frequent handling and manipulation of uncured adhesives in occupational settings.

2.2.4. *Clinical manifestations*

The clinical presentation of allergic reactions to RBCs depends on the type of hypersensitivity, the site of exposure, and whether the affected individual is a patient or a dental professional.

Cutaneous manifestations are the most frequently documented, particularly among dental personnel. Allergic contact dermatitis typically presents as erythema, pruritus, vesiculation, or fissuring of the hands and fingers, most often in areas of repeated contact with uncured resin or adhesive. Chronic exposure may result in eczematous, lichenified lesions or periungual dermatitis. In some cases, facial involvement such as eyelid or perioral dermatitis has been observed, usually due to airborne exposure or inadvertent hand-to-face contact (24-26).

Oral and perioral manifestations occur predominantly in patients. Allergic contact stomatitis presents with burning sensations, erythema, edema, or ulceration of the oral mucosa in direct contact with the restoration. Lichenoid reactions adjacent to composite restorations, cheilitis, and angular stomatitis are also reported presentations. These lesions may clinically resemble other oral diseases, complicating diagnosis without the use of patch testing (23).

Respiratory manifestations are less common but clinically relevant, especially in sensitized dental personnel. Reported symptoms include rhinitis, conjunctivitis, and occupational asthma triggered by inhalation of methacrylate vapors or dust from composite finishing and polishing. Exacerbation of pre-existing asthma has also been described (28).

Systemic manifestations are rare but potentially severe. Immediate type I hypersensitivity may present as generalized urticaria, angioedema, or, in exceptional cases, anaphylaxis. Hallström (29) described a case of a 6-year-old girl who developed anaphylactic shock after fissure sealant placement, followed by urticaria, blistering, and intraoral lesions; symptoms resolved following removal of the material.

2.2.5. *Summary*

Allergic reactions to RBCs are well documented but remain uncommon in patients and significantly more prevalent among dental professionals due to repeated occupational exposure. The clinical spectrum ranges from localized dermatitis and oral mucosal lesions to systemic manifestations such as asthma or, in rare cases, anaphylaxis.

HEMA is the principal sensitizer, whereas BisGMA, UDMA and TEGDMA are less frequently implicated but can still trigger allergic reactions in susceptible individuals.

Considering this, clinicians should remain aware of the allergenic potential of methacrylates, employ protective measures (adequate curing, high-volume suction, nitrile gloves, avoidance of direct contact with uncured resins), and consider patch testing in cases of suspected allergy.

2.3. Local effects

RBCs are intended to remain in close and often prolonged contact with the oral environment. Their presence is not confined to the visible interface with enamel, dentin, or gingival tissues, but also extends indirectly to deeper structures, as substances released from these materials can diffuse through dentinal tubules into the pulp or mix with saliva and interact with soft tissues (30).

The oral mucosa, particularly its non-keratinized regions, exhibits a degree of permeability that may permit monomers to penetrate the epithelium and reach underlying tissues. In vitro studies have demonstrated that several dental monomers exert cytotoxic, genotoxic, and pro-inflammatory effects on mucosal epithelial cells (31), suggesting a theoretical risk of local irritation or chronic low-grade inflammation. However, in vivo, this risk is often mitigated by the transient and diluted nature of exposure, due to the buffering effect of saliva and the high regenerative capacity of mucosal tissues. Clinical manifestations of mucosal toxicity remain rare and are typically limited to allergic reactions, such as oral lichenoid lesions.

In contrast, the diffusion of monomers through dentinal tubules toward the dental pulp represents a more clinically significant concern, especially in deep cavities where the remaining dentin thickness is reduced. Dentinal tubules provide a direct anatomical pathway for low-molecular-weight substances to reach the pulp chamber (2), where resident cells such as odontoblasts and fibroblasts may be exposed. Numerous studies have shown that common resin monomers can induce oxidative stress, mitochondrial dysfunction, and apoptosis in pulpal cells (32). Unlike the mucosa, the pulp resides in a confined environment with limited capacity to buffer toxic stimuli. Even subclinical irritation may compromise pulp vitality over time and increase the risk of irreversible damage, potentially necessitating endodontic intervention (33).

For these reasons, the dentin–pulp pathway is considered the most critical route of concern, as the interface between RBCs and vital pulp tissue is where biocompatibility becomes most clinically relevant.

2.3.1. *Toxicity*

Advances in material development in the industry regarding resin formulations have reduced the proportion of unbound monomers released after curing; however, the problem has not been fully resolved. Complete conversion during polymerization is unattainable, and residual methacrylate groups typically account for 1.5–5% of the final material. Although these amounts may appear small, they are sufficient to produce measurable biological effects (Goldberg, 2008).

The cytotoxicity and genotoxicity of substances released by RBCs have been extensively investigated over the past two decades. Much of this research has focused on the effects of resin monomers on fundamental cellular processes, including proliferation, enzyme activity, morphology, membrane integrity, and essential metabolic functions such as DNA, RNA, and protein synthesis (32, 34, 35).

Overall, most studies agree that RBCs are capable of releasing compounds with varying degrees of cytotoxicity. Highly toxic monomers include BisGMA, UDMA, and TEGDMA, whereas others, such as HEMA and photoinitiators like CQ, are generally considered moderately cytotoxic (7, 36).

Among the substances released from dental composites, the major (co)monomers have been identified as the primary contributors to cytotoxicity (36). Their toxic concentrations (TC_{50} , the concentration that causes a toxic effect in 50% of the exposed cells) have been assessed in a wide range of in vitro models, including permanent cell lines (such as 3T3 and L929 fibroblasts, V79 Chinese hamster lung fibroblasts, HaCaT keratinocytes, and THP-1 monocytes) as well as primary human-derived cells, including pulp, periodontal, gingival, and skin fibroblasts. These studies consistently reveal considerable variability in cellular sensitivity. Nevertheless, in vitro experiments consistently confirm that BisGMA, UDMA, TEGDMA, and HEMA induce dose- and time-dependent reductions in the viability of human dental pulp cells (hDPCs), including fibroblasts and stem cells.

Biological Effects of Substances Released by Resin-Based Composites

Experimental exposures generally range from low micromolar to millimolar concentrations, applied for periods from several hours up to three days. Cell viability is usually assessed using standard tetrazolium salt-based assays (MTT, XTT, WST-1) or by measuring lactate dehydrogenase (LDH) release, all of which are considered gold-standard methods for cytotoxicity testing and are recommended by ISO 10993-5.

From this body of evidence, a consistent toxicity hierarchy emerges: BisGMA > UDMA > TEGDMA > HEMA. Most of these studies have been conducted in gingival fibroblasts (37-41), whereas surprisingly fewer reports exist for hDPCs (39, 42). The results show striking differences: BisGMA and UDMA reduce cell viability by 50% at sub-millimolar concentrations, while TEGDMA requires mid-millimolar concentrations, and HEMA an order of magnitude higher to achieve comparable effects. For example, Reichl et al. (38) reported that gingival fibroblasts TC_{50} are 0.11 mM for Bis-GMA and 0.27 mM for UDMA, compared with 3.7 mM for TEGDMA and 11.9 mM for HEMA, with no significant differences observed when compared to hDPCs. Similarly, Kwon et al. (42) confirmed that TEGDMA was approximately twice as toxic as HEMA in hDPCs, with TC_{50} values of ~4 mM and ~8 mM, respectively.

These toxicity trends have been linked to physicochemical properties, particularly lipophilicity. Yoshii (43) demonstrated an inverse correlation between TC_{50} values and logP (the logarithm of the partition coefficient, describing how a molecule distributes between an organic solvent) for acrylates, methacrylates, and ethylmethacrylates with ether substituents, suggesting that higher lipophilicity (and lower water solubility) enhances toxicity. Issa et al. (44) corroborated this relationship for BisGMA and TEGDMA, showing an inverse correlation between TC_{50} and logP values. Nevertheless, additional factors such as molecular structure and electrical charge also play important roles in determining cytotoxicity (43).

Taken together, these findings provide critical insight into how resin monomers interact with dental pulp cells in vitro. The consistent ranking of toxicity, coupled with mechanistic links to lipophilicity and molecular structure, suggests that pulpal injury is not a random occurrence but rather a predictable biological consequence of monomer

diffusion through dentin. While fibroblast-based models offer valuable baseline data, studies on hDPCs are particularly relevant, as they better reflect the clinical scenario in which residual monomers can compromise pulp vitality.

2.3.2. *Molecular mechanisms of toxicity*

Building on previous research, an increasing number of studies have focused on elucidating the key molecular mechanisms and signaling pathways involved in the cytotoxicity and genotoxicity induced by RBCs. This shift reflects a growing recognition that understanding how monomers exert their biological effects is essential both for predicting clinical outcomes and for guiding the development of safer materials.

Among the various methacrylate monomers used in RBCs, BisGMA and TEGDMA are particularly noteworthy. They serve not only as the principal structural components of most resin matrices but are also among the most extensively studied with respect to their biological activity.

Accordingly, this thesis will focus on the molecular mechanisms by which Bis-GMA and TEGDMA affect cells, with particular emphasis on cytotoxic and genotoxic outcomes, the range of adaptive cellular responses that may modulate toxicity, and the pathways regulating apoptosis versus necrosis.

2.3.2.a. *Molecular mechanisms of TEGDMA toxicity*

TEGDMA is one of the most extensively studied monomers with respect to biocompatibility, as it is readily released from polymerized composites into aqueous environments and accounts for the majority of unreacted double bonds (45, 46). Furthermore, its widespread use as a diluent monomer, at concentrations ranging from 25% to 50% in many commercial formulations, further highlights its clinical significance (47).

From a biological standpoint, the physicochemical properties of TEGDMA contribute to its toxic potential. Owing to its lipophilic nature, TEGDMA can diffuse across cell membranes and accumulate within the cytosol and lipid compartments, thereby directly interacting with critical intracellular targets (Engelmann et al., 2001).

On a cellular level, TEGDMA-induced toxicity has been associated with several interrelated events: glutathione (GSH) depletion, oxidative stress, mitochondrial dysfunction, cellular stress response, activation of various signaling pathways (e.g., redox-sensitive kinases and transcription factors), DNA damage (genotoxicity), and ultimately, the induction of apoptosis and/or necrosis.

2.3.2.a.i. *Depletion of GSH and generation of oxidative stress*

One of the earliest cellular events following TEGDMA exposure is a rapid and pronounced depletion of intracellular GSH. GSH is a tripeptide (γ -glutamyl-cysteinyl-glycine) and the most abundant intracellular non-protein thiol. It acts as a major antioxidant and redox buffer, protecting cells by detoxifying reactive oxygen species (ROS) and electrophilic compounds, and by maintaining the redox state of proteins. Numerous in vitro studies on oral cell types have shown that even low millimolar concentrations of TEGDMA can markedly reduce the cellular GSH pool within minutes to hours (48, 49). In primary human gingival and pulp fibroblasts, Stanislawski and colleagues (48) reported that GSH levels begin to decline within 15–30 minutes of treatment and are almost completely depleted after 4–6 hours.

Notably, this depletion does not appear to result from an oxidative process but rather from a direct chemical interaction between TEGDMA and GSH. Unlike ROS, which oxidize GSH to its disulfide form (GSSG), TEGDMA does not significantly increase GSSG formation (49). Instead, mass spectrometry has revealed the formation of a GSH–TEGDMA adduct, confirming that the monomer, owing to its electrophilic nature, reacts with GSH via a Michael addition reaction (50).

GSH is the cell's primary non-enzymatic redox buffer, and its depletion has profound downstream consequences. Mitochondria are entirely dependent on the cytosolic pool for their GSH supply (51); thus, when cytosolic levels fall, mitochondrial antioxidant capacity is also compromised. Under physiological conditions, mitochondria are the main cellular source of ROS, as electrons leak from complexes I and III of the electron transport chain (ETC) during oxidative phosphorylation, producing superoxide ($O_2^{\cdot-}$). This superoxide is normally dismutated to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutases (SODs) (52, 53), and subsequently detoxified to water by GSH-dependent glutathione peroxidase or by catalase (Fig.7.1) (52).

When cells are exposed to TEGDMA, however, early GSH depletion compromises peroxide detoxification. As a result, H_2O_2 accumulates, shifting the redox balance toward oxidative stress. This loss of redox control coincides with a late "burst" of ROS, typically observed 3–4 hours after exposure in gingival and pulp fibroblasts, as detected by DCFH-DA fluorescence (see "method used in this thesis") (48, 54, 55). The temporal relationship between GSH depletion and ROS escalation strongly supports the view that loss of GSH is a permissive event in the development of oxidative stress.

However, GSH depletion alone does not fully account for TEGDMA's cytotoxicity. For example, Martins et al. (56), demonstrated that artificially elevating intracellular GSH in periodontal ligament fibroblasts did not prevent cell death, suggesting that additional mechanisms are involved. One such mechanism is a direct effect of TEGDMA on mitochondrial electron transport. Mikulás et al. (57) showed, using isolated mitochondria and permeabilized hDPCs, that TEGDMA inhibits Complex I (Fig.7.1). This inhibition promotes electron leakage and premature reduction of oxygen to superoxide, thereby amplifying mitochondrial ROS generation.

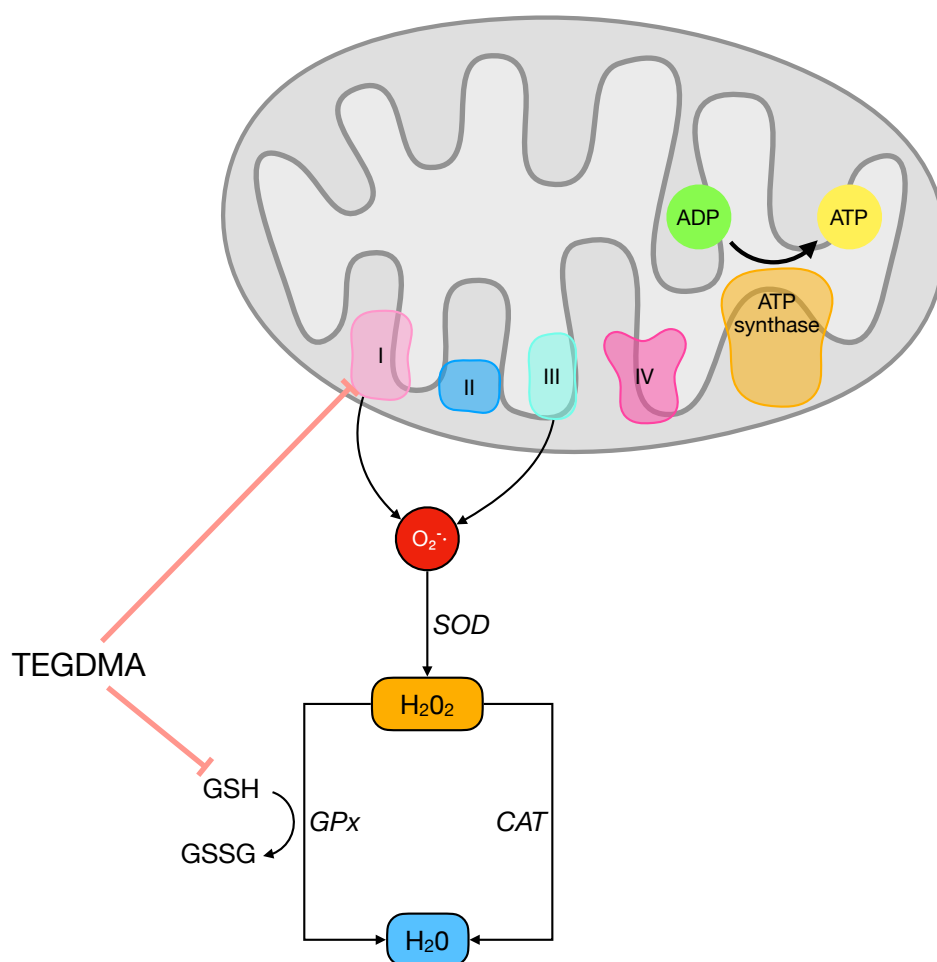


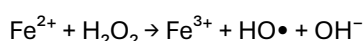
Figure 7.1 : The electron transport chain is composed of four complexes (I,II,III,IV) that collaborate to generate energy within the mitochondria. Superoxide ($O_2^{\cdot-}$) are generated by the mitochondria as a consequence of cellular respiration. In the presence of superoxide dismutase (SOD), $O_2^{\cdot-}$ undergoes catalysis to form hydrogen peroxide (H_2O_2). Glutathione (GSH) dependent glutathione peroxidase (GPx), or catalase (CAT) functions to counteract H_2O_2 by converting it into water, thereby assisting in the regulation of oxidative stress. When TEGDMA is introduced into cells, it can deplete GSH through adduct formation and/or directly inhibit complex I of the mitochondrial electron transport chain, thereby amplifying oxidative stress.

In summary, TEGDMA exerts a dual assault on cellular redox homeostasis: it depletes GSH through direct chemical interaction and impairs mitochondrial respiration by inhibiting Complex I. Together, these mechanisms drive excessive mitochondrial ROS production and H₂O₂ accumulation, culminating in increased oxidative stress.

2.3.2.a.ii. *Mitochondrial dysfunction*

Following the onset of oxidative stress, one of the first critical downstream events in TEGDMA-induced toxicity is the disruption of mitochondrial integrity, characterized by an early collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) in oral cell models. In human gingival fibroblasts, Lefeuvre et al. (58) reported that millimolar concentrations of TEGDMA caused a rapid dissipation of $\Delta\Psi_m$, reflecting an acute breakdown of the proton gradient across the inner mitochondrial membrane.

Mitochondria are not only the primary source of excessive ROS under stress but also their principal target (59). Once H₂O₂ accumulates due to impaired detoxification, it can react with ferrous iron (Fe²⁺) to generate hydroxyl radicals (HO•) via the Fenton reaction (60):



Although short-lived, HO• is extremely reactive and initiate lipid peroxidation within mitochondrial membranes (61). This process compromises membrane fluidity and integrity, amplifying mitochondrial dysfunction. Among the various lipid peroxidation products, malondialdehyde (MDA) is the most abundant and widely used as a biomarker of oxidative stress (62).

In line with this mechanism, Lefeuvre et al. (58) observed a significant increase in lipid peroxidation in TEGDMA-treated gingival fibroblasts, detectable within three hours. Similar findings were later confirmed in odontoblast-like cells, where Baldion et al. (63) reported MDA formation following TEGDMA exposure. Importantly, in both studies, lipid peroxidation was accompanied by mitochondrial dysfunction and increased

cytotoxicity, as indicated by loss of mitochondrial membrane potential and elevated LDH release.

This temporal overlap strongly suggests that lipid peroxidation acts as a key mediator linking ROS overproduction to mitochondrial dysfunction and eventual cell death under TEGDMA stress.

2.3.2.a.iii. *Cellular stress responses*

Following mitochondrial dysfunction, ROS may accumulate to levels that exceed the organelle's antioxidant buffering capacity. Under these conditions, ROS can diffuse into the cytosol, where they activate a range of redox-sensitive signaling pathways. Such responses aim to restore redox homeostasis and support cell survival; however, under sustained oxidative stress, they may also drive apoptotic or necrotic cell death.

Nrf2-Keap-1 activation and antioxidant enzyme modulation

Central to the antioxidant defense is the Nrf2–Keap1 system, the master regulator of cytoprotective gene expression under conditions of oxidative or electrophilic stress (64). Under basal conditions, Nrf2 is sequestered by Keap1 and continuously targeted for proteasomal degradation. In response to oxidative stress, Nrf2 becomes stabilized, translocate to the nucleus, and binds to antioxidant response elements (AREs) to induce a broad array of cytoprotective genes. These include enzymes such as GPx, CAT and SOD (Fig. 7.2) (He et al., 2020).

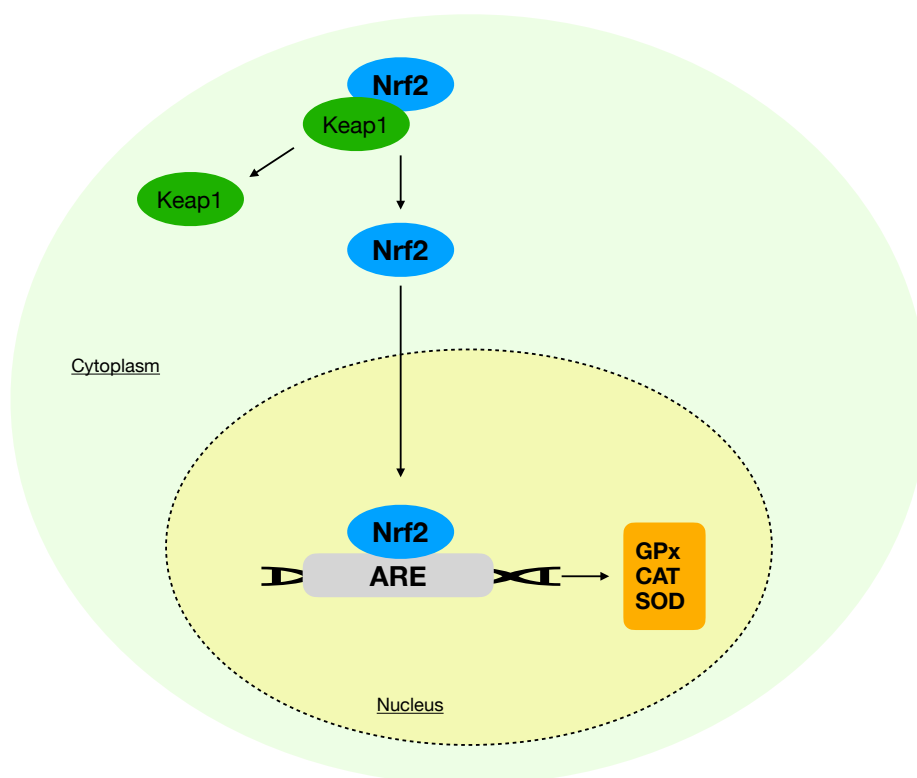


Figure 7.2 : Mechanisms of Nrf2 activation. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1 and targeted for ubiquitination and proteasomal degradation. During oxidative stress, the Nrf2–Keap1 interaction is disrupted, leading to Nrf2 stabilization, phosphorylation, and nuclear translocation. In the nucleus, Nrf2 binds to antioxidant response elements (AREs) in the promoters of target genes, initiating the transcription of cytoprotective enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT).

These enzymatic antioxidants form the first line of defense against oxidative stress. SOD catalyzes the dismutation of superoxide into H_2O_2 , which is subsequently detoxified to water and oxygen by CAT and GPx (65). In hDPCs exposed to TEGDMA, Jiao et al. (66) reported an imbalance in antioxidant enzyme activity: CAT activity was upregulated, whereas SOD and GPx activities were reduced. Because GPx requires

GSH as a substrate, its inhibition is likely a direct consequence of TEGDMA-induced GSH depletion (Fig 7.3).

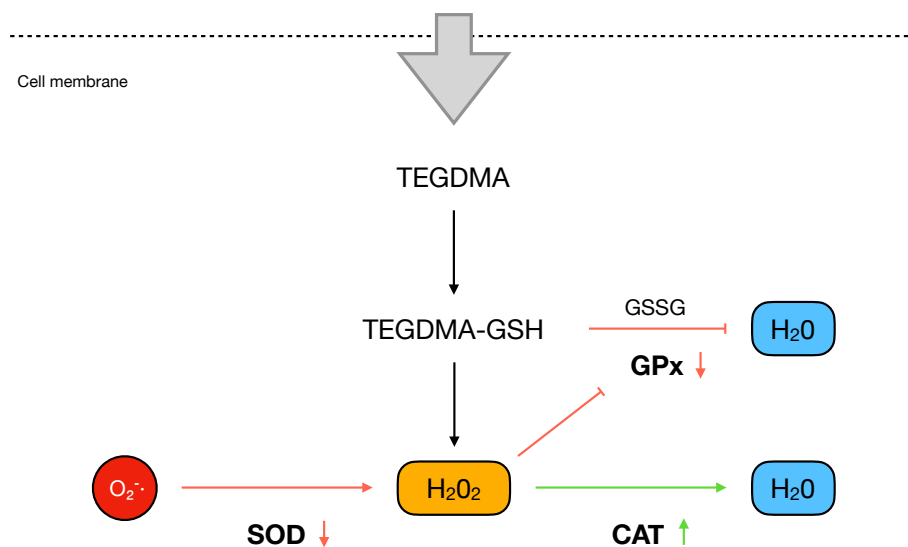


Figure 7.3 : Adaptive redox regulation in cells exposed to TEGDMA. The current results suggest that TEGDMA binds to glutathione (GSH) resulting in a decrease in GSH levels, which causes down-regulation of the expression of glutathione peroxidase (GPx). As a consequence, increased H₂O₂ formation leads to the induction of catalase and a feedback inhibition of superoxide dismutase (SOD) expression.

The compensatory increase in CAT activity, together with diminished SOD and GPx activities, suggests a transient disruption of the antioxidant defense system and implies that Nrf2-driven synthesis of new enzymes may be required to restore redox balance.

