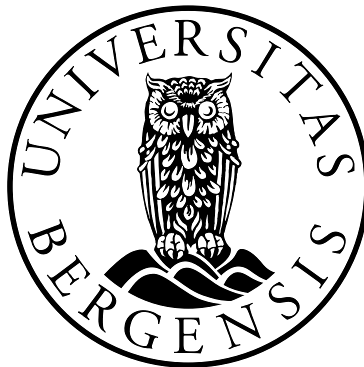
In vitro toxicity of filler particles and methacrylates used in dental composite materials

Cytokine release and cell death

Vibeke Ansteinsson



Dissertation for the degree philosophiae doctor (PhD)
Department of Clinical Dentistry − Biomaterials
University of Bergen
and
Nordic Institute of Dental Materials (NIOM)

Norway 2013

SCIENTIFIC ENVIROMENT

The present work was performed in collaboration with the Biomaterial Research Group at the Faculty of Medicine and Dentistry, University of Bergen and Nordic Institute of Dental Materials (NIOM). Most of the practical biological laboratory work is done at NIOM. Particle characterization has mainly been done at the University of Bergen. The two institutions have jointly participated in the fulfillment of the present work.

ACKNOWLEDGEMENT

The funding and support provided by the Faculty of Dentistry and Medicine, department of Clinical Dentistry at the University of Bergen made this work possible. The experimental work has primarily been carried out at Nordic Institute of Dental Materials (NIOM). I wish to thank co-supervisor, Professor and Director Jon E. Dahl for his support and for allowing me to conduct my work at NIOM.

I will express my gratitude to my main supervisor Professor Nils Roar Gjerdet for excellent guiding and support, both in Bergen and at NIOM. When spending time in Bergen, Nils Roar has always made my time easier by taking me out for a beer or dinner. I will further thank co-supervisor Dr. Jan Tore Samuelsen for all your expertise and for withstand sharing office with me for the last few years.

Thanks to Dr Rune Becher for introducing me to NIOM and to the staff at NIOM for contributing to a positive environment and excellent working condition. A special thanks to co-author Else Morrisbak for excellent guidance in the lab and for always smiling. Thanks to my co-authors: Dr Jørn A Holme, Dr Hilde Kopperud, Dr Anita Solhaug, Dr Anette Bølling and Dr Gro H. Mathisen.

To Audun, thank you for helping me through tough periods, for being an excellent dad and for deliver and pick up in the kindergarten whenever I was too busy. Thanks to my boys Simen and Vinter for taking my mind off work. To the rest of my family, Mom, Dad, Nina, Henriette, Endre, Bjørg and Liv Ingrid, thank you for helping out whenever needed.

To my friends from FHI, Dr Kirsten Rakkestad and Dr Nina Landvik for inspiring me, and to the rest of my friends for celebrating good results and for taking my mind off bad, or no results at all.

LIST OF PUBLICATIONS

I. **Filler Particles Used in Dental Biomaterials Induce Production and Release of Inflammatory mediators *in Vitro***. Ansteinsson VE, Samuelsen JT, Dahl JE. Journal of Biomedical Materials Research B Applied Biomaterials. 2009, 89 (1):86-92.

II. **Cell toxicity of methacrylate monomers – the role of glutathione adduct formation**. Ansteinsson V, Kopperud HB, Morisbak E, and Samuelsen JT. Journal of Biomedical Material Research A. Accepted for publication, February 2013.

III. **DNA-damage, cell-cycle arrest and apoptosis induced in BEAS-2B cells by 2-hydroxyethyl methacrylate (HEMA).** Ansteinsson V, Solhaug A, Samuelsen JT, Holme JA, Dahl JE. Mutation Research. 2011, 16;723(2):158-64.

IV. **TEGDMA and filler particles used in dental composites additively attenuate LPS-induced cytokine release from the monocyte-macrophage cell line RAW 264.** Mathisen GH, Ansteinsson V, Samuelsen JT, Dahl JE, Becher R, Bølling AK. Submitted.

LIST OF ABBREVATIONS

ATM	ataxia teleangiectasia mutated
BisGMA	bisphenol A-glycidyl dimethacrylate
BisEMA	bisphenol A-etyl dimethacrylate
CHK	checkpoint kinase
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DSB	double strand break
ELISA	enzyme-linked immunosorbent assay
GDMA	glycerol dimethacrylate
GSH	glutathione
GSSG	glutathione disulfide
HEMA	2-hydroxyethylmethacrylate
IL	interleukin
LC/MS	liquid chromatography–mass spectrometry
MAPK	mitogen activated protein kinases
MMA	methyl methacrylate
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
PCR	polymerase chain reaction
SDH	succinate dehydrogenase
ROS	reactive oxygen species
SEM	scanning electron microscopy
TEGDMA	triethyleneglycol-dimethacrylate
TEM	transmission electron microscopy
TNF	tumor necrosis factor alpha
UDMA	urethane dimethacrylate

ABSTRACT

Dental polymer-based composite materials are complex materials consisting of several components, the main components being filler particles (inorganic component) and polymer matrix (organic component). The organic component consists of monomers that usually are polymerized upon activation by visible light illumination.

The polymerization process is never complete, and leakage of unreacted methacrylate monomers occurs during clinical service. Degradation processes may weaken the bond between the fillers and the matrix, leading to release of fillers particles and ions in addition to the organic components. Clinical handling during placement and finishing has been shown to cause release of particulate matter to the air in dental clinics, which could be followed by exposure to tissue and saliva of the patients. Thus, both dental personnel and patients could be exposed to components of polymer-based dental materials.

The main objective of the thesis was to characterize the toxic potential of selected inorganic filler particles (barium glass particles and silica particles) and methacrylates (HEMA, TEGDMA, BisGMA, GDMA, MMA) used in dental composite filling materials. The results showed that both nano- and micro-sized filler particles could modulate the release of inflammatory mediators *in vitro*. The methacrylates investigated induced cytotoxicity, but the potency and the mechanism involved seemed to differ between the methacrylates. HEMA was found to induce apoptosis and DNA damage followed by activation of DNA damage response. To address possible exposure to multiple components of polymer-based dental composite materials the effect of co-exposure to components was