Evidence from resin monomer studies indicates that Nrf2 is activated in TEGDMA-exposed cells as a compensatory defense mechanism (Kaufman & Skrtic,

2020). Consistently, pharmacological inhibition of the Nrf2 pathway exacerbates mitochondrial oxidative damage in pulp cells treated with TEGDMA, underscoring the protective role of this signaling axis (Wang et al., 2021).

Endoplasmic reticulum stress

In addition to activating classical antioxidant defenses, oxidative stress can also disrupt protein homeostasis within the endoplasmic reticulum (ER). When unfolded or misfolded proteins accumulate in the ER lumen, cells initiate the unfolded protein response (UPR), a signaling network that initially acts to restore balance (67).

Oxidative stress contributes to ER dysfunction by destabilizing the redox environment required for correct protein folding. The formation and rearrangement of disulfide bonds, catalyzed by ER-resident chaperones, is highly sensitive to redox imbalance. Excess ROS can oxidize critical thiol groups, disrupt calcium signaling, and directly damage amino acid side chains, thereby impairing folding efficiency. As a result, misfolded or unfolded proteins accumulate and activate the UPR.

When ER stress becomes prolonged or severe, the adaptive UPR switches to a pro-apoptotic program (68). A central mediator of this transition is caspase-12, an ER-resident caspase that links unresolved ER dysfunction to the activation of downstream apoptotic cascades.

Although direct evidence for ER stress in TEGDMA-treated cells is still limited, studies with structurally related monomers support its involvement. For example, HEMA has been shown to activate UPR signaling and induce apoptosis under sustained stress conditions (69). Consistent with this, Lovász et al. (70) demonstrated caspase-12 activation in pulp cells exposed to TEGDMA, strongly implicating ER dysfunction in its cytotoxic effects. More broadly, the ability of excessive ROS to promote protein misfolding provides a mechanistic link between oxidative stress, ER stress, and

apoptosis, suggesting that ER dysfunction is an integral component of resin monomer-induced toxicity.

NOX4 Activation

Beyond mitochondria, NADPH oxidase 4 (NOX4) has emerged as an additional source of ROS during TEGDMA-induced toxicity. NOX4 is a member of the NADPH oxidase enzyme family, which catalyzes the transfer of electrons from NADPH to molecular oxygen, generating superoxide and hydrogen peroxide as primary products (71). Notably, NOX4 expression is upregulated during endoplasmic reticulum (ER) stress, underscoring the mechanistic cross-talk between these pathways (72)

Experimental evidence supports a role for NOX4 in resin monomer cytotoxicity. Yeh et al. (73) demonstrated that TEGDMA markedly increased NOX4 mRNA and protein levels in oral cell cultures. Pharmacological inhibition of NOX enzymes or selective NOX4 inhibition significantly reduced ROS levels. Furthermore, siRNA-mediated NOX4 knockdown almost completely abolished TEGDMA-induced apoptosis, enhancing cell survival and reducing caspase-9 activation.

Collectively, these findings position NOX4-derived ROS upstream of mitochondrial cytochrome c release and apoptosis, suggesting a self-reinforcing loop in which ER stress induces NOX4, thereby amplifying ROS production and further impairing mitochondrial function.

Mitogen-Activated Protein Kinase Signaling

The accumulation of ROS resulting from both mitochondrial dysfunction and NOX4 activation also engages the mitogen-activated protein kinase (MAPK) cascades. These kinases are classical redox-sensitive sensors that modulate the balance between cell survival and cell death (74). Resin monomers, including TEGDMA, have been shown to differentially activate MAPK pathways in oral cells (54). However, pharmacological

inhibition of these kinases does not abolish monomer-induced apoptosis (75). This indicates that MAPK activation is not the primary apoptotic trigger but rather reflects a broader cellular adaptation to oxidative stress, integrating both pro-survival and pro-apoptotic signaling. Consequently, MAPK activation is best viewed as a downstream consequence of the oxidative burden amplified by ER stress and NOX4 activity, reinforcing the concept that TEGDMA toxicity operates through interconnected redox-sensitive pathways.

Summary

Exposure to TEGDMA elicits a spectrum of adaptive cellular responses. Early events include activation of the Nrf2-Keap-1 pathway and modulation of antioxidant enzyme activities, reflecting attempts to restore redox homeostasis. Under sustained oxidative conditions, ER stress develops, leading to activation of the unfolded protein response. In parallel, NOX4 upregulation establishes a self-reinforcing loop that amplifies ROS production and further compromises mitochondrial function. Collectively, these interconnected pathways underscore oxidative stress as a central driver of TEGDMA-induced cytotoxicity.

2.3.2.a.iv. Genotoxicity

As discussed above, treatment with TEGDMA can overwhelm cellular antioxidant defenses, and the resulting accumulation of ROS, together with secondary lipid peroxidation products such as MDA, induces a broad spectrum of endogenous DNA lesions (Schweikl et al., 2006). These include oxidized bases, apurinic/apyrimidinic sites, strand breaks, and in some cases mutations that compromise genome stability if left unrepaired.

Among these lesions, a hallmark oxidative lesion is 8-oxoguanine (8-oxoG), the most common marker of DNA base oxidation. Guanine has the lowest redox potential of the four DNA bases, making it especially vulnerable to ROS attack (particularly hydroxyl radicals). Oxidation at the C8 position generates 8-oxoG, which is problematic because during replication it can mispair with adenine instead of cytosine. This mispairing leads to G:C → T:A transversion mutations, one of the most frequent mutation types linked to oxidative stress and a well-recognized source of genomic instability (76). In THP-1 cells, 24–48 h of TEGDMA exposure significantly increased 8-oxoG levels in a dose-dependent manner. Co-treatment with the antioxidant N-acetylcysteine (NAC) lowered 8-oxoG accumulation, confirming ROS involvement in this process (77). Although 8-oxoG is normally repaired by the base excision repair (BER) pathway, excessive damage can overwhelm repair capacity.

Oxidized bases such as 8-oxoG often lead to single-strand breaks (SSBs), which occur either directly through ROS attack or indirectly as BER intermediates. Under physiological conditions, these SSBs is an intentional repair intermediate and are promptly sealed (76). However, under sustained oxidative stress, repair capacity is overwhelmed, causing SSBs to accumulate, a phenomenon that can be detected by alkaline comet assays, a sensitive method for SSB detection (78). Several in vitro studies confirm that, using alkaline comet assays, TEGDMA induces strand breaks: Kleinsasser et al. (79) showed that TEGDMA significantly enhanced DNA migration in peripheral lymphocytes as compared to the negative controls. More recently, Ginzkey et al. (80) demonstrated significant DNA damage in lymphocytes exposed to sub-toxic concentrations of TEGDMA.

If not efficiently repaired, accumulated SSBs may collapse into double-strand breaks (DSBs), one of the most severe forms of DNA damage (81). Phosphorylation of the histone H2AX at sites of DNA damage produces γ H2AX foci, which are widely used as a sensitive and specific marker of DSBs (82). Consistent with this, Urcan et al. (37) first reported that within 6 h of exposure, TEGDMA induced γ H2AX foci in human gingival fibroblasts in a dose-dependent manner. Lottner et al. (83) confirmed these findings

and showed that co-treatment with NAC or ascorbate reduced DSB formation by approximately 50%. This antioxidant protection strongly indicates that TEGDMA-induced DNA breaks are mediated by ROS. Similarly, Schweikl et al. (2007) demonstrated that NAC suppressed DNA damage in V79 fibroblasts, reducing comet tails in neutral comet assay, which is a well-established method specifically used to detect DSBs (78).

Beyond base lesions and strand breaks, TEGDMA also produces chromosomal-level alterations. Two types of evidence are particularly noteworthy: (1) the formation of micronuclei, small extranuclear bodies reflecting chromosomal fragments or whole chromosomes excluded from mitosis, and (2) large-scale gene deletions. Classic studies in V79 fibroblasts demonstrated a dose-dependent increase in micronuclei upon TEGDMA exposure (84, 85). The formation of micronuclei reflects chromosome breakage or mis-segregation and therefore provides cytological evidence of clastogenic potential. In addition, Schweikl and Schmalz (86) reported that most TEGDMA-induced mutants in the HPRT gene carried multi-exon deletions, in contrast to spontaneous mutants, which were predominantly point mutations. This shift toward large-scale deletions indicates that TEGDMA does not simply cause base substitutions but promotes structural chromosomal alterations. Later review confirmed that TEGDMA induces “large deletions of DNA sequences,” a hallmark of clastogenic or crosslinking activity (87).

Taken together, these findings demonstrate that exposure to resin monomers such as TEGDMA elicits substantial oxidative stress, overwhelming cellular antioxidant defenses and DNA repair pathways. The resulting lesions range from oxidized bases like 8-oxoG to single- and double-strand breaks, ultimately progressing to chromosomal-level alterations when repair capacity is exceeded. Key genotoxicity endpoints, including comet assays, γ H2AX foci, and micronucleus formation, consistently reveal the damaging potential of TEGDMA. Importantly, the protective effects of antioxidants such as N-acetylcysteine confirm the central role of ROS in mediating this damage. Taken together, these findings highlight TEGDMA as not only cytotoxic but also

genotoxic, posing a significant risk to genome stability through oxidative DNA damage and its downstream consequences.

Potential Role of Covalent DNA Adducts

Most of the DNA damage caused by TEGDMA can be attributed to its ability to induce oxidative stress. Excessive ROS lead to oxidative base modifications such as 8-oxoguanine, the accumulation of single- and double-strand breaks, and ultimately chromosomal alterations.

Nevertheless, oxidative stress alone does not fully account for all observed lesions. In particular, the occurrence of large multi-exon deletions is inconsistent with the more localized damage typically caused by ROS, and instead aligns more closely with mechanisms involving covalent DNA modifications or crosslinking events such as those generated by alkylating agents (88, 89).

This has led to the hypothesis that TEGDMA, owing to its chemical reactivity, might directly interact with DNA. The monomer's bifunctional acrylate structure contains two electrophilic double bonds that can undergo Michael addition (32, 34). In cellular systems, TEGDMA readily forms thioether adducts with GSH and NAC (90). Because DNA bases contain nucleophilic sites, TEGDMA has the chemical potential to form covalent DNA adducts, a mechanism well described for other alkylating agents (91). Such interactions could block replication and account for the large deletions observed in exposed cells.

At present, direct DNA adducts by TEGDMA remain speculative. Nonetheless, researchers have noted the plausibility of this route. Several indirect observations support their plausibility. Volk et al. (92) showed that TEGDMA induced DNA strand breaks in oral keratinocytes without a corresponding increase in 8-oxoG, suggesting non-oxidative damage. Ansteinsson et al. (93) reported that Trolox (a water-soluble analogue of vitamin E), a pure ROS scavenger, failed to prevent HEMA-induced

cell-cycle arrest, pointing again to a non-oxidative mechanism. By contrast, NAC completely prevented TEGDMA-induced cell cycle delay (Schweikl et al., 2007). This striking difference is explained by NAC's dual mode of action: it not only scavenges ROS (94), but it also directly neutralizes TEGDMA through Michael addition, forming stable NAC–TEGDMA conjugates both inside and outside cells (90). This direct detoxification reduces the pool of free TEGDMA available to interact with DNA, potentially explaining why Trolox is comparatively less effective in mitigating genotoxicity.

Taken together, these results support a unifying model: TEGDMA initially depletes GSH, weakening cellular antioxidant defenses and promoting ROS-mediated lesions. Once GSH reserves are exhausted, unbound TEGDMA can directly attack DNA, producing bulky adducts or crosslinks that overwhelm repair systems. These lesions may cause replication fork collapse and explain the high frequency of large-scale deletions observed in TEGDMA-exposed cells.

Future studies are required to directly demonstrate DNA adduct formation by TEGDMA. Confirmation of such lesions would establish TEGDMA not only as an inducer of oxidative stress but also as a direct genotoxin. Clinically, the formation of covalent adducts in pulp cells that survive the initial exposure could have two possible outcomes: they may trigger cell death, or, if left unrepaired, persist as mutagenic lesions capable of initiating carcinogenic processes. Nevertheless, it is likely that the body's detoxification mechanisms, particularly conjugation with glutathione (GSH), neutralize a substantial fraction of the free monomer before it can reach nuclear DNA.

In conclusion, while ROS-mediated oxidative stress clearly represents the primary driver of TEGDMA-induced genotoxicity, the evidence also suggests a plausible complementary mechanism involving direct covalent DNA adducts. Together, these routes may converge to destabilize the genome, explaining both simple oxidative lesions and the more complex chromosomal deletions associated with TEGDMA exposure.

Activation of DNA Damage Response Pathways and cell cycle arrest.

Cells respond to genotoxic stress through the activation of the DNA damage response (DDR), a coordinated network of signaling pathways designed to detect DNA lesions and initiate either repair or, if necessary, apoptosis. A critical component of DDR is the activation of cell cycle checkpoints, which temporarily halt cell division to prevent the propagation of damage into daughter cells. This protective pause gives cells time to repair DNA lesions before they are converted into lethal breaks or mutations during replication and mitosis.

In agreement with its DNA-damaging properties, TEGDMA has been shown to activate key DDR pathways in several oral cell models. Experimental evidence demonstrates the involvement of the ATM/ATR kinases, central sensors of DNA double-strand breaks and replication stress (77). Downstream of ATM/ATR, histone H2AX phosphorylation is consistently observed in TEGDMA-exposed cells, serving as a sensitive marker of DNA double-strand breaks (37, 83). In addition, TEGDMA triggers the stabilization of the tumor suppressor p53, a master regulator of DDR, which in turn induces transcription of the cyclin-dependent kinase inhibitor p21 (95). Together, these events illustrate that cells not only detect TEGDMA-induced DNA lesions but also engage canonical DDR signaling to mitigate their consequences.

Functionally, the upregulation of p21 leads to cell cycle arrest, particularly at the G1/S and G2/M checkpoints, preventing damaged DNA from being replicated or segregated into daughter cells. Several studies have consistently reported that TEGDMA exposure delays or blocks cell cycle progression in human oral fibroblasts and lymphocytes (85, 95, 96). Such arrest reflects an adaptive attempt to safeguard genome integrity by providing time for repair processes to act.

In summary, DNA breaks and replication stress induced by TEGDMA activate the ATM/ATR kinases, which initiate a cascade stabilizing p53 and promoting p21 expression. The resulting cell cycle arrest prevents entry into mitosis with damaged DNA, thereby serving as a critical protective mechanism. This checkpoint response highlights the fact that TEGDMA-exposed cells are capable of recognizing genotoxic

stress and mounting a defense, although the outcome ultimately depends on the balance between repair efficiency and the extent of the damage.

2.3.2.a.v. Outcomes: apoptosis versus necrosis

If protective mechanisms against DNA damage are successful, they promote cell recovery. If not, the cell progresses to death. In both hDPCs and hGFs, TEGDMA exposure typically results in cell death above a certain threshold.

Few studies have attempted to distinguish whether apoptosis or necrosis is the primary mechanism of cell death and only a handful of those investigated pulp cells. Among them, Janke *et al.* (97) first showed that TEGDMA induces pronounced apoptosis in primary hGFs. Similar findings were later reported in pulp cells, where Yeh *et al.* (73) confirmed apoptosis as the predominant mode of cell death. However, several studies revealed a concentration-dependent shift: at low doses, apoptosis dominates, while higher concentrations or prolonged exposure drive necrosis (98-100). For example, Krifka *et al.* combined viability dye exclusion, nuclear staining, and caspase assays in hDPCs to conclude that TEGDMA causes dose-dependent apoptosis with negligible necrosis until 5 mM, but above 5 mM necrosis sharply rises.

Apoptosis is a regulated, energy-dependent process characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and membrane blebbing while maintaining membrane integrity until late stages (101). At the molecular level, TEGDMA-induced apoptosis proceeds via the intrinsic (mitochondrial) pathway, involving cytochrome c release and caspase-9 activation, which converge on the executioner caspase-3. This was demonstrated by Batarseh *et al.* (102), who reported increased levels of cleaved caspase-9 and caspase-3 in pulp fibroblasts, together with upregulation of the pro-apoptotic proteins Bid and Bim (Bcl-2 family proteins). Similarly, Chang *et al.* (98) confirmed activation of caspase-9 and -3 in cementoblasts

after 24 h exposure, reinforcing the central role of the mitochondrial pathway in TEGDMA cytotoxicity.

In addition, ER stress appears to contribute. Lovász et al. (70) detected increased levels of cleaved caspase-12, together with caspase-9 and -3, in pulp cells exposed to TEGDMA. Caspase-12 is specifically linked to ER stress-mediated apoptosis (103), suggesting that oxidative and proteostatic imbalance induced by TEGDMA extends apoptotic signaling beyond the mitochondrial pathway.

Taken together, the sequential activation of caspase-12 (ER stress mediator), caspase-9 (intrinsic initiator), and caspase-3 (executioner) constitutes a hallmark signature of TEGDMA-induced apoptosis in oral cell models.

In contrast, necrosis arises from overwhelming cellular damage and differs fundamentally from apoptosis. Necrotic cells typically exhibit swelling, organelle rupture, and loss of plasma membrane integrity, which results in the uncontrolled release of intracellular contents. This unregulated process is often triggered by a catastrophic bioenergetic collapse (104). Paradoxically, cells need ATP to maintain their vital functions and to die by apoptosis. When ATP synthesis is severely impaired, apoptotic pathways fail, and cells default to necrotic death. Supporting this, Mikulás et al. (105) reported that high concentrations of TEGDMA disrupt mitochondrial ATP production, precipitating necrosis most likely through the inability to maintain essential ion gradients.

Biological Effects of Substances Released by Resin-Based Composites

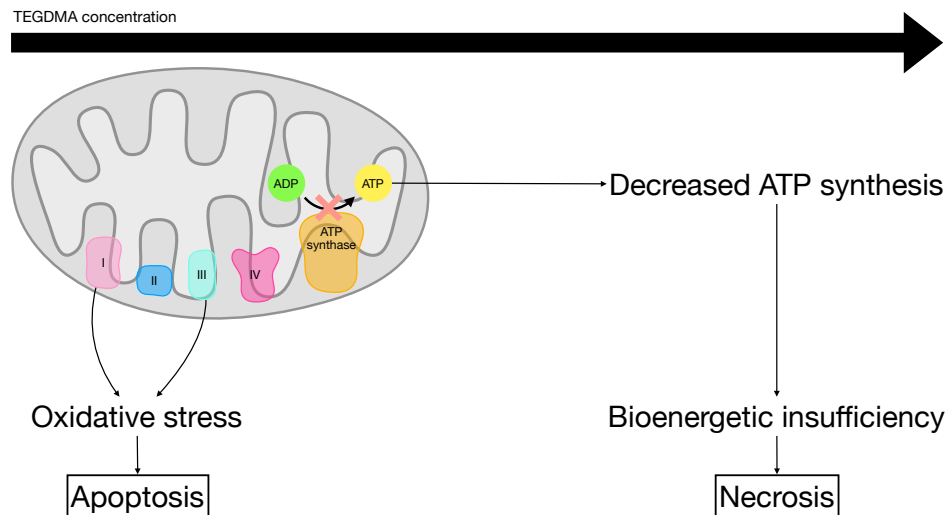


Figure 7.4 : Apoptosis and necrosis induced by TEGDMA. At low concentrations, TEGDMA predominantly induces apoptosis through oxidative stress. However, as TEGDMA concentrations increase, excessive mitochondrial damage leads to severe ATP depletion. Because apoptosis is an energy-dependent process, the loss of ATP synthesis prevents the apoptotic program from being executed. Under these conditions, cells shift toward necrosis, resulting from catastrophic bioenergetic collapse.

In summary, TEGDMA induces cell death in a dose-dependent manner. At low to moderate concentrations, apoptosis predominates, mediated through caspase-dependent pathways involving mitochondria, death receptors, and ER stress. At higher doses, where bioenergetic collapse occurs, necrosis becomes the dominant form of death. This dual outcome underscores that TEGDMA cytotoxicity spans from regulated apoptosis to unregulated necrosis, depending on cellular energy status and the severity of exposure.

2.3.2.b. Molecular mechanisms of BisGMA toxicity

Building on the extensive body of research devoted to TEGDMA, attention has also turned to other major resin monomers, most notably BisGMA. Although less extensively investigated than TEGDMA, numerous *in vitro* studies have examined the toxicity of BisGMA in a variety of human oral cell types, including pulp fibroblasts, gingival fibroblasts, odontoblast-like cells, and keratinocytes. Across these experimental models, BisGMA is consistently reported to be more cytotoxic than TEGDMA (Geurtsen, 2000).

While the two monomers converge on similar downstream pathways, oxidative stress, mitochondrial dysfunction, DNA damage, and cell death, BisGMA frequently produces more pronounced effects. This heightened toxicity is thought to reflect key physicochemical differences: BisGMA's larger molecular size and higher lipophilicity may promote stronger interactions with lipid membranes, leading to greater disruption of membrane integrity and intracellular homeostasis. Its relatively low water solubility could also prolong its retention within cellular compartments, increasing the duration of exposure to sensitive organelles such as mitochondria and the endoplasmic reticulum. Furthermore, structural differences in the methacrylate backbone and bisphenol core may influence its reactivity toward nucleophilic cellular targets, including proteins and nucleic acids, thereby amplifying genotoxic and pro-apoptotic signaling.

Taken together, these observations suggest that although BisGMA and TEGDMA share common toxicological pathways, the magnitude and persistence of BisGMA's effects may be driven by its distinct chemical properties. The following sections will therefore focus specifically on the mechanistic differences that account for BisGMA's higher cytotoxicity compared with TEGDMA, highlighting the unique features that make it a more potent inducer of oxidative stress, organelle dysfunction, and cell death.

2.3.2.b.i. *Lipophilicity and reactivity*

One of the key distinctions between BisGMA and TEGDMA lies in their physicochemical profiles. BisGMA is a bulky, highly lipophilic molecule with low water solubility, properties that favor its accumulation in lipid membranes and prolong its retention within cells. In contrast, TEGDMA is smaller and more hydrophilic, enabling faster diffusion through aqueous environments but also more rapid clearance from cells.

These differences have important toxicological implications. The strong membrane association of BisGMA likely enhances its ability to disrupt lipid bilayers and interfere with organelle function, contributing to more severe and persistent cytotoxic effects. In addition, BisGMA's bisphenol core and reactive methacrylate groups increase its electrophilic potential, raising the likelihood of interactions with nucleophilic cellular targets such as proteins and nucleic acids. Clinically, BisGMA's poor solubility may also slow its elution from resin matrices, leading to more sustained tissue exposure.

2.3.2.b.ii. *Mechanisms of GSH depletion*

Both TEGDMA and BisGMA deplete intracellular GSH and elevate oxidative stress, but they appear to do so through distinct mechanisms. TEGDMA readily undergoes Michael addition with GSH, forming covalent adducts that directly and rapidly consume the cellular antioxidant pool. By contrast, BisGMA does not seem to form such adducts (Ansteinsson et al., 2013), which is somewhat unexpected given its higher electrophilic potential compared to TEGDMA.

This discrepancy suggests that BisGMA-induced GSH depletion is secondary to excessive ROS production rather than direct conjugation. One possible explanation is that BisGMA interacts with mitochondrial components, particularly the electron transport chain, thereby amplifying ROS generation. The resulting oxidative stress would oxidize GSH to its disulfide form (GSSG) and exhaust the antioxidant pool. However, direct experimental confirmation of this mechanism, such as the detection

of increased GSSG, remains lacking, leaving the precise basis of BisGMA-induced GSH depletion unresolved.

2.3.2.b.iii. *Cystine uptake inhibition*

A distinctive mechanism that further differentiates BisGMA from TEGDMA is its ability to block cystine uptake. Cystine import is the rate-limiting step in GSH synthesis, as extracellular cystine must first be transported into the cell and reduced to cysteine before contributing to GSH biosynthesis. Schneider et al. (106) demonstrated that BisGMA, but not TEGDMA, inhibits cystine uptake in human dental pulp cells.

By blocking this pathway, BisGMA not only reduces GSH levels but also impairs their replenishment, thereby prolonging oxidative stress and exacerbating cellular injury. This mechanism, unique to BisGMA, provides a compelling explanation for its more sustained oxidative imbalance and greater cytotoxicity compared with TEGDMA.

2.3.2.b.iv. *Mitochondrial effects*

Mitochondria are a major target of both TEGDMA and BisGMA, but the extent and nature of their effects differ. For TEGDMA, inhibition of ETC complex I has been documented, linking monomer exposure to impaired respiration and ROS accumulation. In contrast, direct evidence for BisGMA's interaction with specific ETC complexes is lacking.

Available studies nevertheless indicate that BisGMA disrupts mitochondrial integrity more severely. In human pulp cells, BisGMA exposure leads to loss of mitochondrial membrane potential and release of pro-apoptotic factors, consistent with activation of the intrinsic apoptotic pathway (107). Its high lipophilicity likely promotes stronger incorporation into mitochondrial membranes, destabilizing their structure and exacerbating dysfunction. While TEGDMA primarily compromises mitochondrial

function through targeted ETC inhibition, BisGMA may act through broader membrane-disruptive mechanisms that produce more extensive damage.

This distinction underscores why BisGMA is often observed to exert greater cytotoxicity: its effects on mitochondria may be more diffuse, persistent, and less amenable to cellular recovery than those caused by TEGDMA.

2.3.2.b.v. *Adaptive responses to oxidative stress*

In contrast to TEGDMA, far less is known about the activation of adaptive antioxidant defenses in response to BisGMA. To date, the only reported study (108) demonstrated overexpression of heme oxygenase-1 (HO-1), a classical Nrf2 target gene. HO-1 catalyzes the degradation of heme into biliverdin and bilirubin, both of which possess antioxidant properties (109). This limited evidence indicates that BisGMA can activate Nrf2-dependent cytoprotective signaling, although the overall adaptive response remains poorly characterized.

2.3.2.b.vi. *DNA damage and cell cycle arrest*

Both TEGDMA and BisGMA induce DNA lesions, but BisGMA consistently shows greater genotoxic potency. In a comprehensive analysis, Drozd et al. (110) demonstrated that BisGMA induces a wide spectrum of DNA damage in human lymphocytes, arising either from ROS-mediated oxidation or from potential direct interactions with DNA. These lesions were accompanied by activation of the DNA damage response and delays in cell cycle progression.

Complementing these findings, Urcan et al. (37) reported that BisGMA at TC_{50} concentrations caused significantly higher levels of DNA DSBs, as indicated by increased γ H2AX foci, than TEGDMA in gingival fibroblasts. This aligns with its consistently higher genotoxic impact. While BisGMA's electrophilic structure suggests

the possibility of direct covalent binding to DNA, experimental evidence for BisGMA–DNA adduct formation is still lacking.

2.3.2.b.vii. *Cell death*

Similar to TEGDMA, BisGMA activates apoptotic pathways at moderate concentrations and shifts toward necrosis at higher concentrations. The key difference lies in the intensity and timing of these responses: apoptosis triggered by BisGMA is accompanied by stronger oxidative stress and more rapid mitochondrial collapse than that observed with TEGDMA (111, 112). These features likely accelerate the transition from regulated apoptosis to uncontrolled necrosis, reinforcing BisGMA's greater cytotoxic potential.

Although BisGMA and TEGDMA share common toxicological pathways, several mechanistic differences explain BisGMA's greater cytotoxicity. Its bulky, lipophilic structure promotes stronger membrane association and prolonged intracellular retention, amplifying organelle disruption. Unlike TEGDMA, which directly conjugates with GSH, BisGMA appears to deplete antioxidants primarily through ROS generation and uniquely blocks cystine uptake, preventing GSH replenishment and sustaining oxidative stress. Mitochondrial damage is also more severe with BisGMA, likely due to broader membrane destabilization, while adaptive antioxidant responses remain poorly characterized. At the genomic level, BisGMA induces more extensive DNA lesions and double-strand breaks, leading to stronger checkpoint activation and cell cycle arrest. Finally, cell death occurs more rapidly and with greater oxidative stress. Together, these differences highlight BisGMA as a more potent inducer of oxidative imbalance, genotoxicity, and cell death compared with TEGDMA.

3. Summary

The comparative analysis of TEGDMA and BisGMA underscores that, while both monomers ultimately converge on similar toxic endpoints, oxidative stress, mitochondrial dysfunction, DNA damage, and cell death, their mechanistic routes and physicochemical profiles confer distinct biological behaviours. TEGDMA, a smaller and more hydrophilic molecule, diffuses rapidly through aqueous environments and is cleared more quickly from cells. Its cytotoxicity is driven primarily by the rapid depletion of intracellular GSH through direct adduct formation, exhausting antioxidant reserves and triggering ROS accumulation.

BisGMA, in contrast, is a bulky, highly lipophilic molecule with low water solubility, favouring its accumulation in cellular membranes and prolonging its interaction with intracellular targets. It does not form stable conjugates with GSH; instead, it elevates ROS production and uniquely inhibits cystine uptake, thereby limiting cysteine availability and preventing GSH resynthesis. This dual mechanism prolongs oxidative imbalance and contributes to its greater cytotoxic potency. Its aromatic backbone and electrophilic double bonds further enhance reactivity toward nucleophilic cellular components, which may explain the higher levels of DNA double-strand breaks observed in exposed gingival fibroblasts.

At the mitochondrial level, both monomers destabilise membrane potential and impair respiration, initiating apoptosis via the intrinsic pathway. However, BisGMA is often associated with an earlier and more pronounced mitochondrial collapse, consistent with its higher lipophilicity and persistence within membranes. Both compounds can also activate caspase-dependent apoptosis and necrosis in a dose-dependent manner, with BisGMA generally producing more persistent and severe effects.

Importantly, their partially distinct mechanisms raise the possibility of synergistic toxicity when both are present, as is the case in many resin formulations. Experimental data confirm that BisGMA/TEGDMA mixtures can be significantly more cytotoxic than either monomer alone, with reduced TC_{50} values in oral cell models (40, 113). Such

synergy likely reflects complementary modes of action, TEGDMA's rapid depletion of antioxidant defences lowering the threshold for BisGMA's sustained oxidative and genotoxic insults, resulting in amplified mitochondrial dysfunction, DNA damage, and inflammatory responses.

From a clinical standpoint, this is highly relevant: patients are exposed not to isolated monomers but to complex mixtures eluted from polymerised composites. In deep cavities or subgingival restorations, residual monomer diffusion through dentin or direct contact with soft tissues may pose a greater risk, particularly in susceptible individuals or in cases of suboptimal polymerisation.

Thus, a deeper understanding of the individual and combined effects of TEGDMA and BisGMA is essential for guiding safer resin chemistry, refining restorative protocols, and improving the long-term biocompatibility of dental materials. Looking forward, these insights should inform both material development and clinical practice. On the research side, priorities include elucidating the molecular basis of monomer interactions, identifying biomarkers of early tissue injury, and designing alternative monomers with reduced cytotoxic potential. On the clinical side, optimising curing protocols, employing barrier techniques, and selecting formulations with lower leachable content represent key strategies to mitigate patient exposure.

4. References

1. Ferracane JL. Resin composite--state of the art. *Dent Mater.* 2011;27(1):29-38.
2. Putzeys E, Duca RC, Coppens L, Vanoirbeek J, Godderis L, Van Meerbeek B, et al. In-vitro transdermal diffusion of monomers from adhesives. *J Dent.* 2018;75:91-7.
3. Cameli N, Mariano M, Pigliacelli F, Silvestri M, Cristaudo A. Occupational Methacrylate Allergy in Dental Personnel. *Allergies.* 2021;1(2):123-7.
4. Kucharska K, Lehmann A, Ortarzewska M, Jankowski J, Nijakowski K. Composite Dust Toxicity Related to Restoration Polishing: A Systematic Review. *Journal of Composites Science.* 2025;9(2).
5. Gupta SK, Saxena P, Pant VA, Pant AB. Release and toxicity of dental resin composite. *Toxicol Int.* 2012;19(3):225-34.
6. Reichl FX, Durner J, Kehe K, Manhart J, Folwaczny M, Kleinsasser N, et al. Toxicokinetic of HEMA in guinea pigs. *J Dent.* 2002;30(7-8):353-8.
7. Bakopoulou A, Papadopoulos T, Garefis P. Molecular toxicology of substances released from resin-based dental restorative materials. *Int J Mol Sci.* 2009;10(9):3861-99.
8. Ponce-Bravo S, Ledesma-Montes C, Martinez-Rivera JL, Garces-Ortiz M. Toxicity test of a dental commercial composite. *J Clin Exp Dent.* 2015;7(2):e289-92.
9. Steiro A, Strøm V, Hafstad E, Reinart LM. Harmful health effects of dental composite resins. A systematic review. 2016.
10. Maserejian NN, Shrader P, Brown OA, Trachtenberg FL, Soncini J, Hauser R, et al. Dental sealants and composite restorations and longitudinal changes in immune function markers in children. *Int J Paediatr Dent.* 2014;24(3):215-25.
11. Yildiz M, Alp HH, Gul P, Bakan N, Ozcan M. Lipid peroxidation and DNA oxidation caused by dental filling materials. *J Dent Sci.* 2017;12(3):233-40.
12. Michelsen VB, Kopperud HB, Lygre GB, Bjorkman L, Jensen E, Kleven IS, et al. Detection and quantification of monomers in unstimulated whole saliva after treatment with resin-based composite fillings in vivo. *Eur J Oral Sci.* 2012;120(1):89-95.
13. Cordeiro M, Souza JCM, Gomes A, Correia P, Fidalgo-Pereira R. Toxicity of resin-matrix composites in a dental clinical setting. *Odontology.* 2025.
14. Gul P, Celik N, Ozgeris FB, Demirkaya-Miloglu F, Kiziltunc A, Seven N. Effects of Bisphenol A Released From Composite Fillings on Reproductive Hormone Levels in Men. *Int Dent J.* 2021;71(4):343-51.
15. De Nys S, Duca RC, Vervliet P, Covaci A, Boonen I, Elskens M, et al. Bisphenol A release from short-term degraded resin-based dental materials. *J Dent.* 2022;116:103894.
16. Rochester JR. Bisphenol A and human health: a review of the literature. *Reprod Toxicol.* 2013;42:132-55.
17. Kingman A, Hyman J, Masten SA, Jayaram B, Smith C, Eichmiller F, et al. Bisphenol A and other compounds in human saliva and urine associated with the placement of composite restorations. *J Am Dent Assoc.* 2012;143(12):1292-302.

18. Arenholt-Bindslev D, Breinholt V, Preiss A, Schmalz G. Time-related bisphenol-A content and estrogenic activity in saliva samples collected in relation to placement of fissure sealants. *Clin Oral Investig.* 1999;3(3):120-5.
19. Joskow R, Barr DB, Barr JR, Calafat AM, Needham LL, Rubin C. Exposure to bisphenol A from bis-glycidyl dimethacrylate-based dental sealants. *J Am Dent Assoc.* 2006;137(3):353-62.
20. Zimmerman-Downs JM, Shuman D, Stull SC, Ratzlaff RE. Bisphenol A blood and saliva levels prior to and after dental sealant placement in adults. *J Dent Hyg.* 2010;84(3):145-50.
21. Lee JH, Yi SK, Kim SY, Kim JS, Son SA, Jeong SH, et al. Salivary bisphenol A levels and their association with composite resin restoration. *Chemosphere.* 2017;172:46-51.
22. Efsa Panel on Food Contact Materials E, Processing A, Lambre C, Barat Baviera JM, Bolognesi C, Chesson A, et al. Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. *EFSA J.* 2023;21(4):e06857.
23. Syed M, Chopra R, Sachdev V. Allergic Reactions to Dental Materials-A Systematic Review. *J Clin Diagn Res.* 2015;9(10):ZE04-9.
24. Goon AT, Isaksson M, Zimerson E, Goh CL, Bruze M. Contact allergy to (meth)acrylates in the dental series in southern Sweden: simultaneous positive patch test reaction patterns and possible screening allergens. *Contact Dermatitis.* 2006;55(4):219-26.
25. Ramos L, Cabral R, Goncalo M. Allergic contact dermatitis caused by acrylates and methacrylates--a 7-year study. *Contact Dermatitis.* 2014;71(2):102-7.
26. Lugović-Mihic L, Filija E, Varga V, Premuž L, Parać E, Tomašević R, et al. Unwanted Skin Reactions to Acrylates: An Update. *Cosmetics.* 2024;11(4).
27. Rai R, Dinakar D, Kurian SS, Bindoo YA. Investigation of contact allergy to dental materials by patch testing. *Indian Dermatol Online J.* 2014;5(3):282-6.
28. Jaakkola MS, Leino T, Tammilehto L, Ylostalo P, Kuosma E, Alanko K. Respiratory effects of exposure to methacrylates among dental assistants. *Allergy.* 2007;62(6):648-54.
29. Hallstrom U. Adverse reaction to a fissure sealant: report of case. *ASDC J Dent Child.* 1993;60(2):143-6.
30. Wiertelak-Makala K, Szymczak-Pajor I, Bociong K, Sliwinska A. Considerations about Cytotoxicity of Resin-Based Composite Dental Materials: A Systematic Review. *Int J Mol Sci.* 2023;25(1).
31. Moharamzadeh K, Brook I, Van Noort R. Biocompatibility of Resin-based Dental Materials. *Materials.* 2009;2(2):514-48.
32. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. *J Dent Res.* 2006;85(10):870-7.
33. Aqili TM, Hakeem MM, Almahmadi DS, Mirah M. Pulpitis tendency in teeth with vital pulp restored with composite resins: a cross-sectional retrospective study. *International Journal of Community Medicine and Public Health.* 2024.
34. Geurtsen W. Biocompatibility of resin-modified filling materials. *Crit Rev Oral Biol Med.* 2000;11(3):333-55.

35. Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. *Biomaterials*. 2013;34(19):4555-63.
36. Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J Biomed Mater Res*. 1998;41(3):474-80.
37. Urcan E, Scherthan H, Styliou M, Haertel U, Hickel R, Reichl FX. Induction of DNA double-strand breaks in primary gingival fibroblasts by exposure to dental resin composites. *Biomaterials*. 2010;31(8):2010-4.
38. Reichl FX, Esters M, Simon S, Seiss M, Kehe K, Kleinsasser N, et al. Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. *Arch Toxicol*. 2006;80(6):370-7.
39. Reichl FX, Seiss M, Marquardt W, Kleinsasser N, Schweikl H, Kehe K, et al. Toxicity potentiation by H₂O₂ with components of dental restorative materials on human oral cells. *Arch Toxicol*. 2008;82(1):21-8.
40. Durner J, Wellner P, Hickel R, Reichl FX. Synergistic interaction caused to human gingival fibroblasts from dental monomers. *Dent Mater*. 2012;28(8):818-23.
41. Reichl FX, Simon S, Esters M, Seiss M, Kehe K, Kleinsasser N, et al. Cytotoxicity of dental composite (co)monomers and the amalgam component Hg(2+) in human gingival fibroblasts. *Arch Toxicol*. 2006;80(8):465-72.
42. Kwon JH, Park HC, Zhu T, Yang HC. Inhibition of odontogenic differentiation of human dental pulp cells by dental resin monomers. *Biomater Res*. 2015;19:8.
43. Yoshii E. Cytotoxic effects of acrylates and methacrylates: Relationships of monomer structures and cytotoxicity. *Journal of Biomedical Materials Research*. 1997;37(4):517-24.
44. Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. *Dent Mater*. 2004;20(1):12-20.
45. Spahl W, Budzikiewicz H, Geurtsen W. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. *J Dent*. 1998;26(2):137-45.
46. Ferracane JL. Hygroscopic and hydrolytic effects in dental polymer networks. *Dent Mater*. 2006;22(3):211-22.
47. Peutzfeldt A. Resin composites in dentistry: the monomer systems. *Eur J Oral Sci*. 1997;105(2):97-116.
48. Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A. TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. *J Biomed Mater Res A*. 2003;66(3):476-82.
49. Engelmann J, Volk J, Leyhausen G, Geurtsen W. ROS formation and glutathione levels in human oral fibroblasts exposed to TEGDMA and camphorquinone. *J Biomed Mater Res B Appl Biomater*. 2005;75(2):272-6.
50. Ansteinsson V, Kopperud HB, Morisbak E, Samuelson JT. Cell toxicity of methacrylate monomers-the role of glutathione adduct formation. *J Biomed Mater Res A*. 2013;101(12):3504-10.

51. Fernandez-Checa JC, Garcia-Ruiz C, Colell A, Morales A, Mari M, Miranda M, et al. Oxidative stress: role of mitochondria and protection by glutathione. *Biofactors*. 1998;8(1-2):7-11.
52. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003;552(Pt 2):335-44.
53. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417(1):1-13.
54. Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R. Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. *Dent Mater*. 2007;23(1):34-9.
55. Schweikl H, Hiller KA, Eckhardt A, Bolay C, Spagnuolo G, Stempfl T, et al. Differential gene expression involved in oxidative stress response caused by triethylene glycol dimethacrylate. *Biomaterials*. 2008;29(10):1377-87.
56. Martins CA, Leyhausen G, Geurtsen W, Volk J. Intracellular glutathione: a main factor in TEGDMA-induced cytotoxicity? *Dent Mater*. 2012;28(4):442-8.
57. Mikulas K, Hermann P, Gera I, Komlodi T, Horvath G, Ambrus A, et al. Triethylene glycol dimethacrylate impairs bioenergetic functions and induces oxidative stress in mitochondria via inhibiting respiratory Complex I. *Dent Mater*. 2018;34(7):e166-e81.
58. Lefeuvre M, Amjaad W, Goldberg M, Stanislawski L. TEGDMA induces mitochondrial damage and oxidative stress in human gingival fibroblasts. *Biomaterials*. 2005;26(25):5130-7.
59. Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Arch Toxicol*. 2013;87(7):1157-80.
60. Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett*. 1995;82-83:969-74.
61. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev*. 2014;2014:360438.
62. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis*. 2005;15(4):316-28.
63. Baldion PA, Velandia-Romero ML, Castellanos JE. Dental resin monomers induce early and potent oxidative damage on human odontoblast-like cells. *Chem Biol Interact*. 2021;333:109336.
64. Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, et al. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol*. 2006;26(1):221-9.
65. Miller AF. Superoxide dismutases: ancient enzymes and new insights. *FEBS Lett*. 2012;586(5):585-95.
66. Jiao Y, Ma S, Wang Y, Li J, Shan L, Liu Q, et al. N-Acetyl Cysteine Depletes Reactive Oxygen Species and Prevents Dental Monomer-Induced Intrinsic Mitochondrial Apoptosis In Vitro in Human Dental Pulp Cells. *PLoS One*. 2016;11(1):e0147858.

67. Chen X, Shi C, He M, Xiong S, Xia X. Endoplasmic reticulum stress: molecular mechanism and therapeutic targets. *Signal Transduct Target Ther.* 2023;8(1):352.
68. Urrea H, Dufey E, Lisbona F, Rojas-Rivera D, Hetz C. When ER stress reaches a dead end. *Biochim Biophys Acta.* 2013;1833(12):3507-17.
69. Diamanti E, Mathieu S, Jeanneau C, Kitraki E, Panopoulos P, Spyrou G, et al. Endoplasmic reticulum stress and mineralization inhibition mechanism by the resinous monomer HEMA. *Int Endod J.* 2013;46(2):160-8.
70. Lovasz BV, Berta G, Lempel E, Setalo G, Jr., Vecsernyes M, Szalma J. TEGDMA (Triethylene Glycol Dimethacrylate) Induces Both Caspase-Dependent and Caspase-Independent Apoptotic Pathways in Pulp Cells. *Polymers (Basel).* 2021;13(5).
71. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev.* 2007;87(1):245-313.
72. Bhattarai KR, Riaz TA, Kim HR, Chae HJ. The aftermath of the interplay between the endoplasmic reticulum stress response and redox signaling. *Exp Mol Med.* 2021;53(2):151-67.
73. Yeh CC, Chang JZ, Yang WH, Chang HH, Lai EH, Kuo MY. NADPH oxidase 4 is involved in the triethylene glycol dimethacrylate-induced reactive oxygen species and apoptosis in human embryonic palatal mesenchymal and dental pulp cells. *Clin Oral Investig.* 2015;19(6):1463-71.
74. Averill-Bates D. Reactive oxygen species and cell signaling. Review. *Biochim Biophys Acta Mol Cell Res.* 2024;1871(2):119573.
75. Krifka S, Hiller KA, Bolay C, Petzel C, Spagnuolo G, Reichl FX, et al. Function of MAPK and downstream transcription factors in monomer-induced apoptosis. *Biomaterials.* 2012;33(3):740-50.
76. David SS, O'Shea VL, Kundu S. Base-excision repair of oxidative DNA damage. *Nature.* 2007;447(7147):941-50.
77. Eckhardt A, Gerstmayr N, Hiller KA, Bolay C, Waha C, Spagnuolo G, et al. TEGDMA-induced oxidative DNA damage and activation of ATM and MAP kinases. *Biomaterials.* 2009;30(11):2006-14.
78. Rojas E, Lopez MC, Valverde M. Single cell gel electrophoresis assay: methodology and applications. *J Chromatogr B Biomed Sci Appl.* 1999;722(1-2):225-54.
79. Kleinsasser NH, Schmid K, Sassen AW, Harreus UA, Staudenmaier R, Folwaczny M, et al. Cytotoxic and genotoxic effects of resin monomers in human salivary gland tissue and lymphocytes as assessed by the single cell microgel electrophoresis (Comet) assay. *Biomaterials.* 2006;27(9):1762-70.
80. Ginzkey C, Zinnitsch S, Steussloff G, Koehler C, Hackenberg S, Hagen R, et al. Assessment of HEMA and TEGDMA induced DNA damage by multiple genotoxicological endpoints in human lymphocytes. *Dent Mater.* 2015;31(8):865-76.
81. Bohgaki T, Bohgaki M, Hakem R. DNA double-strand break signaling and human disorders. *Genome Integr.* 2010;1(1):15.
82. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998;273(10):5858-68.

83. Lottner S, Shehata M, Hickel R, Reichl FX, Durner J. Effects of antioxidants on DNA-double strand breaks in human gingival fibroblasts exposed to methacrylate based monomers. *Dent Mater.* 2013;29(9):991-8.
84. Schweikl H, Schmalz G, Spruss T. The induction of micronuclei in vitro by unpolymerized resin monomers. *J Dent Res.* 2001;80(7):1615-20.
85. Schweikl H, Hartmann A, Hiller KA, Spagnuolo G, Bolay C, Brockhoff G, et al. Inhibition of TEGDMA and HEMA-induced genotoxicity and cell cycle arrest by N-acetylcysteine. *Dent Mater.* 2007;23(6):688-95.
86. Schweikl H, Schmalz G. Triethylene glycol dimethacrylate induces large deletions in the hprt gene of V79 cells. *Mutat Res.* 1999;438(1):71-8.
87. Geurtsen W, Leyhausen G. Chemical-Biological Interactions of the resin monomer triethyleneglycol-dimethacrylate (TEGDMA). *J Dent Res.* 2001;80(12):2046-50.
88. Cho E, Allemang A, Audebert M, Chauhan V, Dertinger S, Hendriks G, et al. AOP report: Development of an adverse outcome pathway for oxidative DNA damage leading to mutations and chromosomal aberrations. *Environ Mol Mutagen.* 2022;63(3):118-34.
89. Beranek DT. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat Res.* 1990;231(1):11-30.
90. Spagnuolo G, Desiderio C, Riviello V, Amato M, Rossetti DV, D'Anto V, et al. In vitro cellular detoxification of triethylene glycol dimethacrylate by adduct formation with N-acetylcysteine. *Dent Mater.* 2013;29(8):e153-60.
91. Lawley PD, Phillips DH. DNA adducts from chemotherapeutic agents. *Mutat Res.* 1996;355(1-2):13-40.
92. Volk J, Leyhausen G, Geurtsen W. Glutathione level and genotoxicity in human oral keratinocytes exposed to TEGDMA. *J Biomed Mater Res B Appl Biomater.* 2012;100(2):391-9.
93. Ansteinsen V, Solhaug A, Samuelsen JT, Holme JA, Dahl JE. DNA-damage, cell-cycle arrest and apoptosis induced in BEAS-2B cells by 2-hydroxyethyl methacrylate (HEMA). *Mutat Res.* 2011;723(2):158-64.
94. Tenório M, Graciliano NG, Moura FA, Oliveira ACM, Goulart MOF. N-Acetylcysteine (NAC): Impacts on Human Health. *Antioxidants (Basel).* 2021;10(6).
95. Mavrogonatos E, Eliades T, Eliades G, Klefsas D. The effect of triethylene glycol dimethacrylate on p53-dependent G2 arrest in human gingival fibroblasts. *Biomaterials.* 2010;31(33):8530-8.
96. Schweikl H, Altmannberger I, Hanser N, Hiller KA, Bolay C, Brockhoff G, et al. The effect of triethylene glycol dimethacrylate on the cell cycle of mammalian cells. *Biomaterials.* 2005;26(19):4111-8.
97. Janke V, von Neuhoff N, Schlegelberger B, Leyhausen G, Geurtsen W. TEGDMA causes apoptosis in primary human gingival fibroblasts. *J Dent Res.* 2003;82(10):814-8.
98. Chang HH, Chang MC, Huang GF, Wang YL, Chan CP, Wang TM, et al. Effect of triethylene glycol dimethacrylate on the cytotoxicity, cyclooxygenase-2 expression and prostanooids production in human dental pulp cells. *Int Endod J.* 2012;45(9):848-58.

99. Spagnuolo G, Galler K, Schmalz G, Cosentino C, Rengo S, Schweikl H. Inhibition of phosphatidylinositol 3-kinase amplifies TEGDMA-induced apoptosis in primary human pulp cells. *J Dent Res*. 2004;83(9):703-7.
100. Krifka S, Petzel C, Bolay C, Hiller KA, Spagnuolo G, Schmalz G, et al. Activation of stress-regulated transcription factors by triethylene glycol dimethacrylate monomer. *Biomaterials*. 2011;32(7):1787-95.
101. Mustafa M, Ahmad R, Tantry IQ, Ahmad W, Siddiqui S, Alam M, et al. Apoptosis: A Comprehensive Overview of Signaling Pathways, Morphological Changes, and Physiological Significance and Therapeutic Implications. *Cells*. 2024;13(22).
102. Batarseh G, Windsor LJ, Labban NY, Liu Y, Gregson K. Triethylene glycol dimethacrylate induction of apoptotic proteins in pulp fibroblasts. *Oper Dent*. 2014;39(1):E1-8.
103. Shiraishi H, Okamoto H, Yoshimura A, Yoshida H. ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. *J Cell Sci*. 2006;119(Pt 19):3958-66.
104. Kushnareva Y, Newmeyer DD. Bioenergetics and cell death. *Ann N Y Acad Sci*. 2010;1201:50-7.
105. Mikulas K, Komlodi T, Foldes A, Svab G, Horvath G, Nagy AM, et al. Bioenergetic Impairment of Triethylene Glycol Dimethacrylate- (TEGDMA-) Treated Dental Pulp Stem Cells (DPSCs) and Isolated Brain Mitochondria are Amended by Redox Compound Methylene Blue (dagger). *Materials (Basel)*. 2020;13(16).
106. Schneider TR, Hakami-Tafreshi R, Tomasino-Perez A, Tayebi L, Lobner D. Effects of dental composite resin monomers on dental pulp cells. *Dent Mater J*. 2019;38(4):579-83.
107. Zhu Y, Gu YX, Mo JJ, Shi JY, Qiao SC, Lai HC. N-acetyl cysteine protects human oral keratinocytes from Bis-GMA-induced apoptosis and cell cycle arrest by inhibiting reactive oxygen species-mediated mitochondrial dysfunction and the PI3K/Akt pathway. *Toxicol In Vitro*. 2015;29(8):2089-101.
108. Chang MC, Lin LD, Chan CP, Chang HH, Chen LI, Lin HJ, et al. The effect of BisGMA on cyclooxygenase-2 expression, PGE2 production and cytotoxicity via reactive oxygen species- and MEK/ERK-dependent and -independent pathways. *Biomaterials*. 2009;30(25):4070-7.
109. Pae HO, Chung HT. Heme oxygenase-1: its therapeutic roles in inflammatory diseases. *Immune Netw*. 2009;9(1):12-9.
110. Drozd K, Wysokinski D, Krupa R, Wozniak K. Bisphenol A-glycidyl methacrylate induces a broad spectrum of DNA damage in human lymphocytes. *Arch Toxicol*. 2011;85(11):1453-61.
111. Li YC, Kuan YH, Huang FM, Chang YC. The role of DNA damage and caspase activation in cytotoxicity and genotoxicity of macrophages induced by bisphenol-A-glycidyl dimethacrylate. *Int Endod J*. 2012;45(6):499-507.
112. Huang FM, Chang YC, Su CH, Wu SW, Lee SS, Lee MW, et al. Rutin-protected BisGMA-induced cytotoxicity, genotoxicity, and apoptosis in macrophages through the reduction of the mitochondrial apoptotic pathway and induction of antioxidant enzymes. *Environ Toxicol*. 2021;36(1):45-54.

Biological Effects of Substances Released by Resin-Based Composites

113. Ratanasathien S, Wataha JC, Hanks CT, Dennison JB. Cytotoxic interactive effects of dentin bonding components on mouse fibroblasts. *J Dent Res.* 1995;74(9):1602-6.

VIII. Objectives

This thesis is guided by the central question:

How can the biological risks of RBCs be reduced without compromising their clinical performance?

To address this, the research pursues three objectives:

Evaluate the mechanical and biological performance of contemporary RBCs, using bulk-fill formulations as a model to study polymerization efficiency and curing depth in commercial materials.

Quantify monomer elution and its biological consequences, with emphasis on oxidative stress and metabolic activity.

Explore strategies to enhance material biocompatibility, focusing on the incorporation of the antioxidant NAC and assessing its dual impact on cytotoxicity and composite properties.

Within this framework, two experimental avenues are pursued. **Bulk-fill composites are employed as a testing platform**: their increased increment thickness (up to 4 mm versus 2 mm for conventional RBCs) provides a practical model for examining depth-dependent variations in curing quality, mechanical stability, and biological responses. Importantly, bulk-fills are not the main focus of this thesis, but rather an investigative tool to assess how incomplete polymerization influences material performance.

Second, **adjunctive approaches to improve composite biocompatibility are explored**, with NAC serving as a proof-of-concept additive. By serving as a glutathione precursor, exerting antioxidant activity and directly neutralizing resin monomers through adduct formation, NAC has shown promise in mitigating resin-induced oxidative stress and cytotoxicity; however, its impact on polymerization kinetics and mechanical integrity remains insufficiently defined and requires evaluation.

IX. Methods used in this thesis

This thesis employed an integrated methodological framework to evaluate RBCs, combining mechanical, chemical, and biological assays. The choice of tests was guided by the *Academy of Dental Materials* recommendations for standardized mechanical characterization, which prioritize flexural strength, modulus, and hardness as clinically relevant measures of material performance (1). In addition, all assays were conducted, where possible, in accordance with relevant international standards, namely ISO 4049 for mechanical testing of dental composites and the ISO 10993 series for biological evaluation of medical devices, ensuring reproducibility, comparability, and regulatory compliance.

1. Three-Point Bending (Flexural Strength and Modulus)

Flexural strength and modulus were determined using a universal testing machine (Lloyd LRX Plus, Lloyd Instruments, UK) equipped with a load cell of suitable capacity. The machine operates by applying a controlled force through a moving crosshead, while recording the resulting deformation of the specimen. In the three-point bending setup, rectangular specimens (25 × 2 × 2 mm) were supported at two points separated by a 20 mm span. A loading nose was positioned at the midpoint between the supports, delivering a downward force at a constant crosshead speed of 0.75 mm/min until specimen fracture occurred.

The principle of this test relies on generating tensile, compressive, and shear stresses within the specimen simultaneously: the upper surface of the bar experiences compression, while the lower surface is subjected to tension. The magnitude of stress is calculated from the applied load, span length, and specimen geometry. The flexural

strength (σ , MPa) represents the maximum stress sustained before fracture, while the flexural modulus (E, GPa) is derived from the slope of the initial, linear portion of the load–deflection curve, reflecting the material’s stiffness.

Universal testing machines measure force using a load cell that converts mechanical deformation into an electrical signal, typically via strain gauges mounted on an elastic element. Simultaneously, specimen deflection is tracked either by crosshead displacement or by extensometers. The resulting load–deflection data are continuously recorded and plotted, allowing precise determination of mechanical properties according to ISO 4049:2000.

This method is widely adopted because flexural strength and modulus are ranked as priority mechanical properties by the Academy of Dental Materials, and because the three-point bending test subjects the specimen to combined stresses that approximate clinical conditions in which dental restorations fail due to complex loading.

2. Vickers Microhardness

Vickers microhardness was measured using a diamond pyramidal indenter mounted on a hardness tester (Durimet, Leitz, Germany). The machine functions by pressing the indenter into the polished surface of the specimen under a known static load, here either 200 g or 500 g depending on the study, applied for 30 s. The load is transmitted through a mechanical lever system to ensure precise and reproducible force delivery.

When the indenter penetrates the specimen, it leaves a square-shaped impression with diagonals that can be measured optically under a microscope integrated into the machine. The Vickers Hardness Number (VHN, kgf/mm^2) is calculated by dividing the applied load by the surface area of the indentation, which is determined from the diagonal lengths. Harder materials produce smaller indentations for the same applied force.

This method is particularly suited for dental composites because it is sensitive to differences in polymerization efficiency: insufficiently cured material will yield lower hardness values, especially at greater depths. In addition, repeating the test after immersion in ethanol provides insight into material degradation and softening under chemical challenge, simulating intraoral exposure.

3. Electron Paramagnetic Resonance (EPR) Spectroscopy for Trapped Free Radicals

EPR spectroscopy was employed to quantify trapped free radicals remaining after photo-polymerization. The spectrometer (MINISCOPE MS200, Magnettech, Germany) detects unpaired electrons, which exhibit magnetic moments. When placed in a magnetic field and irradiated with microwave radiation, these unpaired electrons can absorb energy by flipping their spin states.

The instrument generates a magnetic field sweep, while a detector records the resonance absorption as peaks corresponding to specific g-factors. In cured RBCs, free radicals trapped in the polymer matrix produce a signal at $g = 2.02$. The height of the fourth peak in the resonance spectrum is proportional to radical concentration. Because radicals are essential intermediates in polymerization, their presence after curing provides information on the completeness and stability of the curing reaction.

4. Degree of Conversion by Micro-Raman Spectroscopy

Raman spectroscopy was used to determine the DC of RBCs at various depths. Raman spectroscopy relies on the inelastic scattering of monochromatic light, a phenomenon first described by C.V. Raman in 1928. When a laser beam interacts with molecular bonds, most photons are scattered elastically (Rayleigh scattering), but a small fraction

undergoes a change in energy corresponding to the vibrational energy levels of the molecule. This shift in wavelength, known as the Raman shift, provides a fingerprint of the molecule's vibrational modes.

In these studies, a 780 nm diode laser was directed onto the specimen surface through a high-magnification objective (50×). The scattered light was collected and passed through a diffraction grating to resolve the spectrum. Specific peaks in the spectrum were monitored: the aliphatic carbon-carbon double bond (C=C) at 1640 cm^{-1} , which decreases during polymerization, and the aromatic carbon-carbon stretch at 1610 cm^{-1} , which remains constant and serves as an internal reference. By comparing the ratio of these peaks before and after curing, the DC was calculated as the percentage of reactive double bonds converted into polymer.

This technique is highly sensitive, non-destructive, and allows depth-resolved analysis by focusing the laser at different distances below the surface (0, 2, 4, and 6 mm in this thesis). It provides direct insight into polymerization efficiency, which is a critical determinant of both mechanical performance and residual monomer release.

5. Chemical Analyses for Monomer and Photoinitiator Elution

Ultra-High-Performance Liquid Chromatography coupled to Tandem Mass Spectrometry (UHPLC-MS/MS) was employed to quantify the release of residual monomers and photoinitiators into aqueous medium.

UHPLC principle: Liquid chromatography separates compounds based on their affinity for a stationary phase (column packing) and a mobile phase (solvent). Under high pressure (up to 1000 bar), the mobile phase pushes analytes through a tightly packed column with small particle size ($1.8\text{ }\mu\text{m}$ in this study), achieving very high resolution.

Compounds with different polarities and molecular structures elute at different retention times.

MS/MS principle: As compounds elute, they are ionized in an electrospray ionization (ESI) source, generating charged molecular species. These ions enter the first quadrupole mass filter (Q1), which selects a specific parent ion (precursor) based on mass-to-charge ratio (m/z). The ion is then fragmented in a collision cell (Q2) by collision-induced dissociation, and the resulting fragments are analyzed by a second mass analyzer (Q3). This tandem approach increases sensitivity and specificity by monitoring multiple reaction monitoring (MRM) transitions unique to each analyte.

Workflow in this thesis: Conditioned medium from composite disks was spiked with an internal standard, 1,6-hexanediol dimethacrylate (HDDMA), filtered, and injected into a C8 column under isocratic methanol/water elution with ammonium acetate. Target analytes included TEGDMA, BisGMA, DMAEMA, CQ, TPO, and NAC. Each analyte was quantified by comparing the peak area of its quantifier multiple reaction monitoring (MRM) transition to calibration curves, while qualifier transitions confirmed identity.

This combined UHPLC–MS/MS method is considered the gold standard for trace-level quantification of leachable substances from dental composites, as it combines chromatographic separation of structurally similar compounds with highly selective mass-based detection.

6. Biological Properties

6.1. Cell metabolic activity – MTS Assay

The metabolic activity of human dental pulp cells (hDPCs) exposed to conditioned media was assessed using the MTS assay (CellTiter 96® Aqueous One Solution, Promega, USA). This colorimetric method is based on the conversion of a tetrazolium

salt (MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) into a soluble formazan product by cellular enzymes, primarily mitochondrial dehydrogenases.

When viable cells with intact metabolic pathways are present, NAD(P)H-dependent oxidoreductase enzymes reduce MTS to formazan. The reaction occurs extracellularly, producing a water-soluble dye that accumulates in the culture medium. The amount of formazan formed is directly proportional to the number of metabolically active (viable) cells.

Absorbance of the formazan was measured at 490 nm in a microplate reader (SpectraMax M2, Molecular Devices). Cells exposed to conditioned medium for 24 h were compared with untreated controls (100% viability reference). This method is widely used in biomaterials research because it provides a sensitive, quantitative, and non-radioactive measure of cell viability and proliferation.

6.2. Reactive Oxygen Species Quantification

Intracellular oxidative stress was evaluated using a fluorescent ROS-sensitive probe, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA). This non-fluorescent compound is cell-permeable and enters live cells, where intracellular esterases cleave the diacetate groups, trapping it as H₂DCF inside the cytoplasm.

In the presence of reactive oxygen species, H₂DCF undergoes oxidation to form 2',7'-dichlorofluorescein (DCF), a highly fluorescent molecule. The intensity of the fluorescence signal is directly proportional to the intracellular ROS levels. Fluorescence was measured at an excitation wavelength of 495 nm and an emission wavelength of 525 nm using a microplate reader (SpectraMax M2).

Positive controls were included by exposing cells to tert-butyl hydroperoxide (TBHP, 100 μ M), a known inducer of oxidative stress, to confirm the responsiveness of the assay. Results were normalized against untreated cell controls to express relative ROS production.

Methods used in this thesis

This assay provides a direct measure of oxidative stress, a key mediator of cytotoxicity in dental materials. It is particularly relevant for evaluating the protective effects of antioxidants such as NAC incorporated into composites.

7. References

1. Ilie N, Hilton TJ, Heintze SD, Hickel R, Watts DC, Silikas N, et al. Academy of Dental Materials guidance-Resin composites: Part I-Mechanical properties. Dent Mater. 2017;33(8):880-94.

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

This chapter is based on the following published article:

Gilli M, Hollaert TG, Setbon HM, des Rieux A, Leprince JG. Quality of cure in depth of commercially available bulk-fill composites: A layer-by-layer mechanical and biological evaluation. *Oper Dent.* 2022;47(4):437-48. doi:10.2341/21-084-L.

Abstract

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. 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 20s with Bluephase G2 (Ivoclar-Vivadent, irradiance=1050mW/cm²). The following physico-mechanical properties were measured for the upper (0-2mm), intermediate (2-4mm) and lower (4-6mm) layers: degree of conversion using Raman Spectrometry (DC, in %), microhardness using a Vickers micro-indenter before (VHN dry) and after a 24h storage in ethanol (VHN EtOH), 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 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 4mM 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

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

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 with respect to layer thickness. Finally, the use of NAC appeared as beneficial to reduce the risk of harmful effect 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.

1. 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 intrinsic need to layer the composite restorations,(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. First, changing the photoinitiator to significantly reduce the curing time (below 5s) while maintaining, or in some cases improving, mechanical and biological key material properties.(7-10) The second strategy was 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 materials 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. 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 (4mm bulk-fill RBC covered with 2mm conventional RBC) to a classic incremental filling, in class I and II cavities. Over the 6-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-

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

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 the past to evaluate the “depth of cure”,(16, 17) 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 over-estimation of the true value.(18) Therefore, a combination of various methods is more likely to provide a more complete assessment of the quality of cure in depth.(16) Based on a systematic review and meta-analysis,(19) the two main reasons for the short-term failure of RBC restorations were identified: endodontic causes (<1year post-placement) and fractures (2-3 years post-placement). Hence, besides the mechanical properties which are well-known to be important for material performance, biological aspects must be taken into account as well when attempting to evaluate *in vitro* the performance of thick RBC restorations. Notably, RBCs can release various active compounds such as monomers or photoinitiators,(20) which can reach the pulp, especially in deep cavities, and induce undesirable biological responses.(21) The latter have been so far mostly attributed to an oxidative stress through the generation of reactive oxygen species.(22)

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.

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

2. Materials and Methods

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).

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 (Alstatten, Switzerland)	Dual-cure bulk-fill flowable composite	Universal	F33233	Arbitrary thickness owing to its dual curing properties

Table 1 : List of tested materials

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

For the evaluation of flexural properties (modulus and strength) in depth, the composites were placed into 3 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 20s on the upper side, with the light tip in close contact with the polyester film. 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/cm² as measured by Bluephase Meter (Ivoclar-Vivadent, Schaan, Liechtenstein)).

The three 2mm-thick layers – 0-2mm (upper layer), 2-4mm (intermediate layer), 4-6mm (lower layer) – were removed from the molds and polished using SiC paper grit 1000 then placed in distilled water in the dark during a week at 37°C. They were then submitted to a three-point bend test in a universal testing machine (LRX Plus, Lloyd Instrument) at a strain rate of 0.75mm/min until fracture occurred (n=5). Flexural modulus and strength were calculated as previously described.⁽¹⁵⁾

For the measurement of microhardness and DC, 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 20s irradiation with the same parameters described above. The 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 780nm by a frequency-stabilized single mode diode laser through a microscope objective (50x) and spectra were obtained in the region 1600 cm⁻¹, with the following conditions: microhole: 50; irradiation time: 60s; number of accumulations: 5; 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

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

uncured sample; the aromatic peak at 1610 cm^{-1} was used as the internal standard.(23) On the same samples, Vickers microhardness was measured in dry conditions (VHN dry; $n=3$) along the side of the sample at similar depth (0, 2, 4 and 6mm) using a Durimet microhardness tester (Leitz, Wetzlar, Germany) and applying a 200 g load for 30s. The samples were then immersed in pure ethanol for 24h, before re-measuring the microhardness (VHN EtOH; $n=3$).⁽¹⁵⁾ In order to enable comparison with flexural properties, an average value was calculated between 0 and 2mm (upper layer), 2 and 4 mm (intermediate layer), 4 and 6mm (lower layer).

For the study of human dental pulp cell (hDPC) viability, conditioned medium was produced by incubating composite disks into cell culture medium (Dulbecco's Modified Eagle Medium – supplemented with 10% bovine serum, L-Glutamine, 100 U/ml penicillin and $100\mu\text{g/ml}$ streptomycin, Thermo Fischer-Scientific, Waltham, Massachusetts, USA). The composites were packed into 3 superimposed and aligned white Teflon molds (5mm diameter, 2mm 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 2mm-thick disks – 0-2mm (upper layer), 2-4mm (intermediate layer), 4-6mm (lower layer) – was then placed in 1mL of culture medium in a 24-well plate and incubated during 7 days at 5% CO_2 , 95% humidity (Figure 1c). After 1 week, conditioned media were collected and incubated with hDPC 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 40mm^3 corresponding to the volume of the mold was injected directly into the well.

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

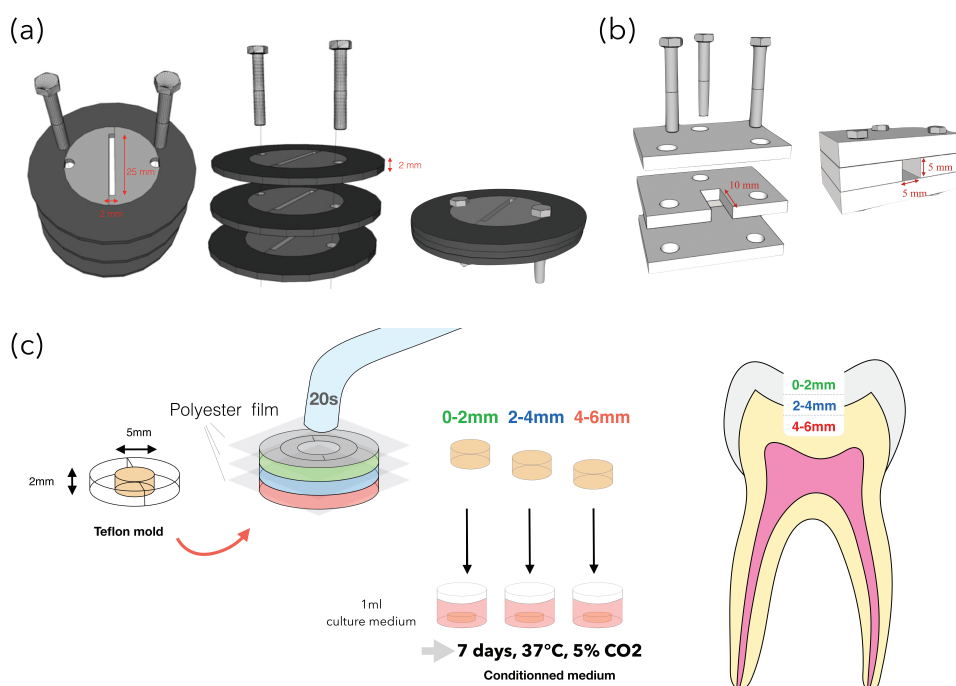


Figure 1 : Experimental setups for layer-by-layer polymerization: (a) molds for samples for 3-point-bending; (b) molds for samples for DC and VHN measurements; and (c) measurement of cell toxicity. (c) presents how each layer of the composite cylinder was then used to produce the conditioned medium.

To evaluate the effect of the components released by the materials on cell viability, hDPC were seeded onto 96-well plates at 10^4 cells per well (3.03×10^4 cells/cm²). The culture was maintained with 5% CO₂, 37°C during 24h to allow cell adhesion. After that, cell culture medium was removed and replaced by the conditioned medium for 24h at 5% CO₂, 37°C, standard culture medium was used as control. Cell viability was assessed using a MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, Wisconsin, USA) as per supplier instructions. Briefly, the culture medium was removed from the wells, which were washed 3 times with PBS. MTS solution (100 µl) was then added to each well during 30 minutes at 5% CO₂, 37°C.

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

The absorbance of each well was then determined using a microplate reader (SpectraMax M2, Molecular devices) at a wavelength of 490nm.

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 4mM (n=4 wells per condition).

The cells 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. hDPC were isolated by the outgrowth method.(24) Briefly, after surface disinfection, the teeth were split in order to recover the pulp tissue, which was then rinsed in growth medium (see above), minced into small pieces, and placed in 6-well plates. Growth medium was changed every 2-3 days. After 10 days, the cells were harvested using Acutase (Life Technologies, Gent, Belgium), centrifugated, 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 2.5×10^5 cells/T75 flask for subsequent use. For all experiments performed in the present work, the cells were used at passage 6.

Statistical analyses were preformed using JMP software. 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, i.e. 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.

3. Results

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 systematically for the two conventional materials (Grandio and GrandioF) for all properties (Figure 2).

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

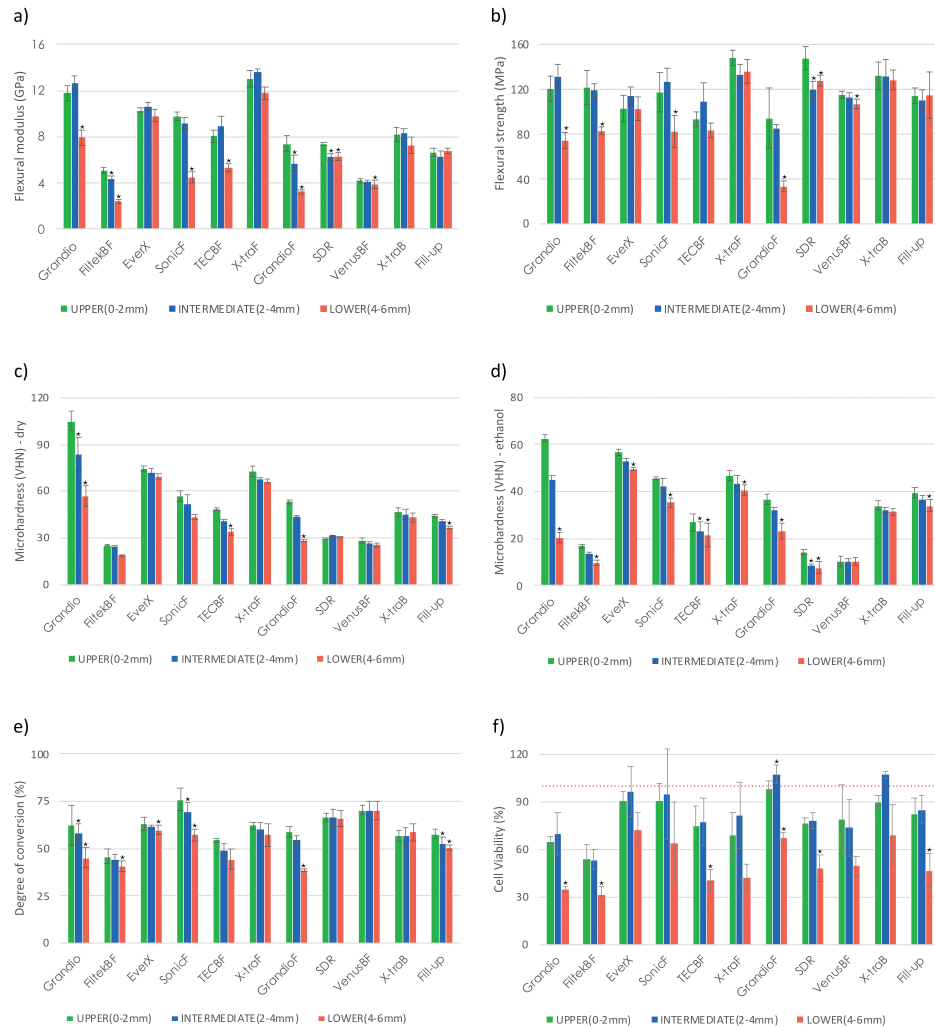


Figure 2 : Mechanical and biological properties for each material according to the three layers (upper, intermediate, and lower): (a) flexural modulus; (b) flexural strength; (c) microhardness after dry storage; (d) microhardness after 24 hours of ethanol storage; (e) degree of conversion; and (f) human 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.

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

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 on hDPC viability ($p < 0.05$; Table 2), with values ranging from 54% (FiltekBF) to 98.7% (GrandioF).

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.

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

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 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 conditioned medium prepared with uncured composites compared to the lower composite layer ($p < 0.05$ for all materials except SonicF, TECBF and VenusBF). Average

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

relative values for uncured composites ranged from 0 to 60.3% compared to the standard growth medium (Figure 3). The addition of 4mM 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 to 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.

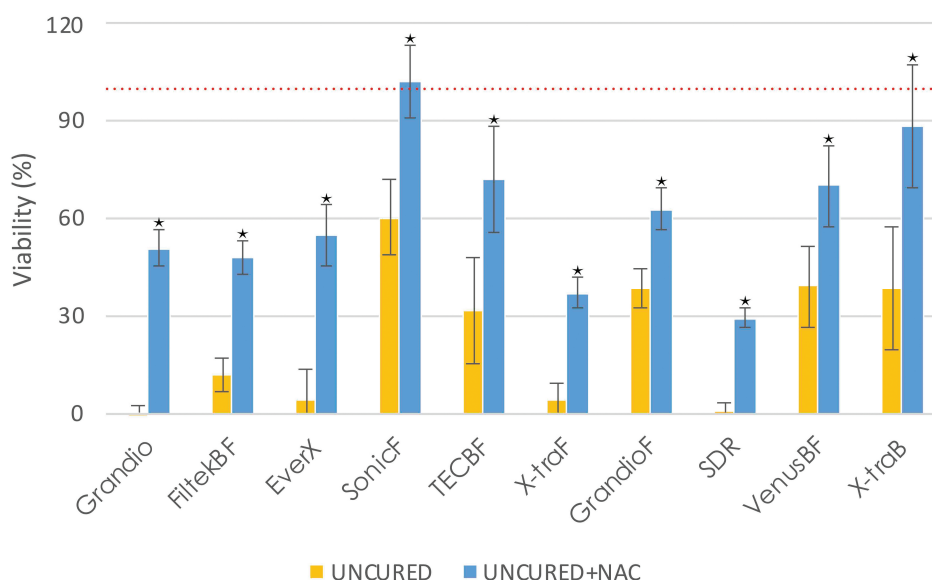


Figure 3 : Relative cell viability of cells grown in conditioned medium prepared with uncured composites, with and without the addition of 4 mM of NAC; 100% viability (horizontal dotted line) corresponds to the values measured for the cells cultivated in regular growth medium. *Identifies a statistical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

4. 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.⁽²⁵⁾ 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 material 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,⁽²⁶⁻³⁰⁾ 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.^(18, 31) 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 (4-6mm) and uncured layers were considered in the present work as worst-case scenarios (incomplete curing or uncured material).

As mentioned above, Bulk-fill composites are usually considered as materials allowing polymerization in 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 ($\leq 4\text{mm}$) for all tested properties, which support the recommendation made in previous work to be cautious with the bulk fill

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

“label” (28). In fact, this labelling is further challenged by the fact that increasing significantly the curing time (100 s or more) may lead to comparable levels of conversion at 4 mm depth.(11)

Not only the evolution of a given property in depth needs to be considered with regards to the upper layer, but the actual absolute value. To illustrate that, among the four materials strictly complying to “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. This is in line with previous works based mostly on physico-mechanical aspects,(15, 25) 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,(32) and that substances can be released that have harmful effects on human pulp cells.(33) The cytotoxicity of the composites leachates depends both on the nature and the amount of the released molecules.(34) Monomers and compounds of the photoinitiator system represent most of the released molecules,(35) 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 0.087mM for BisGMA to 11.53mM for HEMA and 3.460mM for TEGDMA.(34) Hence, a first possible explanation of the inter-material differences observed here regarding hDPC viability is therefore 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.(36) However, an additional parameter is essential regarding the cytotoxicity of a molecule, i.e. its solubility in aqueous medium. In order to induce cell damage, they must indeed be soluble in the aqueous growth medium. For example, the solubility of BisGMA in water is 9.5ug/mm³ as compared to 27.5ug/mm³ for TEGDMA.(37) It was very elegantly demonstrated by Meng et al. that no

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

BisGMA could be detected in water storage after 1 week, while the monomer was detected in large amounts one week later once the solvent was switched to ethanol.(38) Consequently, the measurement of EC50 of hydrophobic monomers such as BisGMA required their prior dissolution into DMSO and subsequent dilution in aqueous cell growth medium.(34) Since growth medium without DMSO was used in the present work (aqueous media, 7days 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.

Lastly, although most of the cytotoxicity of RBCs has been attributed to monomers via an oxidative stress (22), 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.(39, 40) The presence of unconverted photoinitiator molecules was shown to result from insufficient light exposure,(41) which may be the case beyond 4mm 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 due to the fact that their precise composition is rarely disclosed.(42) While a thorough quantification of released monomers would be useful to discuss some hypotheses, this was beyond the scope of the present work, and would in any case much more accurate if the experiment was repeated with experimental materials with 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.(43) Hence, the first and most obvious explanation for the reduction in cell viability beyond 4mm thickness would be the

reduction in monomer conversion, which is known to be inversely correlated with monomer elution.(44) This is globally in line with the present data, and in agreement with a previous work, including one Bulk-fill and 3 conventional composites, and reporting no differences in viability until 4mm depth.(45)

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.(32) Consequently, even at similar DC, a different local cross-linking density can be measured.(18, 31) 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 4mM 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.(46, 47) Nevertheless, under the present experimental conditions, the addition of NAC at maximum non-cytotoxic concentration did not allow a full recovery of hDPCs viability except for SonicF. This could first be explained by the fact that the maximum concentration of NAC remains too low (4mM) 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 increased levels of reactive oxygen species similar to those of induced by 2-hydroxyethyl methacrylate were not associated to an increase in cell death or cell growth inhibition, unlike when the monomer was present.(48) This tends to indicate that monomer-induced cell damage may not be caused exclusively by the increase in reactive oxygen species.

5. Conclusions

Within the limitations of this work, and with regards to 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 regards both their quality of cure in depth, which is material and property specific, and their intrinsic properties when “optimally” cured
- (2) Not only the evolution of a given property in depth needs to be considered with regards to the upper layer, but the actual absolute value, since significant inter-material differences were observed
- (3) The use of NAC appears as beneficial to reduce the risk of harmful effect to dental pulp cells, especially in case of excessive thickness use, and may be of potential interest as an additive to composites in the future.

6. References

1. Laske M, Opdam NJM, Bronkhorst EM, Braspenning JCC, Huysmans M. Ten-Year Survival of Class II Restorations Placed by General Practitioners. *JDR Clin Trans Res.* 2016;1(3):292-9.
2. Pallesen U, van Dijken JW, Halken J, Hallonsten AL, Hoigaard R. Longevity of posterior resin composite restorations in permanent teeth in Public Dental Health Service: a prospective 8 years follow up. *J Dent.* 2013;41(4):297-306.
3. Van Nieuwenhuysen JP, D'Hoore W, Carvalho J, Qvist V. Long-term evaluation of extensive restorations in permanent teeth. *J Dent.* 2003;31(6):395-405.
4. Opdam NJ, Bronkhorst EM, Roeters JM, Loomans BA. A retrospective clinical study on longevity of posterior composite and amalgam restorations. *Dent Mater.* 2007;23(1):2-8.
5. Park J, Chang J, Ferracane J, Lee IB. How should composite be layered to reduce shrinkage stress: incremental or bulk filling? *Dent Mater.* 2008;24(11):1501-5.
6. Kwon Y, Ferracane J, Lee IB. Effect of layering methods, composite type, and flowable liner on the polymerization shrinkage stress of light cured composites. *Dent Mater.* 2012;28(7):801-9.
7. Randolph LD, Steinhilber J, Moginger B, Gallez B, Stansbury J, Palin WM, et al. Photopolymerization of highly filled dimethacrylate-based composites using Type I or Type II photoinitiators and varying co-monomer ratios. *Dent Mater.* 2016;32(2):136-48.
8. Randolph LD, Palin WM, Bebelman S, Devaux J, Gallez B, Leloup G, et al. Ultra-fast light-curing resin composite with increased conversion and reduced monomer elution. *Dent Mater.* 2014;30(5):594-604.
9. Ilie N, Watts DC. Outcomes of ultra-fast (3 s) photo-cure in a RAFT-modified resin-composite. *Dent Mater.* 2020;36(4):570-9.
10. Leprince JG, Hadis M, Shortall AC, Ferracane JL, Devaux J, Leloup G, et al. Photoinitiator type and applicability of exposure reciprocity law in filled and unfilled photoactive resins. *Dent Mater.* 2011;27(2):157-64.
11. Palin WM, Leprince JG, Hadis MA. Shining a light on high volume photocurable materials. *Dent Mater.* 2018;34(5):695-710.
12. Atabek D, Aktas N, Sakaryali D, Bani M. Two-year clinical performance of sonic-resin placement system in posterior restorations. *Quintessence Int.* 2017;48(9):743-51.
13. Canali GD, Ignacio SA, Rached RN, Souza EM. One-year clinical evaluation of bulk-fill flowable vs. regular nanofilled composite in non-carious cervical lesions. *Clin Oral Investig.* 2018.
14. van Dijken JW, Pallesen U. Bulk-filled posterior resin restorations based on stress-decreasing resin technology: a randomized, controlled 6-year evaluation. *Eur J Oral Sci.* 2017;125(4):303-9.
15. Leprince JG, Palin WM, Vanacker J, Sabbagh J, Devaux J, Leloup G. Physico-mechanical characteristics of commercially available bulk-fill composites. *J Dent.* 2014;42(8):993-1000.

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

16. Leprince JG, Palin WM, Hadis MA, Devaux J, Leloup G. Progress in dimethacrylate-based dental composite technology and curing efficiency. *Dent Mater.* 2013;29(2):139-56.
17. Ferracane JL, Hilton TJ, Stansbury JW, Watts DC, Silikas N, Ilie N, et al. Academy of Dental Materials guidance-Resin composites: Part II-Technique sensitivity (handling, polymerization, dimensional changes). *Dent Mater.* 2017;33(11):1171-91.
18. Leprince JG, Leveque P, Nysten B, Gallez B, Devaux J, Leloup G. New insight into the "depth of cure" of dimethacrylate-based dental composites. *Dent Mater.* 2012;28(5):512-20.
19. Opdam NJ, van de Sande FH, Bronkhorst E, Cenci MS, Bottenberg P, Pallesen U, et al. Longevity of posterior composite restorations: a systematic review and meta-analysis. *J Dent Res.* 2014;93(10):943-9.
20. Van Landuyt KL, Nawrot T, Geebelen B, De Munck J, Snauwaert J, Yoshihara K, et al. How much do resin-based dental materials release? A meta-analytical approach. *Dent Mater.* 2011;27(8):723-47.
21. Bakopoulou A, Papadopoulos T, Garefis P. Molecular toxicology of substances released from resin-based dental restorative materials. *International journal of molecular sciences.* 2009;10(9):3861-99.
22. Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. *Biomaterials.* 2013;34(19):4555-63.
23. Pianelli C, Devaux J, Bebelman S, Leloup G. The micro-Raman spectroscopy, a useful tool to determine the degree of conversion of light-activated composite resins. *J Biomed Mater Res.* 1999;48(5):675-81.
24. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, et al. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. *Cell Tissue Res.* 2013;353(1):65-78.
25. Van Ende A, De Munck J, Lise DP, Van Meerbeek B. Bulk-Fill Composites: A Review of the Current Literature. *J Adhes Dent.* 2017;19(2):95-109.
26. Yu P, Yap A, Wang XY. Degree of Conversion and Polymerization Shrinkage of Bulk-Fill Resin-Based Composites. *Oper Dent.* 2017;42(1):82-9.
27. Lima RBW, Troconis CCM, Moreno MBP, Murillo-Gomez F, De Goes MF. Depth of cure of bulk fill resin composites: A systematic review. *Journal of esthetic and restorative dentistry : official publication of the American Academy of Esthetic Dentistry [et al].* 2018;30(6):492-501.
28. Goncalves F, Campos LMP, Rodrigues-Junior EC, Costa FV, Marques PA, Francci CE, et al. A comparative study of bulk-fill composites: degree of conversion, post-gel shrinkage and cytotoxicity. *Braz Oral Res.* 2018;32:e17.
29. Miletic V, Pongprueksa P, De Munck J, Brooks NR, Van Meerbeek B. Curing characteristics of flowable and sculptable bulk-fill composites. *Clin Oral Investig.* 2017;21(4):1201-12.
30. Al-Ahdal K, Ilie N, Silikas N, Watts DC. Polymerization kinetics and impact of post polymerization on the Degree of Conversion of bulk-fill resin-composite at clinically relevant depth. *Dent Mater.* 2015;31(10):1207-13.

31. Asmussen E, Peutzfeldt A. Polymer structure of a light-cured resin composite in relation to distance from the surface. *Eur J Oral Sci.* 2003;111(3):277-9.
32. Barszczewska-Rybarek IM. A Guide through the Dental Dimethacrylate Polymer Network Structural Characterization and Interpretation of Physico-Mechanical Properties %M doi:10.3390/ma12244057 %U <https://www.mdpi.com/1996-1944/12/24/4057>. Materials %@ 1996-1944. 2019;12(24):4057.
33. Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *J Biomed Mater Res.* 1998;41(3):474-80.
34. Reichl FX, Esters M, Simon S, Seiss M, Kehe K, Kleinsasser N, et al. Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. *Arch Toxicol.* 2006;80(6):370-7.
35. Durner J, Spahl W, Zaspel J, Schweikl H, Hickel R, Reichl FX. Eluted substances from unpolymerized and polymerized dental restorative materials and their Nernst partition coefficient. *Dent Mater.* 2010;26(1):91-9.
36. Durner J, Wellner P, Hickel R, Reichl FX. Synergistic interaction caused to human gingival fibroblasts from dental monomers. *Dent Mater.* 2012;28(8):818-23.
37. Gajewski VE, Pfeifer CS, Froes-Salgado NR, Boaro LC, Braga RR. Monomers used in resin composites: degree of conversion, mechanical properties and water sorption/solubility. *Braz Dent J.* 2012;23(5):508-14.
38. Meng J, Yang H, Cao M, Li L, Cai Q. Correlating cytotoxicity to elution behaviors of composite resins in term of curing kinetic. *Mater Sci Eng C Mater Biol Appl.* 2017;78:413-9.
39. Van Landuyt KL, Krifka S, Hiller KA, Bolay C, Waha C, Van Meerbeek B, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. *Dent Mater.* 2015;31(8):916-27.
40. Manojlovic D, Dramicanin MD, Miletic V, Mitic-Culafic D, Jovanovic B, Nikolic B. Cytotoxicity and genotoxicity of a low-shrinkage monomer and monoacylphosphine oxide photoinitiator: Comparative analyses of individual toxicity and combination effects in mixtures. *Dent Mater.* 2017;33(4):454-66.
41. Hadis MA, Shortall AC, Palin WM. Competitive light absorbers in photoactive dental resin-based materials. *Dent Mater.* 2012;28(8):831-41.
42. Leprince J, Palin WM, Mullier T, Devaux J, Vreven J, Leloup G. Investigating filler morphology and mechanical properties of new low-shrinkage resin composite types. *J Oral Rehabil.* 2010;37(5):364-76.
43. Mohsen NM, Craig RG, Hanks CT. Cytotoxicity of urethane dimethacrylate composites before and after aging and leaching. *J Biomed Mater Res.* 1998;39(2):252-60.
44. Ferracane JL. Elution of leachable components from composites. *J Oral Rehabil.* 1994;21(4):441-52.
45. Marigo L, Spagnuolo G, Malara F, Martorana GE, Cordaro M, Lupi A, et al. Relation between conversion degree and cytotoxicity of a flowable bulk-fill and three conventional flowable resin-composites. *Eur Rev Med Pharmacol Sci.* 2015;19(23):4469-80.

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

46. Krifka S, Seidenader C, Hiller KA, Schmalz G, Schweikl H. Oxidative stress and cytotoxicity generated by dental composites in human pulp cells. *Clin Oral Investig.* 2012;16(1):215-24.
47. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. *J Dent Res.* 2006;85(10):870-7.
48. Morisbak E, Ansteinsson V, Samuelsen JT. Cell toxicity of 2-hydroxyethyl methacrylate (HEMA): the role of oxidative stress. *Eur J Oral Sci.* 2015;123(4):282-7.

XI. Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

This chapter is based on the following manuscript:

Gilli M, Grutle LA, Bebelman S, Uhlig S, Kopperud HM, Leprince JG. Effect of N-acetylcysteine Incorporation into Resin-Based Composites on Chemo-Mechanical Properties, Human Dental Pulp Cell Metabolic Activity and ROS Production. *Manuscript submitted for publication.*

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

Abstract

Objectives: To investigate whether incorporation of N-acetylcysteine (NAC) into resin-based composites (RBCs) enhances their biocompatibility toward human dental pulp cells (hDPCs) while preserving polymerization efficiency and mechanical performance, with consideration of different photoinitiator systems.

Methods: Experimental RBCs were formulated with either camphorquinone/amine (CQ, type II) or diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO, type I) initiators. NAC was incorporated at 0, 0.5, or 1 wt%. Material properties (degree of conversion [DC], free radical concentration, microhardness, elastic modulus, flexural strength) were assessed. *Ultra-High-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry* quantified NAC and monomer elution. Eluates obtained from the composite materials were applied to hDPCs to evaluate metabolic activity (MTS assay) and reactive oxygen species (ROS) generation.

Results: In CQ-based composites, NAC reduced free radical concentration and significantly reduced flexural strength, elastic modulus, Vickers microhardness, and DC at 0.5% NAC and partial recovery at 1%. TEGDMA release was highest at 0.5% NAC. In TPO-based composites, NAC incorporation had minimal adverse effects and was associated with a slight increase in DC. NAC release was dose-dependent, greater in uncured materials, and sufficient to reduce ROS levels in nearly all conditions. Metabolic activity of hDPCs remained at ~80-90% across cured composites, while uncured composites caused substantial cytotoxicity (~20% viability) irrespective of NAC content.

Significance: NAC incorporation consistently decreased ROS production in both CQ- and TPO-based composites. However, this antioxidant effect was not accompanied by a significant improvement in hDPC metabolic activity under the tested conditions. These findings suggest that ROS scavenging alone is insufficient to counteract monomer-related cytotoxicity, emphasizing the need to consider additional mechanisms beyond oxidative stress when assessing the biocompatibility of modified RBCs.

1. Introduction

Dental resin-based composites (RBCs) are widely used in restorative dentistry due to their excellent aesthetic characteristics, favorable mechanical properties, and strong adhesion to dental tissues, making them the material of choice for direct restorations(1, 2). Nevertheless, despite these advantages, RBCs exhibit certain limitations. A key drawback is the incomplete monomer-to-polymer conversion, even under optimal curing conditions, primarily due to vitrification of the resin matrix, which restricts further polymerization at intraoral temperatures (3). This incomplete conversion results in the leaching of unreacted monomers, such as triethylene glycol dimethacrylate (TEGDMA) and bisphenol A glycidyl methacrylate (Bis-GMA) (4), which are known to negatively impact cellular viability through mechanisms including reactive oxygen species (ROS) generation and glutathione (GSH) depletion (5). GSH is the most important non-enzymatic thiol involved in maintaining cellular redox balance (6). These cytotoxic effects raise concerns about the cytocompatibility of RBCs, specifically regarding their impact on dental pulp cells.

To mitigate the biological impact of monomer elution, the use of compounds acting as ROS scavengers, such as N-acetylcysteine (NAC), ascorbic acid or vitamin E, has been explored (7). NAC, a potent antioxidant and GSH precursor, has shown promise in reducing resin-induced cytotoxicity by scavenging ROS, enhancing intracellular GSH levels, and potentially binding with monomers to reduce their bioavailability (7). In vitro studies have demonstrated that the addition of NAC to the culture medium significantly decreases the cytotoxicity of uncured RBCs on human dental pulp cells, although the extent of protection depends on the specific material tested (8). However, this approach is not clinically practical; instead, incorporating NAC directly into the material presents a more convenient alternative (9, 10).

In this context, the incorporation of 1 wt% NAC into a commercial RBC has been shown to reduce cytotoxic and genotoxic responses in human gingival fibroblasts under specific conditions (9). Conversely, another study reported that the same

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

concentration of NAC can interfere with the polymerization process, affecting key material properties such as the degree of conversion and monomer elution, with effects again being material-dependent (10). These findings underscore the importance of evaluating the effects of NAC simultaneously on both cellular response and material properties, in an integrated manner rather than separately.

To date, investigations into the influence of NAC on polymerization efficiency have focused solely on composites formulated with camphorquinone (CQ), by far the most widely used photoinitiator in commercially available RBCs. CQ is a type-II photoinitiator that requires a co-initiator, typically an amine (11) to initiate polymerization. However, alternative photoinitiator systems exist, such as type-I initiators like phosphine oxides, which function via a different mechanism and do not require co-initiators (12). Given the potential interaction of NAC with free radicals at the initiation stage, it is important to determine whether its incorporation also affects these alternative initiation systems. Experimental composites were formulated using either CQ or TPO as the photoinitiator.

Therefore, the objective of this study was to investigate whether the incorporation of NAC into RBCs: (a) improves their cytocompatibility toward human dental pulp cells (hDPCs), and (b) does so without compromising key chemo-mechanical properties of the material. By evaluating polymerization efficiency, mechanical properties, monomer elution and the effect of eluates on hDPC metabolic activity and ROS production, this study aimed to elucidate – in an integrated manner – the dual effect of NAC incorporation into RBCs formulated with different photoinitiator systems.

2. Materials and methods

2.1. Experimental composites preparation

To maintain precise control over composition, RBCs were synthesized in-house using two different photoinitiator systems, either a combination of camphorquinone (CQ, CAS number 10334-26-6, Sigma-Aldrich, St. Louis, MO, USA) and 2-(dimethylamino)ethyl methacrylate (DMAEMA, CAS nr. 2867-47-2, Sigma-Aldrich), or diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO, CAS nr. 75980-60-8, Sigma-Aldrich) as described in previous works (13, 14). Briefly, the resin blend was composed of 70/30 (w/w) bisphenol A glycerolate dimethacrylate (Bis-GMA, CAS nr. 1565-94-2, Sigma-Aldrich) and triethylene glycol dimethacrylate (TEGDMA, CAS nr. 109-16-0, Sigma-Aldrich), and contained either CQ/DMAEMA (0.2/0.8 %) or TPO (0.42 %) as photoinitiator. In addition to the resin blend, nanofillers (methacrylsilane treated fumed silica, 12 nm, AEROSIL® R 7200, Evonik Industries, Germany) were incorporated at a concentration of 10% (w/w) and left to rest overnight in the dark at room temperature to ensure particle wetting. NAC (CAS nr. 616-91-1, Sigma-Aldrich) was finely ground and pre-mixed with microfillers (65%, w/w, silanated barium glass - G018-186/K6, $d_{50} = 3 \pm 1$ micrometer, Schott AG, Landshut, Germany) in proportions of 0.5 or 1% relative to the total material weight. The maximum NAC concentration (1 wt%) was determined during preliminary material preparation, as higher levels led to poor handling properties, i.e. crumbly and unworkable materials. The microfiller mixture containing NAC was then incorporated into the nanofiller-resin blend. Formulations without NAC served as control materials.

2.2. Sample preparation

Two series of samples were produced: one for studying the material properties (degree of conversion, Vickers microhardness, trapped free radical concentration, elastic modulus, and flexural strength), and another for investigating monomer and photoinitiator elution, cytotoxicity and ROS quantification (Fig. 1).

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

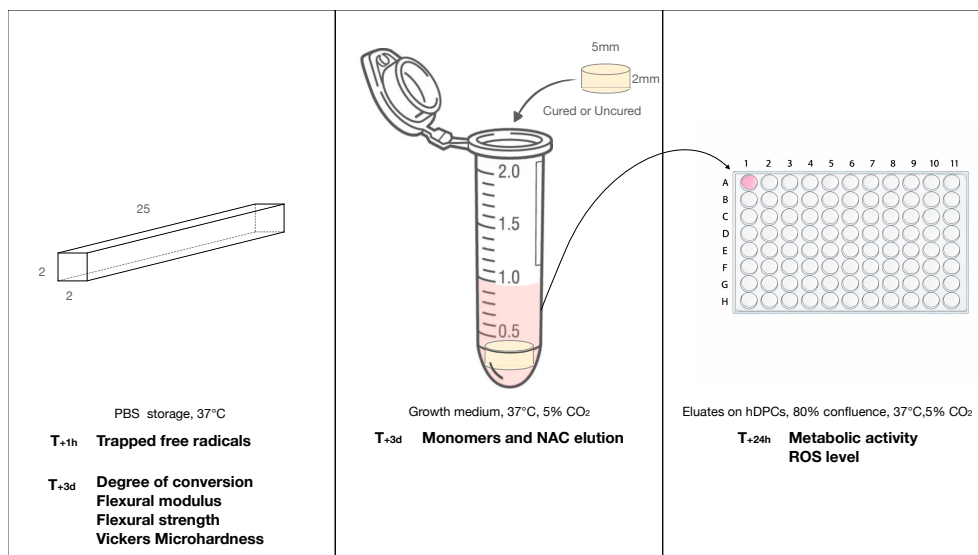


Figure 1 - Schematic representation of the experimental design, illustrating sample preparation, eluate production, and exposure of hDPSCs for the assessment of material properties, metabolic activity, and ROS production.

Samples for the material properties ($n = 15$) were prepared by packing the composites in $25 \times 2 \times 2$ mm molds (according to ISO 4049). The molds were covered with a transparent polyester film, and light-curing applied using an ultra-high power fiber-coupled LED (Prizmatix, Israel) at two narrow spectral peaks corresponding to the absorption ranges of the respective photoinitiators (CQ: peak = 470 nm, peak width = 30nm; TPO: peak = 405 nm, peak width = 22nm). A collimator was employed to expand the beam diameter from 5 mm to 25 mm, ensuring complete coverage of the samples. Calibration curves were generated, and the final irradiance was adjusted to 800 mW/cm² for both wavelengths. Samples were therefore photopolymerized by a single irradiation of 20 s for CQ and 5 s for TPO-based RBCs, based on the optimal photopolymerization parameters identified from previous work (14). For the chemical analyzes and *in vitro* toxicology, samples were prepared in cylindrical molds of 5 mm diameter and 2 mm thickness under the same photopolymerization conditions as those described above (Fig.1).

2.3. Material properties

Polymerization efficiency was evaluated based on two key properties. First, the concentration of trapped free radicals within the polymerized resin-based composites was evaluated using electron paramagnetic resonance using a MINISCOPE MS200 EPR spectrometer (Magnettech, Germany), equipped with a rectangular cavity (TE102). The parameters used were identical with those reported in earlier studies (15, 16). Briefly, the relative concentration of trapped propagating radicals was evaluated based on the height of the fourth peak of the EPR signal (from 336.1 to 336.6 mT of the magnetic field, g-factor 2.02) (17), which was acquired precisely 1 h after the end of photopolymerization. The samples were then polished with 1000-grit SiC paper and stored in distilled water at 37°C in the dark for 1 week. Second, the degree of conversion (DC, in %) was assessed on both the upper and lower surfaces of the samples using a Raman spectrometer (DXR Raman microscope, Thermo Scientific, Madison, WI, USA), as previously described (18). Briefly, samples were excited at 780 nm with a frequency-stabilized single-mode diode laser through a microscope objective (50× magnification), and spectra were obtained in the 1600 cm^{-1} region under the following conditions: microhole size of 50 μm , irradiation time of 60 s, 5 accumulations, and a grating of 400 lines/mm. DC values were calculated based on the intensity decrease of the methacrylate vinyl (C=C) peak at 1640 cm^{-1} , relative to the uncured sample, using the aromatic peak at 1610 cm^{-1} as an internal standard.

A 3-point bend-test was performed as previously described (19) using an universal testing machine (Lloyd LRX Plus, Lloyd Instruments, Fareham, UK), with a 20-mm-span between supports and 0.75 mm/min crosshead speed until sample fracture. Flexural modulus (determined from the tangent of the initial slope) and strength were calculated following ISO 4049 specifications.

Microhardness was then measured on the same samples ($n = 15$) using a Vickers microhardness tester (Durimet, Leitz, Wetzlar, Germany), with a load of 500 g applied for 30 s on the upper surface, following a previously described methodology (18).

2.4. Chemical analyses

Ultra-High-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (UHPLC-MS/MS) analyses were performed to monitor elution from RBCs into the cell culture medium (Dulbecco's Modified Eagle Medium with 10% bovine serum, L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, Thermo Fischer Scientific, Waltham, MA, USA). Each composite disk was placed in a microcentrifuge tube containing 1 mL of cell culture medium, to produced eluates. Three specimen replicates per composite type (cured and uncured CQ- and TPO-containing composites with 0, 0.5 and 1% NAC) were included. The specimens were incubated at 37°C for 72 h (ES-20 orbital shaker-incubator, SIA Biosan, Riga, Latvia). After 72 h, 100 µL of the medium was collected, vortex-mixed with 300 µL methanol, and then centrifuged at $9447 \times g$ for 5 min. The supernatant was transferred to a Costar® Spin-X® centrifuge tube filter with a 0.45 µm nylon filter membrane (Corning Inc., Corning, NY, USA), and centrifuged at $9447 \times g$ for 5 min. An aliquot (200 µL) of the filtrate was transferred to a HPLC vial and diluted with 800 µL methanol. Calibration standards were prepared from individual stock solutions in methanol, and 1,6-hexanediol dimethacrylate (HDDMA, CAS Number 6606-59-3, Sigma Aldrich) was used as internal standard (IS). For NAC, all stock solutions were made in water, without addition of an IS. The concentration ranges of the calibration standards were 0.2–20 ng/mL for TEGDMA, BisGMA and TPO, 5–300 ng/mL for DMAEMA, 20–400 ng/mL for CQ, and 5–1000 ng/mL for NAC.

Instrumental analyses were performed using an Agilent 1290 Infinity II UHPLC system, consisting of a G7167B multisampler, a G7120A high speed pump and a G7116B multicolumn thermostat, coupled to an Agilent G6470A triple quadrupole mass spectrometer via an Agilent Jet Stream (AJS) electrospray ionization (ESI) interface (all Agilent Technologies, Santa Clara, California, USA). The system was operated using MassHunter Acquisition software version B.08.00. Data were processed using MassHunter Quantitative Analysis software version B.07.01 and MassHunter Qualitative Analysis software version B.07.00 (all Agilent Technologies). The ESI

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

interface was operated using the following parameters: drying gas (nitrogen) flow 12.5 L/min, drying gas temperature 200 °C, nebulizer pressure 45 psi, sheath gas flow 12 L/min, sheath gas temperature 390 °C, capillary voltage 3000 V, and nozzle voltage 1000 V. Chromatography was performed using a Zorbax Eclipse Plus C8 RRHD column (2.1 x 100 mm, 1.8 µm particles, Agilent Technologies), at a temperature of 35°C. The column was eluted isocratically using 4:1 methanol/water (v/v), containing 10 mM ammonium acetate. The flow rate was 0.4 mL/min, and the injection volume was 0.2 µL. The MassHunter Acquisition Optimizer software was used to determine the optimal settings for multiple reaction monitoring (MRM), including precursor and product ion *m/z*, fragmentor voltages and collision energies. One MRM ion transition was used for quantification (“quantifier ion”), while the peak area ratio with a second ion transition (“qualifier ion”) was used to confirm the presence of each analyte. For NAC, only one ion transition was monitored because of the absence of a suitable qualifier ion signal (see Supplementary material for LC-MS/MS parameters).

2.5. Biological properties

Two properties were considered key with regards to the effect of monomers on hDPCs: their metabolic activity and their ROS production. To investigate these effects, eluates was prepared by incubating the composite disks in cell culture medium. Material samples were incubated in 1 ml of culture medium for 3 days at 37°C (20). Positive controls were prepared by incubating an equivalent volume of uncured materials. The eluates produced were then used as growth medium for hDPCs.

Primary hDPC cultures were isolated from impacted wisdom teeth by the outgrowth method (21) and used as previously described between passage 3 and 5 (8). Cells were seeded into 96-well plates at a density of 2.04×10^4 cells per well (6.06×10^4 cells/cm²). The plates were maintained at 37°C in 5% CO₂ for 24 h to allow cell adhesion.

2.6. Metabolic activity

To study metabolic activity changes related to monomer exposure, the culture medium was then replaced with the different eluates for a 24 h incubation under the same conditions, with standard culture medium used as a control. A tetrazolium assay employing MTS as colorimetric dye (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was then used according to the manufacturer's instructions. Following medium removal and three PBS washes, MTS solution (100 µl) was added to each well and incubated for 30 min at 37°C in 5% CO₂. Absorbance was measured at 490 nm using a microplate reader (SpectraMax M2, Molecular Devices, LLC, San Jose, CA, USA).

2.7. ROS production

ROS production measurements were performed using the cell-permeant fluorescent probe 2',7'-dichlorofluorescein (DCF, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Similarly to the MTS assay, cells were seeded into 96-well plates at a density of 2.04×10^4 cells per well (6.06×10^4 cells/cm²). The plates were maintained at 37°C in 5% CO₂ for 24 h to allow cell adhesion. Thereafter, the culture medium was replaced with a 20 µM DCF solution for 60 minutes. After incubation, the DCF solution was removed, and the cells were incubated with prewarmed eluates at 37°C in 5% CO₂ for an additional 24 hours. Exposure to tert-butyl hydroperoxide (TBHP, Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 100 µM was used as a positive control. Cells were analyzed using a microplate reader (SpectraMax M2, Molecular Devices, LLC, San Jose, CA, USA), with excitation and emission wavelengths set at 495 nm and 525 nm, respectively. Fluorescence intensity values were normalized to those of untreated control samples.

2.8. Statistical analysis

Statistical analyses were performed using JMP Pro 17.2.0 (SAS Institute, Cary, NC, USA). A Shapiro-Wilk test was used to test the normality of the distributions. One-way

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

ANOVA was performed in case of normal distribution followed by Tukey's test for multiple comparisons. When normality could not be verified, a Kruskal-Wallis test was performed, followed by Dunn's test for multiple comparison. The level of statistical significance was set at 0.05.

3. Results

3.1. Material properties

Before comparing DC across test conditions, a preliminary analysis was conducted to confirm the absence of a gradient between the upper and lower surfaces of the specimens in all groups, thereby excluding depth-related sample heterogeneity. No significant differences in upper-to-lower surface DC ratio were observed. Hence, only the DC values of the upper surface are presented and considered as representative of the whole sample.

A clear trend was observed when comparing material properties between CQ- and TPO-containing RBCs. Among the CQ formulations, a statistically significant decrease in all material properties was observed for CQ-0.5%NAC compared to CQ-0%NAC. The CQ-1%NAC formulation showed intermediate values for all parameters, with statistically significant differences from the other two conditions in all properties except for DC, which was comparable to that of CQ-0%NAC (Fig. 2). In contrast, TPO composites demonstrated smaller variations across all formulations (0%, 0.5%, and 1% NAC); however, some differences between groups were still statistically significant (Fig. 2). In particular, a clear trend was observed for DC, with a significant increase at higher NAC concentrations.

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

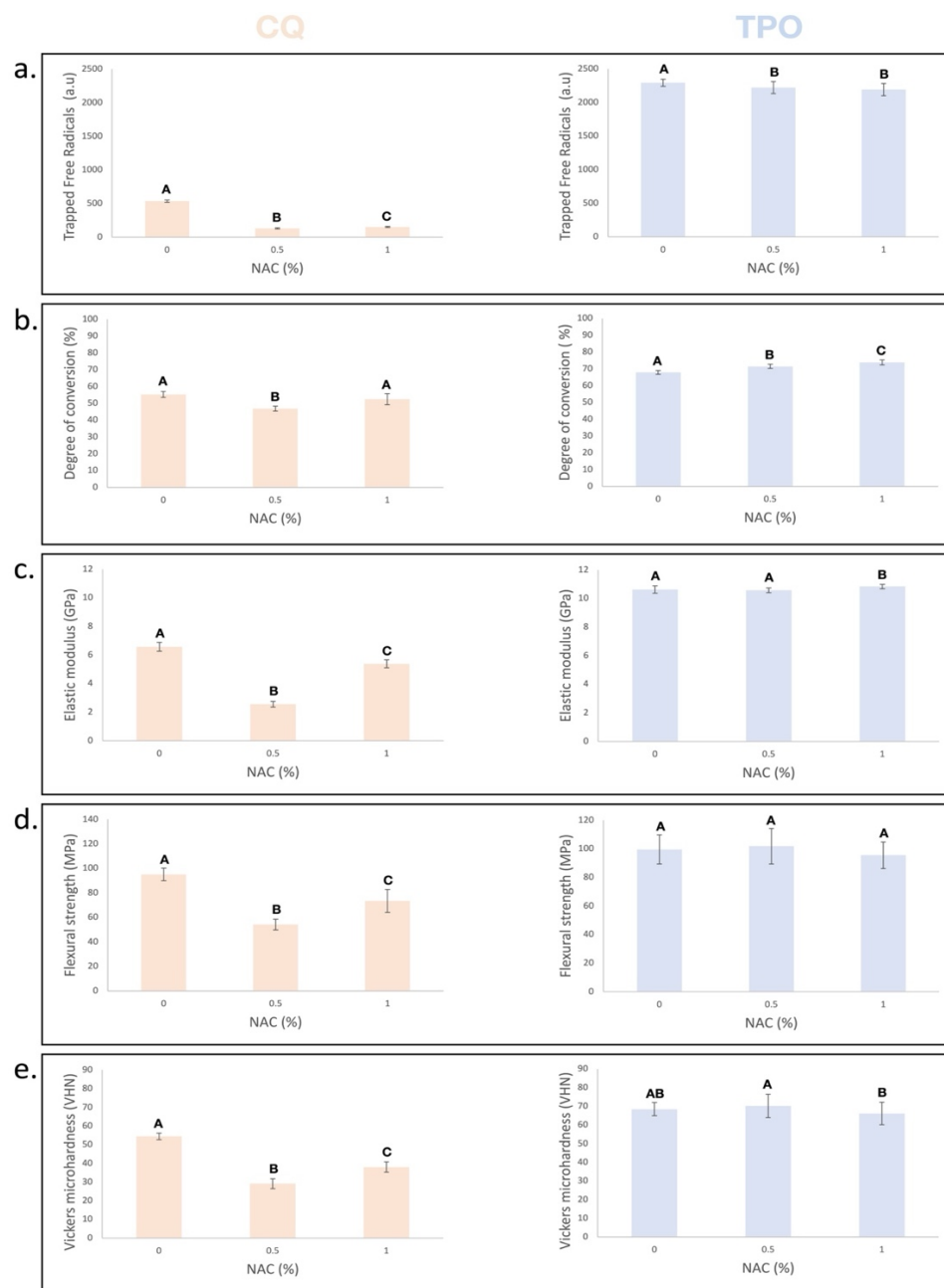


Figure 2 – Effect of NAC incorporation (0.5 or 1 %) on material properties. (a) Trapped free radicals concentration, (b) Degree of conversion, (c) Elastic modulus, (d) Flexural strength, (e) Vickers microhardness. Data are presented as means $\pm$ SD ($n = 15$). Different letters indicate statistically significant differences.

3.2. Chemical analyses

3.2.1. NAC Elution

The elution of NAC followed parallel trends in both CQ and TPO composites, with significant increases in NAC levels correlating with its content in the formulation. This increase was particularly prominent in non-polymerized composites, underscoring the higher release of NAC in unpolymerized conditions (Fig.3).

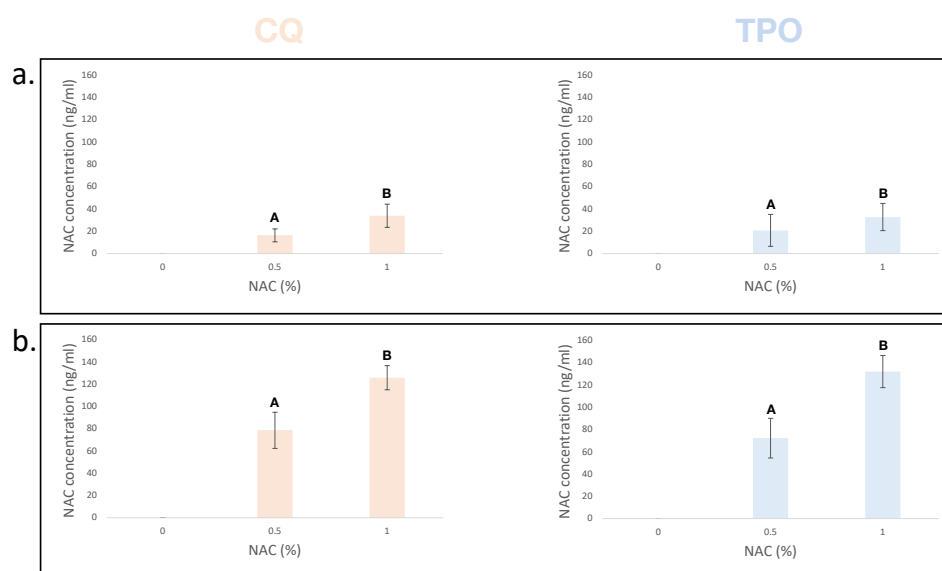


Figure 3 – Concentration of eluted NAC from CQ and TPO composites supplemented with NAC (0.5 or 1%) from (a) polymerized samples, and (b) uncured samples. Data are presented as means $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences.

3.2.2. Elution of residual monomers and photoinitiators

Regarding the elution of photoinitiators, concentrations were below the limit of quantification for RBCs containing either CQ or TPO (data not shown).

The elution of residual TEGDMA and BisGMA in polymerized RBCs was comparable for CQ-containing RBCs that contained 0% NAC and 1% NAC, whereas the specimens containing 0.5% NAC displayed a significantly higher TEGDMA release (Fig. 4a).

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

Unpolymerized composites showed a consistent trend for detection of residual monomers regardless of the type of photoinitiator: TEGDMA levels declined significantly as NAC concentration increased, whereas no significant variations in BisGMA concentrations were observed across all NAC formulations (Fig. 4b).

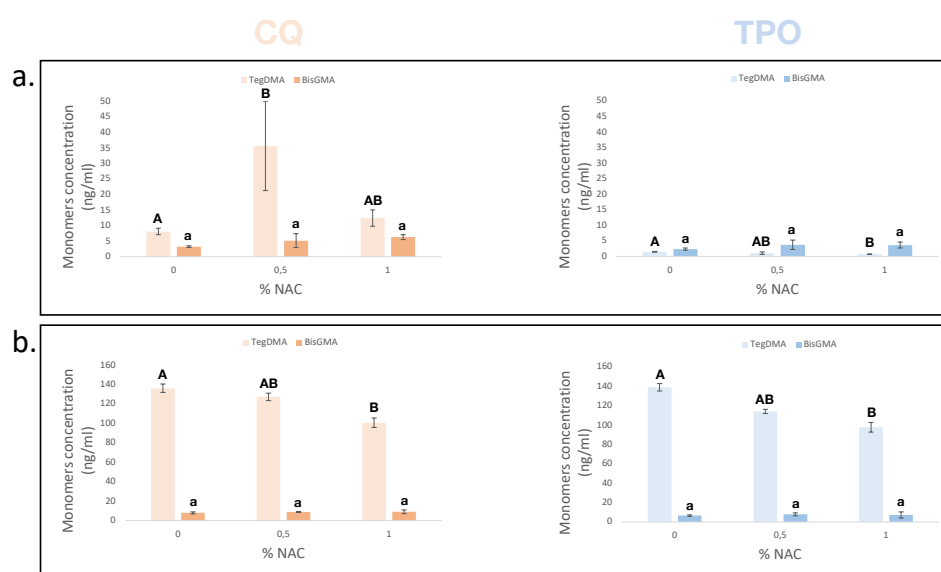


Figure 4 - Concentration of eluted monomers (BisGMA and TEGDMA) from CQ and TPO composites supplemented with NAC (0.5 or 1 %). (a) Polymerized samples, (b) Uncured samples. Data are presented as means $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences.

3.3. Biological Properties

3.3.1. Metabolic activity

Exposure of hDPC cells to eluates of polymerized composites revealed comparable levels of metabolic activity of the cells ($p > 0.05$), reaching approximately 80% relative to the control, with minimal variability across formulations – except for CQ-1%NAC, which showed a slightly higher metabolic activity ($p < 0.05$) (Fig.5.a). For non-polymerized composites, however, a substantial reduction in metabolic activity was observed, with values approximating 20% of the control. No significant differences

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

were found between the different NAC concentrations (0, 0.5 and 1%) within either CQ and TPO formulations (Fig.5.b).

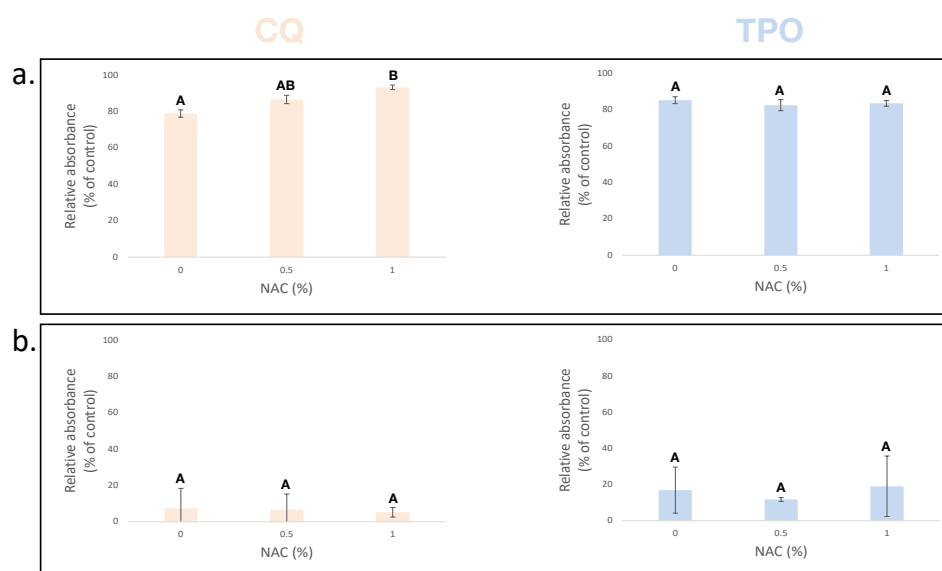


Figure 5 – Results from the MTS assay after 24h exposure presented as percentage of metabolic activity relative to the control group (cells with standard growth medium, set to 100%) for CQ and TPO composites supplemented with NAC (0.5 or 1 %) compared to 0% NAC from (a) polymerized samples, and (b) non-polymerized samples. Absorbance was measured at 490 nm using a microplate reader. Data are presented as means $\pm$ SD ($n = 4$). Different letters indicate statistically significant differences.

3.3.2. ROS production

Incubation of the hDPSCs with the composites, all NAC-supplemented composites (cured or uncured, at 0.5% or 1%) showed a statistically significant decrease in ROS levels compared to the non-supplemented ones, except for CQ-0.5%NAC(Fig.6).

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

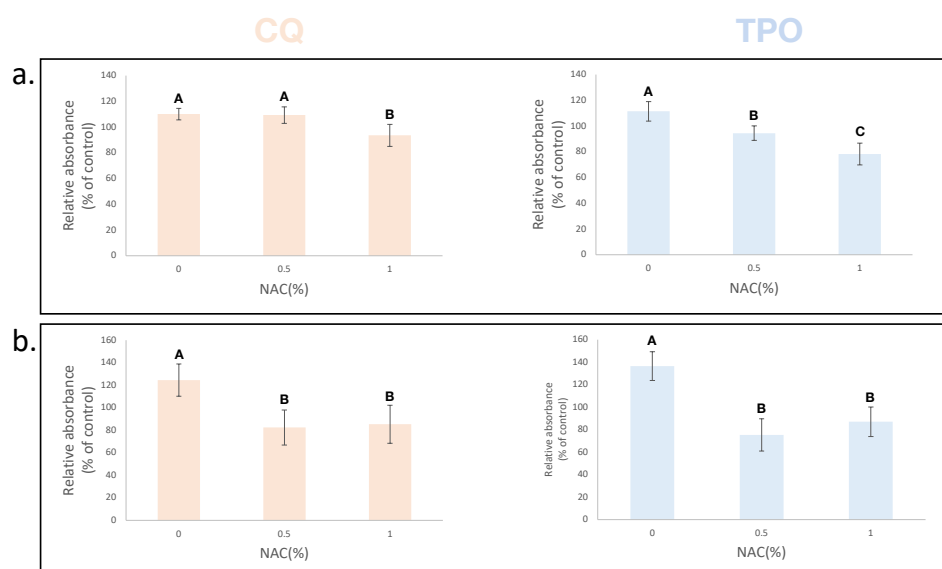


Figure 6 - Results of ROS quantification after 24h exposure presented as percentage of ROS production relative to the control group (cells with standard growth medium, set as 100) for CQ and TPO composites supplemented with NAC (0.5 or 1 %) compared to 0% NAC from (a) polymerized samples, and (b) non-polymerized samples., fluorescence was measured, using a microplate reader, with excitation and emission wavelengths set at 495 nm and 525 nm, respectively. Data are presented as means $\pm$ SD ($n = 4$). Different letters indicate statistically significant differences.

4. Discussion

Recent research has increasingly explored the incorporation of bioactive or naturally derived agents into resin-based composites to reduce their biological impact and improve cellular responses. Various antioxidant and antibacterial additives have been investigated(22, 23). However, their integration into methacrylate-based systems remains challenging, as they may interfere with free-radical polymerization and compromise material properties. Therefore, assessing both material performance and cellular response in an integrated manner is essential. Within this context, NAC, a well-known antioxidant and glutathione precursor, represents a promising candidate due to its ability to modulate oxidative stress.

The present study investigated the effects of NAC incorporation into RBCs formulated with two distinct photoinitiator systems (CQ and TPO). Under the tested conditions, NAC-supplemented RBCs significantly reduced ROS levels in all groups except CQ-0.5%NAC. However, it had no substantial impact on hDPCs metabolic activity in either the cured or uncured states – apart from a marginal yet statistically significant increase observed in the polymerized CQ-1%NAC group, which was accompanied by a compromise in key material properties. It should be noted that the biological evaluation was limited to a 24-hour exposure period, consistent with ISO 10993-5 recommendations for in vitro cytotoxicity testing. Since oxidative stress and mitochondrial dysfunction represent early cellular responses to resin-derived eluates, this time point was considered appropriate for assessing the acute effects of NAC incorporation. Nonetheless, longer incubation periods may be required to investigate delayed effects on proliferation or differentiation and should be addressed in future studies. Interestingly, although NAC incorporation did not produce significant measurable biological benefits in TPO-based composites, it underscored the critical role of the photoinitiator type in modulating the effects of NAC on the material itself. Indeed, NAC-supplemented TPO-based RBCs exhibited only minor disruptions in initiation efficiency and in the associated mechanical properties compared to their CQ-based counterparts. Moreover, NAC incorporation led to a major decrease in trapped

free radicals for CQ composites, whereas only a slight decrease was observed for TPO composites (Fig. 2a). Thiol groups, like in the NAC molecule, are known for their potential as radical scavengers (24). Therefore, the pronounced effect of NAC on CQ-based RBCs is not unexpected and is likely due to interference with the radical initiation process, as evidenced by the significant reduction in trapped free radical concentration in these formulations. These concentrations were shown to serve as an indicator of the growth centers formed during the auto-acceleration stage of polymerization (16). The present data suggest that NAC disrupted radical formation in CQ-based systems, likely by interfering with the CQ/amine photoinitiator mechanism. On the contrary, it can be hypothesised that the free electron in TPO is not as easily accessible for NAC compared to the CQ/amine, which are both comparably simple molecules. This discrepancy between the two photoinitiator systems regarding NAC's effect on free radical generation suggests a disturbance in the interaction between CQ and the amine, in this case, DMAEMA. As mentioned earlier, while TPO is a type-I photoinitiator that generates free radicals via photocleavage of a C-P bond, CQ is a type-II photoinitiator that requires a co-initiator, usually an amine (11, 12). After the CQ triplet state is formed upon photon absorption, it abstracts a proton from the amine, leading to the formation of an amine radical, which initiates polymerization (11, 25, 26). Given NAC's known ability to donate protons through its thiol group (27), it is plausible that NAC competes with the amine for proton donation, thereby diminishing the efficiency of radical initiation and, consequently, the polymerization process. As a result, NAC incorporation significantly impacted the DC, especially in CQ 0.5%, where pronounced differences were observed (Fig. 2b). This reduction in DC following NAC addition to CQ-based commercial RBCs has been previously reported, although the effect appears to be material-dependent (10). Surprisingly, this reduction in DC was less pronounced in CQ-1%NAC (Fig. 2b). This could be explained by the contribution of the thiol itself (as thiyl radicals) to the initiation of polymerization via a thiol-ene mechanism (28). Thiol-ene polymerization, which can be initiated by light or heat, proceeds via initiation, propagation, chain transfer, and termination steps, similar to conventional radical polymerization, and can occur in parallel. Additionally, it is largely unaffected by

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

environmental factors such as oxygen or humidity. Likewise, NAC incorporation into TPO-based formulations resulted in a slight but significant increase in DC (Fig. 2b), which may also be attributed to the thiol-ene mechanism. This finding represents a major outcome of this study, as it has not been previously reported and could open new perspectives for incorporating antioxidant molecules into RBCs.

As reviewed by Leprince et al. (2013) (3), DC is a critical material property, positively correlated with mechanical characteristics such as microhardness, elastic modulus, and flexural strength (18, 29-33), and negatively correlated with monomer elution (4, 30, 34). In the present study, observed trends in microhardness, modulus, flexural strength, and TEGDMA elution were consistent with the impact of NAC on DC.

It is well-established that DC in dimethacrylate-based composites typically remains well below 100% at ambient or oral temperature, even under optimal irradiation, due to vitrification, which limits further polymerization (Leprince et al. 2013). As polymerization progresses, the glass transition temperature of the material increases (35), eventually approaching the curing temperature (slightly above oral temperature) (36, 37). At this point, vitrification occurs, drastically reducing molecular mobility and thereby arresting the reaction, aside from limited post-polymerization that may continue for up to 24 hours (16, 38, 39). This physical restriction on monomer-to-polymer conversion leaves unreacted components (primarily monomers and initiators) prone to elution. Accordingly, as mentioned above, a lower DC is typically associated with greater monomer release.

In the present work, cured CQ-based composites exhibited higher TEGDMA and Bis-GMA elution than their TPO-based counterparts, consistent with previous reports (14, 40). The highest TEGDMA release was observed in the CQ composite containing 0.5% NAC, which also exhibited the lowest DC, further confirming the inverse correlation between DC and monomer elution. In contrast, no significant differences in Bis-GMA elution were observed among the various NAC formulations, despite differences in DC. This may suggest a saturation effect, potentially resulting from a combination of factors, including the limited molecular mobility of Bis-GMA within the cured matrix and

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

its low solubility in aqueous media, as elegantly demonstrated by Meng et al. (2007) (41). Regarding (co-)initiator components, their concentrations remained below the quantification limits in all samples and are therefore not discussed further.

As for biological effects, the eluted methacrylate monomers, TEGDMA and Bis-GMA in the present study, are known to increase oxidative stress and have been associated with a reduced GSH level (7). This rise in oxidative stress has been reported to induce DNA damage, cell cycle arrest, and cell death (5, 7).

To counteract monomer toxicity arising from oxidative stress, NAC was incorporated into the composite formulations. In agreement with findings by Yang et al. (10), NAC elution from the RBCs in this study was dose-dependent in both CQ- and 2,4,6-trimethylbenzoyl diphenylphosphine oxide (TPO)-based composites (Fig. 3), confirming that the experimental materials were capable of releasing increasing NAC concentrations into the medium.

The antioxidant property of NAC is linked to its capacity to directly scavenge ROS. The associated significant reduction of ROS levels at 24 h observed with NAC-supplemented composites (Fig. 6) confirms that the released NAC concentrations were sufficient to exert an antioxidant effect, albeit without any significant change in cell metabolic activity under most conditions, as will be discussed below.

Regarding metabolic activity of cells exposed to eluates of cured composites, levels were comparable for both CQ- and TPO-based RBCs, except for CQ-1%NAC, which showed a slight increase. However, even though metabolic activity remained relatively stable, they consistently remained below control levels (approximately 80%) and this despite notable differences in eluted monomers across all tested formulations.

At first glance, this reduction might suggest cytotoxicity. However, the MTS assay, which measures mitochondrial SDH activity, does not exclusively signal cell death. Rather, it can also reflect slowed proliferation or cell cycle arrest (20). This is important to consider, since earlier works have shown that methacrylate monomers can elicit a

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

variety of cellular responses, from cell cycle arrest to apoptosis and necrosis (42-44). As discussed above, any of these mechanisms could contribute to reduce MTS signals.

In our study, metabolic activity remained at approximately 80%, without a significant increase in ROS levels (CQ-0.5%NAC), or even with a significant decrease (CQ-1%NAC, TPO-0.5%NAC and TPO-1%NAC). Since the monomer concentrations are far below those reported to induce measurable cell death (45), the reduction in MTS signal is unlikely due to a cytotoxic effect. Instead, sub-toxic monomer levels are more likely to induce cell cycle arrest or inhibit proliferation (46).

This observation raises a broader question about how methacrylates, here TEGDMA and Bis-GMA, exert their genotoxic effects. While oxidative stress is widely recognized as a central mechanism, it remains unclear whether these monomers, or their metabolites, may also form direct covalent adducts with DNA. Such a mechanism would help explain DNA damage occurring independently of oxidative stress. This interpretation aligns with the findings of Volk et al. (47) who reported that TEGDMA caused DNA damages without a corresponding increase in oxidative stress markers. Moreover, a study by Ansteinsson et al. (48) showed that the antioxidant trolox did not counteract the HEMA-induced cell-cycle arrest, which indicates that the DNA damage is of non-oxidative origin. Moreover, the findings reported by Chang et al. (2010) (49) further support this view, demonstrating that Bis-GMA exposure reduced SDH activity, like in the present work, and induced cell cycle arrest, without a corresponding increase in ROS production.

Regarding CQ-1%NAC formulation (Fig. 5), we observed an increase in MTS signal, accompanied by a significant reduction in ROS levels (Fig. 6). This supports the idea that NAC can exert beneficial biological effects (9), although such effects appear to be limited to very specific conditions. As noted by Schweikl et al. (2006), the molecular mechanisms by which NAC influences various redox-sensitive systems are complex and not yet fully understood (7). Moreover, as previously mentioned, this modest improvement in cell metabolic activity came at the cost of a significant reduction in key

mechanical properties (Fig. 2), further underscoring the need for an integrated evaluation that considers both biological and material performance.

While cured composites provide insight into typical clinical performance under proper usage conditions, uncured materials represent a worst-case exposure scenario—such as when excessive composite thickness prevents adequate polymerization at depth. To our knowledge, the potential of NAC to mitigate the biological impact of inadequate polymerization has not been previously investigated. Therefore, the following section focuses on the biological impact of uncured RBCs. These materials exhibited significantly higher monomer elution than their cured counterparts, resulting in a dramatic reduction in cell viability (~10% of control). In both CQ- and TPO-based formulations, NAC incorporation at 0.5% and 1% concentrations led to a decrease in TEGDMA elution (approximately 30 % for 1%NAC formulations). In addition to its antioxidative properties, NAC could form chemical adducts with most commonly used methacrylate monomers, with the exception of Bis-GMA in both cell and cell free systems(50, 51), thereby potentially reducing their bioavailability. In both CQ- and TPO-based formulations, NAC incorporation at 0.5% and 1% concentrations led to a significant decrease in detectable TEGDMA levels in eluates from uncured composites, while Bis-GMA concentrations remained unchanged (Fig. 5b). This finding indicates that the presence of NAC was associated with a reduced availability of TEGDMA in the extraction medium. The present study did not directly investigate adduct formation or DNA-related effects. Therefore, the observed reduction in TEGDMA concentrations should be interpreted as an analytical outcome of NAC incorporation rather than as evidence of monomer detoxification via covalent binding or DNA protection. Further dedicated chemical and biological analyses would be required to elucidate the underlying mechanisms responsible for this reduction.

Like in cured RBCs, NAC incorporation in uncured materials resulted in a significant reduction in ROS production, even below control level (fig.6). Yet, despite a significantly reduced TEGDMA bioavailability and ROS production, NAC addition did not significantly improve cell viability in uncured composites compared to NAC-free

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties and Cellular Toxicity of Resin-Based Composites

controls. This may be partly explained by the low NAC concentrations released into the medium, which averaged between approximately 70 and 130 ng/mL and therefore not sufficient to form enough covalent adducts to lead to an increase in the measured metabolic activity. Despite incorporating the maximum amount of NAC that could be stably integrated into the RBCs, the released concentrations remained two to three orders of magnitude lower than those used in our previous work (8), where 4 mM NAC, the highest non-toxic concentration, was directly added to the eluates. In that study, we showed a beneficial effect of NAC on hDPC viability. However, even under those conditions, NAC was not sufficient to completely prevent monomer-induced cytotoxicity in most tested materials.

Our observations from uncured RBCs confirm that reducing ROS production alone is insufficient to mitigate monomer-induced cytotoxicity under the present conditions, indicating the involvement of additional cytotoxic mechanisms, presumably the direct interaction of monomers with DNA as discussed above.

Although not part of the initial objectives of this study, our findings highlight the importance of considering cytotoxic pathways beyond oxidative stress.

5. Conclusion

Incorporation of NAC into resin-based composites interfered with free-radical initiation and polymerization efficiency in camphorquinone-based systems, resulting in reduced degree of conversion, inferior mechanical properties, and increased monomer elution. These effects were largely absent in TPO-based formulations, where polymerization was preserved.

Although NAC-supplemented composites significantly reduced ROS production, this effect did not translate into a consistent improvement in human dental pulp cell metabolic activity under the experimental conditions used. These findings suggest that optimizing polymerization efficiency and minimizing monomer release remain more effective strategies for improving short term cytocompatibility than incorporating antioxidant additives. Nevertheless, future studies should explore longer-term cellular responses and additional biological endpoints, including the assessment of DNA double-strand breaks, as previous studies have shown that NAC at concentrations as low as 50 μ M can reduce genotoxic damage induced by methacrylate monomers(52), particularly in photoinitiator systems that maintain polymerization performance.

6. References

1. Ferracane JL. Resin composite--state of the art. *Dent Mater.* 2011;27(1):29-38.
2. Ilie N, Hickel R. Resin composite restorative materials. *Aust Dent J.* 2011;56 Suppl 1:59-66.
3. Leprince JG, Palin WM, Hadis MA, Devaux J, Leloup G. Progress in dimethacrylate-based dental composite technology and curing efficiency. *Dent Mater.* 2013;29(2):139-56.
4. Ferracane JL. Elution of leachable components from composites. *J Oral Rehabil.* 1994;21(4):441-52.
5. Samuelsen JT, Dahl JE. Biological aspects of modern dental composites. *Biomater Investig Dent.* 2023;10(1):223223.
6. Fernandez-Checa JC, Garcia-Ruiz C, Colell A, Morales A, Mari M, Miranda M, et al. Oxidative stress: role of mitochondria and protection by glutathione. *Biofactors.* 1998;8(1-2):7-11.
7. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. *J Dent Res.* 2006;85(10):870-7.
8. Gilli M, Hollaert TG, Setbon HM, des Rieux A, Leprince JG. Quality of Cure in Depth of Commercially Available Bulk-fill Composites: A Layer-by-layer Mechanical and Biological Evaluation. *Oper Dent.* 2022;47(4):437-48.
9. Yang Y, Wang L, Huang Z, Ge L, Shi J. N-acetylcysteine as a novel methacrylate-based resin cement component: effect on cell apoptosis and genotoxicity in human gingival fibroblasts. *BMC Oral Health.* 2024;24(1).
10. Yang Y, Reichl F-X, Ilie N, Shi J, Dhein J, Hickel R, et al. Antioxidants as a novel dental resin-composite component: Effect on elution and degree of conversion. *Dent Mater.* 2019;35(4):650-61.
11. Cook WD. Photopolymerization Kinetics of Dimethacrylates Using the Camphorquinone Amine Initiator System. *Polymer.* 1992;33(3):600-9.
12. Neumann MG, Schmitt CC, Ferreira GC, Correa IC. The initiating radical yields and the efficiency of polymerization for various dental photoinitiators excited by different light curing units. *Dent Mater.* 2006;22(6):576-84.
13. Hardy CMF, Bebelman S, Leloup G, Hadis MA, Palin WM, Leprince JG. Investigating the limits of resin-based luting composite photopolymerization through various thicknesses of indirect restorative materials. *Dent Mater.* 2018;34(9):1278-88.
14. Randolph LD, Palin WM, Bebelman S, Devaux J, Gallez B, Leloup G, et al. Ultra-fast light-curing resin composite with increased conversion and reduced monomer elution. *Dent Mater.* 2014;30(5):594-604.
15. Leprince J, Lamblin G, Truffier-Boutry D, Demoustier-Champagne S, Devaux J, Mestdagh M, et al. Kinetic study of free radicals trapped in dental resins stored in different environments. *Acta Biomaterialia.* 2009;5(7):2518-24.
16. Leprince JG, Lamblin G, Devaux J, Dewaele M, Mestdagh M, Palin WM, et al. Irradiation modes' impact on radical entrapment in photoactive resins. *J Dent Res.* 2010;89(12):1494-8.

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties
and Cellular Toxicity of Resin-Based Composites

17. Truffier-Boutry D, Gallez XA, Demoustier-Champagne S, Devaux J, Mestdagh M, Champagne B, et al. Identification of free radicals trapped in solid methacrylated resins. *Journal of Polymer Science Part A: Polymer Chemistry*. 2003;41(11):1691-9.
18. Leprince JG, Leveque P, Nysten B, Gallez B, Devaux J, Leloup G. New insight into the "depth of cure" of dimethacrylate-based dental composites. *Dent Mater*. 2012;28(5):512-20.
19. Randolph LD, Palin WM, Watts DC, Genet M, Devaux J, Leloup G, et al. The effect of ultra-fast photopolymerisation of experimental composites on shrinkage stress, network formation and pulpal temperature rise. *Dent Mater*. 2014;30(11):1280-9.
20. Standardization IOF. ISO 10993-5:2009. Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity. Geneva: ISO; 2009.
21. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, et al. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. *Cell Tissue Res*. 2013;353(1):65-78.
22. Makvandi P, Jamaledin R, Jabbari M, Nikfarjam N, Borzacchiello A. Antibacterial quaternary ammonium compounds in dental materials: A systematic review. *Dent Mater*. 2018;34(6):851-67.
23. Cherchali FZ, Attik N, Issaadi HM, Abouelleil H, Decoret D, Amirouche-Korichi A, et al. Integration of various forms of *Salvadora Persica* << Siwak >> into dental composites: A pilot in vitro study. *Dent Mater*. 2025;41(9):1120-30.
24. Noguchi N, Saito Y, Niki E. Actions of Thiols, Persulfides, and Polysulfides as Free Radical Scavenging Antioxidants. *Antioxid Redox Signal*. 2023;39(10-12):728-43.
25. Chen YC, Ferracane JL, Pahl SA. Quantum yield of conversion of the photoinitiator camphorquinone. *Dent Mater*. 2007;23(6):655-64.
26. Jakubiak J, Allonas X, Fouassier JP, Sionkowska A, Andrzejewska E, Linden LA, et al. Camphorquinone–amines photoinitiating systems for the initiation of free radical polymerization. *Polymer*. 2003;44(18):5219-26.
27. Zhitkovich A. N-Acetylcysteine: Antioxidant, Aldehyde Scavenger, and More. *Chem Res Toxicol*. 2019;32(7):1318-9.
28. Machado TO, Sayer C, Araujo PHH. Thiol-ene polymerisation: A promising technique to obtain novel biomaterials. *Eur Polym J*. 2017;86:200-15.
29. Lovell LG, Newman SM, Donaldson MM, Bowman CN. The effect of light intensity on double bond conversion and flexural strength of a model, unfilled dental resin. *Dent Mater*. 2003;19(6):458-65.
30. Petronijevic Sarcev B, Balos S, Markovic D, Sarcev I, Vukcevic M, Labus Zlatanovic D, et al. Effect of the Degree of Conversion on Mechanical Properties and Monomer Elution from Self-, Dual- and Light-Cured Core Composites. *Materials (Basel)*. 2021;14(19).
31. Li J, Li H, Fok AS, Watts DC. Multiple correlations of material parameters of light-cured dental composites. *Dent Mater*. 2009;25(7):829-36.
32. Ferracane JL. Correlation between hardness and degree of conversion during the setting reaction of unfilled dental restorative resins. *Dent Mater*. 1985;1(1):11-4.

Effect of N-acetylcysteine Incorporation on the Chemo-Mechanical Properties
and Cellular Toxicity of Resin-Based Composites

33. Ferracane JL, Greener EH. The effect of resin formulation on the degree of conversion and mechanical properties of dental restorative resins. *J Biomed Mater Res*. 1986;20(1):121-31.
34. Pongprueksa P, Miletic V, Janssens H, Van Landuyt KL, De Munck J, Godderis L, et al. Degree of conversion and monomer elution of CQ/amine and TPO adhesives. *Dent Mater*. 2014;30(6):695-701.
35. Lecamp L, Youssef B, Bunel C, Lebaudy P. Photoinitiated polymerization of a dimethacrylate oligomer: 1. Influence of photoinitiator concentration, temperature and light intensity. *Polymer*. 1997;38(25):6089-96.
36. Dewaele M, Asmussen E, Peutzfeldt A, Munksgaard EC, Benetti AR, Finne G, et al. Influence of curing protocol on selected properties of light-curing polymers: degree of conversion, volume contraction, elastic modulus, and glass transition temperature. *Dent Mater*. 2009;25(12):1576-84.
37. Lovell LG, Lu H, Elliott JE, Stansbury JW, Bowman CN. The effect of cure rate on the mechanical properties of dental resins. *Dent Mater*. 2001;17(6):504-11.
38. Dewaele M, Truffier-Boutry D, Devaux J, Leloup G. Volume contraction in photocured dental resins: the shrinkage-conversion relationship revisited. *Dent Mater*. 2006;22(4):359-65.
39. Truffier-Boutry D, Demoustier-Champagne S, Devaux J, Biebuyck JJ, Mestdagh M, Larbanois P, et al. A physico-chemical explanation of the post-polymerization shrinkage in dental resins. *Dent Mater*. 2006;22(5):405-12.
40. Randolph LD, Steinhaus J, Möglinger B, Gallez B, Stansbury J, Palin WM, et al. Photopolymerization of highly filled dimethacrylate-based composites using Type I or Type II photoinitiators and varying co-monomer ratios. *Dent Mater*. 2016;32(2):136-48.
41. Meng J, Yang H, Cao M, Li L, Cai Q. Correlating cytotoxicity to elution behaviors of composite resins in term of curing kinetic. *Mater Sci Eng C Mater Biol Appl*. 2017;78:413-9.
42. Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R. Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. *Dent Mater*. 2007;23(1):34-9.
43. Samuelsen JT, Holme JA, Becher R, Karlsson S, Morisbak E, Dahl JE. HEMA reduces cell proliferation and induces apoptosis in vitro. *Dent Mater*. 2008;24(1):134-40.
44. Spagnuolo G, D'Antò V, Valletta R, Strisciuglio C, Schmalz G, Schweikl H, et al. Effect of 2-hydroxyethyl methacrylate on human pulp cell survival pathways ERK and AKT. *J Endod*. 2008;34(6):684-8.
45. Reichl FX, Esters M, Simon S, Seiss M, Kehe K, Kleinsasser N, et al. Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts. *Arch Toxicol*. 2006;80(6):370-7.
46. Chang HH, Chang MC, Huang GF, Wang YL, Chan CP, Wang TM, et al. Effect of triethylene glycol dimethacrylate on the cytotoxicity, cyclooxygenase-2 expression and prostanooids production in human dental pulp cells. *Int Endod J*. 2012;45(9):848-58.
47. Volk J, Leyhausen G, Geurtsen W. Glutathione level and genotoxicity in human oral keratinocytes exposed to TEGDMA. *J Biomed Mater Res B Appl Biomater*. 2012;100(2):391-9.

48. Ansteinsson V, Solhaug A, Samuelsen JT, Holme JA, Dahl JE. DNA-damage, cell-cycle arrest and apoptosis induced in BEAS-2B cells by 2-hydroxyethyl methacrylate (HEMA). *Mutat Res.* 2011;723(2):158-64.
49. Chang MC, Chen LI, Chan CP, Lee JJ, Wang TM, Yang TT, et al. The role of reactive oxygen species and hemoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by BisGMA. *Biomaterials.* 2010;31(32):8164-71.
50. Spagnuolo G, Desiderio C, Riviaccio V, Amato M, Rossetti DV, D'Anto V, et al. In vitro cellular detoxification of triethylene glycol dimethacrylate by adduct formation with N-acetylcysteine. *Dent Mater.* 2013;29(8):e153-60.
51. Ansteinsson V, Kopperud HB, Morisbak E, Samuelsen JT. Cell toxicity of methacrylate monomers-the role of glutathione adduct formation. *J Biomed Mater Res A.* 2013;101(12):3504-10.
52. Yang Y, He X, Shi J, Hickel R, Reichl FX, Hogg C. Effects of antioxidants on DNA double-strand breaks in human gingival fibroblasts exposed to dental resin co-monomer epoxy metabolites. *Dent Mater.* 2017;33(4):418-26.

Discussion and Perspectives

The central question that underpins this thesis is: *how can the biological effects of RBCs be minimized without compromising their chemo-mechanical performance?* This question arises from the inherent paradox of composites: while they are indispensable in modern restorative dentistry due to their esthetics, adhesion, and versatility, their incomplete polymerization and elution of residual compounds raise concerns for pulp health and long-term biocompatibility.

Two complementary strategies exist to address this challenge. The first focuses on intrinsic modifications of the material itself, modifying resin chemistry, photoinitiator systems, polymerization kinetics, or filler technology to reduce residual monomer release and improve network stability. The second involves extrinsic strategies, including the use of additives such as antioxidants (e.g., NAC), optimization of clinical handling protocols, or protective measures designed to mitigate cytotoxic effects after placement.

This discussion critically evaluates both approaches. It highlights how advances in bulk-fill formulations and photoinitiator systems aim to improve depth of cure and reduce biological risks, while also considering the potential and limitations of adjunctive strategies like antioxidant incorporation. Importantly, it situates these laboratory findings against the reality of clinical practice, where material behavior is shaped by complex and variable conditions. By acknowledging the gap between in vitro evidence and in vivo outcomes, this analysis seeks to define not only what is scientifically achievable but also what is clinically meaningful in the pursuit of safer, high-performance RBCs.

7. Material Properties and Intrinsic Modifications

7.1. Degree of conversion

As discussed in Chapter VI, the DC rarely exceeds 70%, leaving a persistent reservoir of reactive groups. Higher DC correlates with improved hardness, wear resistance, and reduced elution. From the biological perspective, this is highly relevant: Chapter VII demonstrated that residual monomers such as BisGMA and TEGDMA diffuse and can induce oxidative stress and mitochondrial dysfunction even at sub-micromolar concentrations. Thus, improving DC is not merely a goal regarding mechanical performance but a biological imperative.

One pathway toward higher conversion lies in the choice of initiator system. The traditional CQ/amine pair, while robust, is limited in efficiency. In contrast, Type I initiators such as TPO generate a higher radical yield per absorbed photon, often achieving greater polymerization efficiency at lower concentrations. In theory, this reduces the amount of residual initiator available for elution and lowers the overall pool of unreacted monomers. From a practical standpoint, these photoinitiators necessitate activation by light of a different wavelength, for which most dental practices are not yet adequately equipped. Although some recently developed curing units incorporate polywave technology, this limitation continues to hinder widespread clinical adoption. Beyond this, violet light required for TPO penetration is attenuated more rapidly than blue light, risking under-curing in deep increments and paradoxically increasing monomer release.

From a regulatory perspective, TPO has been progressively restricted and ultimately banned in the European Union due to concerns related to its classification as a substance of potential reproductive toxicity. Nevertheless, other Type I photoinitiators, including monoacylphosphine oxide (MAPO), bisacylphosphine oxide (BAPO), and germanium-based initiators such as Ivocerin®, remain authorized and are currently used in commercial materials, including bulk-fill composites such as Tetric PowerFill.

In this context, experimental models based on Type I initiation systems remain scientifically relevant, as they reflect both existing and emerging clinical formulations.

Additional strategies have also been proposed to enhance conversion. Pre-heating of composite resins prior to placement increases monomer mobility, delays vitrification, and facilitates a more complete reaction (1). This technique has been shown to yield higher DC and improved crosslinking without altering the formulation, offering a simple chairside adjunct to reduce residual monomer release. On a more fundamental level, thiol-ene polymerization systems provide an alternative to traditional dimethacrylate chain-growth reactions. As a result, thiol-ene systems could enhance double-bond conversion and substantially limit the pool of leachable compounds that underpin the cytotoxicity (2). However, despite their biological promise, thiol-ene composites remain experimental and face hurdles in formulation stability and clinical handling.

In short, initiator innovation, optimized curing strategies, and alternative polymerization chemistries all contribute to higher DC and reduced leachable content. Yet these approaches are constrained by practical considerations such as equipment availability, stress development, and compatibility with existing materials. Gains in conversion must therefore be critically balanced against mechanical reliability and clinical feasibility.

7.2. Filler phase

Another strategy to reduce the biological impact of composites lies in modifying the filler phase. As highlighted in Chapter VI, fillers occupy the majority of composite volume and play a central role in determining both mechanical and physicochemical properties. Increasing filler loading decreases the proportion of resin matrix, thereby reducing the overall reservoir of unreacted methacrylate monomers available for

elution. This has clear biological relevance, since Chapter VII showed that eluates from the organic matrix are the primary contributors to toxicity.

However, the benefits of increasing filler content are limited by important practical constraints. Excessive filler loading substantially raises paste viscosity, impairing the clinician's ability to adapt the material to cavity walls (3). It also increases light scattering, particularly when particle size is heterogeneous, leading to reduced depth of cure and paradoxically lower DC in deeper layers (4). These trade-offs explain why filler content in commercial composites typically remains below 80 wt%, despite the theoretical benefits of further increasing loading.

Last developments have sought to overcome these limitations not by simply raising filler fraction, but by optimizing filler architecture. Nanohybrids and nanoclusters improve packing density while maintaining workable viscosity, allowing high filler loading without compromising handling (3). Taken together, these innovations illustrate that the biological advantage of fillers derives less from maximizing content than from achieving an optimized distribution and stable interface.

7.3. Alternative monomers

A more radical avenue for reducing the biological impact of RBCs is the substitution of the methacrylate backbone itself. As described in Chapter VII, methacrylate chemistry underpins nearly all current formulations but is intrinsically limited by incomplete polymerization and susceptibility to hydrolytic degradation. Chapter VIII further showed that the major dimethacrylates, BisGMA, UDMA, TEGDMA, and HEMA, differ in cytotoxic potential but all display measurable adverse effects *in vitro*, particularly through oxidative stress and genotoxicity.

Several attempts have been made in order to overcome these problems through the development of new monomer systems, including the so-called expanding monomers

based on epoxide systems (oxiranes, siloranes) that set via cationic polymerization, and multifunctional hyper-branched methacrylic monomers (dendrimers) as alternatives to the conventional dimethacrylic formulation.

- Epoxide systems (oxiranes, siloranes) cure via cationic ring-opening, producing highly crosslinked and hydrophobic networks with reduced shrinkage and water sorption (5). Studies on silorane-based composites confirmed lower solubility, reduced elution, and equivalent or diminished cytotoxicity compared with methacrylate controls (6, 7). Nonetheless, eluates were still detectable under organic solvent storage, and the long-term biological effects of their initiator systems are not fully understood (8).
- Hyper-branched methacrylic monomers, characterized by their dendritic architecture, introduce multiple reactive methacrylate terminals into a single molecule, facilitating the formation of highly crosslinked and homogeneous networks that may reduce residual monomer content and elution. However, even after heat-assisted post-polymerization (15min, 120°C; which is clearly not clinically realistic), DC plateaued around 62–65%, leaving some peripheral groups unreacted and potentially hydrolysis-prone (9). Their high molecular weight could inherently limit diffusion and toxicity, though available data—limited to in vitro biocompatibility assays—are insufficient to fully assess safety (10).

Another promising strategy is thiol–ene polymerization, which differs from both chain-growth methacrylates and cationic systems by following a step-growth mechanism. This allows near-complete double-bond conversion and produces homogeneous, stress-relieved networks. Laboratory studies indicate significantly lower residual monomer release which could potentially lead to reduced cytotoxic and genotoxic effects compared with BisGMA or TEGDMA-based systems (11).

Despite these advances, none of these alternative systems have achieved durable commercial success. Their obstacles are broadly shared: (i) clinical handling problems such as excessive viscosity, or altered working time; (ii) compatibility issues with existing adhesive systems and curing units, which would require dentists to invest in new techniques or equipment; (iii) economic barriers, since the cost of synthesizing and producing these novel monomers is significantly higher than that of conventional dimethacrylates; and (iv) regulatory uncertainty, because the long-term toxicity of novel monomers and their degradation products is not yet sufficiently characterized. Taken together, these barriers explain why conventional methacrylate monomers, despite their imperfections, continue to dominate restorative dentistry: they represent a robust compromise between reactivity, mechanical performance, affordability, and clinical usability.

8. Additives and Protective Strategies

While intrinsic modifications of the composite structure aim to reduce the formation of residual monomers, an alternative approach is to counteract the biological consequences of those molecules once they have diffused into surrounding tissues. As detailed previously, the cytotoxicity of monomers is strongly linked to the induction of oxidative stress, depletion of glutathione, and mitochondrial dysfunction in pulp-derived cells. This has led to growing interest in the incorporation or application of antioxidants as supplementary strategies to mitigate composite-induced toxicity.

8.1. Antioxidants incorporated into resins

Experimental studies have incorporated antioxidants such as NAC and quercetin directly into adhesives or composite matrices (12, 13). These agents can neutralize ROS, restore intracellular glutathione levels, and prevent apoptosis, thereby directly targeting the mechanisms of cytotoxicity described in Chapter VIII.

However, as Chapter VII highlighted, the polymerization of dimethacrylates is highly dependent on radical balance. The addition of free radical scavengers carries the risk

of interfering with the CQ/amine photoinitiator system, lowering the DC, and paradoxically increasing the pool of residual monomers. This creates a delicate trade-off between antioxidant protection and adequate curing.

Among the antioxidants studied, NAC shows the most favorable profile. Beyond its ROS-scavenging properties, NAC possesses a free thiol group that can covalently bind to residual methacrylate monomers, thereby reducing their bioavailability. This dual action is particularly relevant in the context of Chapter VIII, where cytotoxicity was shown to depend not only on oxidative stress but also on the direct reactivity of monomers with cellular components.

This is particularly relevant because, as demonstrated in Chapter XI, well-formulated composites that are adequately polymerized do not necessarily trigger oxidative stress when their eluates are brought into contact with cells. Instead, the predominant biological effect observed was a reduction in cellular metabolic activity without concomitant ROS generation. These findings suggest that limiting the bioavailability of residual monomers may be a more effective strategy than exclusively targeting oxidative pathways. In this context, NAC emerges as a promising adjunct, as it can both scavenge ROS and neutralize unreacted methacrylates, thereby addressing the two principal toxicological mechanisms identified in Chapters VII and IVII. Nonetheless, more detailed investigations, particularly at sub-toxic concentrations and over extended periods, are required to elucidate the broader spectrum of toxicity mechanisms beyond oxidative stress.

8.2. External antioxidant treatments

Another route is the application of antioxidants externally, before composite placement. Antioxidant pretreatments, especially sodium ascorbate, have been widely studied in bleaching and endodontic contexts. They effectively neutralize residual free radicals generated before adhesive application, restoring polymerization and bond strength (14, 15).

In chapter X, a recovery of cellular viability was observed following the addition of NAC to the culture medium. However, this effect cannot be directly attributed to a reduction in reactive oxygen species, as oxidative stress markers were not quantified in that experimental setting. Consequently, the underlying mechanisms responsible for the observed recovery remain speculative within the scope of the present study. Nevertheless, previous investigations have demonstrated that NAC is capable of reducing intracellular oxidative stress and restoring redox balance in pulp-derived cells exposed to resin monomers, supporting a potential link between antioxidant activity and improved cellular outcomes(16).

While these findings are not directly related to resin composite toxicity, where free radicals and monomers are leached after polymerization, they illustrate a strategy that could potentially be adapted to mitigate the biological effects of composite eluates.

9. Clinical Mitigation: Residual Dentin and Operative Conditions

In addition to material design, clinical practice plays a decisive role in shaping the biological impact of RBCs. As discussed in Chapter VIII, the diffusion of residual monomers through dentin is a central pathway by which pulpal cells are exposed to toxic substances. The extent of this diffusion, and thus the biological risk, is strongly modulated by operative conditions.

9.1. Residual dentin thickness

A thicker dentin barrier reduces the permeability of monomers and degradation products toward the pulp chamber. Numerous in vitro perfusion studies have

demonstrated that residual dentin thickness is inversely correlated with the cytotoxic effects of composite eluates on pulp cells (17, 18). This protective effect reflects both the increased diffusion distance and the intrinsic buffering capacity of dentin. However, this protection is not absolute. Dentin permeability varies according to tubule density, sclerosis, and the presence of caries, meaning that two cavities of equal depth may differ substantially in their ability to block monomer penetration. In young patients with wide tubules or in presence of demineralized dentin due to the carious process, residual dentin may provide a weaker barrier compared with older, more sclerotic dentin. Importantly, most available studies have focused on short-term cytotoxicity endpoints, whereas subtle alterations in cellular function may occur at exposure levels that do not compromise cell viability. These sub-toxic effects, which are more likely to reflect clinical exposure scenarios, may nonetheless interfere with regenerative processes within the dentin-pulp complex. Consequently, future studies should aim to combine dentin barrier models with functional assays targeting pulp cell behavior, in order to better assess the biological relevance of residual dentin thickness beyond its role as a purely physical diffusion barrier.

9.2. Use of liners

To enhance pulpal protection in deep cavities, liners such as calcium hydroxide or calcium silicate cements have been recommended after caries excavation (19). These materials act as both physical and chemical barriers between the composite restoration and the pulp, and possess intrinsic abilities to stimulate positive responses from pulp tissues, including the release of bioactive ions (20), upregulation of growth factors such as TGF- β 1 (21), promotion of odontoblast differentiation (22), and stimulation of tertiary dentin or dentin bridge formation (23). Collectively, these effects support pulp healing and repair by reducing inflammation and fostering the deposition of new mineralized tissue. However, this strategy is not universally adopted, largely due to the limited clinical evidence supporting its effectiveness. Furthermore, the use of an intermediate layer may compromise adhesion between the composite and the tooth structure and may also complicate the restorative procedure.

9.3. Curing protocols

Polymerization protocols critically determine the amount of residual monomer in RBCs. Incremental placement (adding composite in thin layers, typically 1–2 mm) associated with optimal light curing enhance light penetration and increase the DC of each layer(24). Nonetheless, the outcome is highly operator-dependent: inadequate exposure times, suboptimal light positioning, inappropriate curing mode, or excessive increment thickness can all result in insufficient polymerization. Such under-curing not only elevates monomer elution but also compromises mechanical properties.

Taken together, clinical factors such as dentin thickness, liner application, and curing protocol can exert a profound influence on the biological risks associated with composites. Yet none of these measures provides an absolute safeguard. The dentin barrier is variable, liners may complicate adhesion and represent additional costs, and curing strategies are sensitive to operator skill. These limitations reinforce the conclusion from Chapters VII and VIII: the biological safety of composites is not determined by material science alone, but by the integration of material properties with careful operative technique.

10. Perspectives

The results of this thesis must be interpreted in the context of the clinical situation in which RBCs are applied. Carious lesions trigger a state of pulp inflammation that depends on the extent and speed of caries progression. Hence, inflammation pre-exists when the restorative treatment takes place. In such conditions, pulp cells are already exposed to oxidative stress, inflammatory mediators, along with reduced regenerative capacity. The subsequent exposure to residual monomers eluted from composites occurs in this compromised microenvironment, where toxic effects may be amplified. Yet, most experimental studies, including those presented here, rely on healthy pulp cell models, which only partially capture the clinical reality. This highlights the need to better integrate inflamed pulp models into future biocompatibility research.

While acute cytotoxicity and genotoxicity of monomers such as TEGDMA and BisGMA are well documented, their potential long-term consequences remain far less understood. Residual monomers may diffuse at low concentrations over prolonged periods, exerting effects that are not immediately lethal but may alter tissue homeostasis. These effects could include persistent low-grade inflammation, subtle disruption of stem cell functions, or gradual accumulation of DNA damage that escapes repair. Such chronic insults, though less dramatic than acute cytotoxicity, may ultimately compromise pulp vitality and predispose the tissue to degeneration or impaired repair. Future research should therefore move beyond short-term assays and incorporate longitudinal models that better reflect chronic exposure.

A particularly underexplored aspect concerns the biological effects of sub-toxic monomer concentrations when defined on the basis of effective free concentrations rather than nominal concentrations calculated from the amount of monomer added to the culture medium. In aqueous biological systems, resin monomers do not remain fully dissolved and freely available: they may bind to serum proteins, adsorb to plastic cultureware, partition into lipid phases, or undergo partial degradation. As a result, the nominal concentration introduced into the medium may substantially overestimate the fraction of monomer that is biologically available to cells. Importantly, the concentration of free monomer present in the culture medium, and therefore capable of interacting with cellular targets, may be significantly lower than the calculated nominal concentration. This discrepancy is rarely accounted for in conventional *in vitro* toxicological studies but may critically influence dose-response relationships and the interpretation of sub-toxic effects. Future studies should therefore aim to distinguish between nominal and bioavailable monomer concentrations, for instance by quantifying free monomer fractions or by designing exposure models that better reflect physicochemical partitioning in biological environments.

These levels, closer to what is expected clinically, may not cause overt cell death but can still impair critical cellular functions. Evidence suggests that pulp stem cells exposed to non-lethal doses may show altered migration, proliferation, and

differentiation potential (25, 26), processes essential for dentinogenesis and pulp repair. Moreover, sub-toxic exposure could modulate inflammatory signaling pathways, tipping the balance between adaptive and destructive responses. The study of these “silent” but biologically significant effects is essential for understanding how composites influence pulp regeneration over the long term.

To address these challenges, more sophisticated experimental models are needed. Conventional two-dimensional cultures of pulp fibroblasts, while useful, cannot reproduce the complexity of the dentin–pulp interface or the dynamics of the oral environment. Emerging systems such as three-dimensional pulp organoids and tooth-on-chip platforms offer promising alternatives (27, 28), as they integrate fluid flow, extracellular matrix, and cell heterogeneity. In addition, co-culture models including immune cells would allow exploration of inflammatory crosstalk. Equally important is the incorporation of dentin barrier models, since dentin modulates the diffusion of monomers and may attenuate or delay their toxic impact. Such approaches will help to bridge the gap between simplified *in vitro* studies and clinical outcomes.

From a translational standpoint, several strategies can help mitigate risks. Clinically, optimising curing protocols to minimise residual monomers, applying protective liners in deep cavities, and selecting formulations with lower leachable content are practical measures. On the materials side, the development of novel monomers with reduced cytotoxicity and genotoxicity should be a priority. Importantly, regulatory standards such as those from ISO currently emphasize cytotoxicity assays, but the findings of this thesis underline the need to expand evaluation criteria to include oxidative DNA damage, and regenerative capacity.

Finally, it is important to recognize that the dental pulp is a living and dynamic tissue capable of repair and regeneration. Even subtle, sublethal disturbances could have significant long-term implications for pulp vitality and reparative dentinogenesis. Future progress in this field depends on integrating mechanistic cellular studies with clinically relevant models, while ensuring that insights directly inform material innovation and clinical practice. By aligning biocompatibility with mechanical

Discussion and Perspectives

performance, the next generation of RBCs can be designed to ensure both restorative success and long-term pulp health.

11. References

1. Daronch M, Rueggeberg FA, De Goes MF. Monomer conversion of pre-heated composite. *J Dent Res*. 2005;84(7):663-7.
2. Lu H, Carioscia JA, Stansbury JW, Bowman CN. Investigations of step-growth thiol-ene polymerizations for novel dental restoratives. *Dent Mater*. 2005;21(12):1129-36.
3. Ferracane JL. Resin composite--state of the art. *Dent Mater*. 2011;27(1):29-38.
4. Leprince JG, Palin WM, Hadis MA, Devaux J, Leloup G. Progress in dimethacrylate-based dental composite technology and curing efficiency. *Dent Mater*. 2013;29(2):139-56.
5. Weinmann W, Thalacker C, Guggenberger R. Siloranes in dental composites. *Dent Mater*. 2005;21(1):68-74.
6. Shafiei F, Tavangar MS, Razmkhah M, Attar A, Alavi AA. Cytotoxic effect of silorane and methacrylate based composites on the human dental pulp stem cells and fibroblasts. *Med Oral Patol Oral Cir Bucal*. 2014;19(4):e350-8.
7. Madhyastha PS, Naik DG, Kotian R, Padma D, Srikant N, Bhat KM. Evaluation of Cytotoxicity of Silorane and Methacrylate based Dental Composites using Human Gingival Fibroblasts. *J Clin Diagn Res*. 2015;9(1):ZC05-8.
8. Kopperud HM, Schmidt M, Kleven IS. Elution of substances from a silorane-based dental composite. *Eur J Oral Sci*. 2010;118(1):100-2.
9. Viljanen EK, Skrifvars M, Vallittu PK. Dendrimer/methyl methacrylate copolymers: residual methyl methacrylate and degree of conversion. *J Biomater Sci Polym Ed*. 2005;16(10):1219-31.
10. Wan Q, Rumpf D, Schricker SR, Mariotti A, Culbertson BM. Influence of hyperbranched multi-methacrylates for dental neat resins on proliferation of human gingival fibroblasts. *Biomacromolecules*. 2001;2(1):217-22.
11. Boulden JE, Cramer NB, Schreck KM, Couch CL, Bracho-Troconis C, Stansbury JW, et al. Thiol-ene-methacrylate composites as dental restorative materials. *Dent Mater*. 2011;27(3):267-72.
12. Yang H, Li K, Yan H, Liu S, Wang Y, Huang C. High-performance therapeutic quercetin-doped adhesive for adhesive-dentin interfaces. *Sci Rep*. 2017;7(1):8189.
13. Yang Y, Wang L, Huang Z, Ge L, Shi J. N-acetylcysteine as a novel methacrylate-based resin cement component: effect on cell apoptosis and genotoxicity in human gingival fibroblasts. *BMC Oral Health*. 2024;24(1):222.
14. Ismail EH, Kilinc E, Hardigan PC, Rothrock JK, Thompson JY, Garcia-Godoy C. Effect of Two-minute Application of 35% Sodium Ascorbate on Composite Bond Strength following Bleaching. *J Contemp Dent Pract*. 2017;18(10):874-80.
15. Ebrahimi-Chaharom ME, Kimyai S, Mohammadi N, Oskoei PA, Daneshpuy M, Bahari M. Effect of sodium ascorbate on the bond strength of all-in-one adhesive systems to NaOCl-treated dentin. *J Clin Exp Dent*. 2015;7(5):e595-9.
16. Schweikl H, Spagnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. *J Dent Res*. 2006;85(10):870-7.

17. Galler K, Hiller KA, Ettl T, Schmalz G. Selective influence of dentin thickness upon cytotoxicity of dentin contacting materials. *J Endod.* 2005;31(5):396-9.
18. Jiang RD, Lin H, Zheng G, Zhang XM, Du Q, Yang M. In vitro dentin barrier cytotoxicity testing of some dental restorative materials. *J Dent.* 2017;58:28-33.
19. European Society of Endodontology developed b, Duncan HF, Galler KM, Tomson PL, Simon S, El-Karim I, et al. European Society of Endodontology position statement: Management of deep caries and the exposed pulp. *Int Endod J.* 2019;52(7):923-34.
20. Camilleri J. Characterization of hydration products of mineral trioxide aggregate. *Int Endod J.* 2008;41(5):408-17.
21. Chatterjee I, Agrawal P, Sinha Y, Majee N, Bansal S. In Vitro Response of Dental Pulp Stem Cells to Calcium Silicate-Based Cements: A Systematic Review and Meta-Analysis of Preclinical Evidence. *Cureus.* 2025;17(7):e88990.
22. Paranjpe A, Zhang H, Johnson JD. Effects of mineral trioxide aggregate on human dental pulp cells after pulp-capping procedures. *J Endod.* 2010;36(6):1042-7.
23. Accorinte ML, Loguercio AD, Reis A, Carneiro E, Grande RH, Murata SS, et al. Response of human dental pulp capped with MTA and calcium hydroxide powder. *Oper Dent.* 2008;33(5):488-95.
24. Torres CRG, Prado TP, Avila D, Pucci CR, Borges AB. Influence of Light-Curing Time and Increment Thickness on the Properties of Bulk Fill Composite Resins With Distinct Application Systems. *Int J Dent.* 2024;2024:2123406.
25. Bakopoulou A, Leyhausen G, Volk J, Tsiftoglou A, Garefis P, Koidis P, et al. Effects of HEMA and TEDGMA on the in vitro odontogenic differentiation potential of human pulp stem/progenitor cells derived from deciduous teeth. *Dent Mater.* 2011;27(6):608-17.
26. Kwon JH, Park HC, Zhu T, Yang HC. Inhibition of odontogenic differentiation of human dental pulp cells by dental resin monomers. *Biomater Res.* 2015;19:8.
27. Xu X, Li Z, Ai X, Tang Y, Yang D, Dou L. Human three-dimensional dental pulp organoid model for toxicity screening of dental materials on dental pulp cells and tissue. *Int Endod J.* 2022;55(1):79-88.
28. Franca CM, Tahayeri A, Rodrigues NS, Ferdosian S, Puppini Rontani RM, Sereda G, et al. The tooth on-a-chip: a microphysiologic model system mimicking the biologic interface of the tooth with biomaterials. *Lab Chip.* 2020;20(2):405-13.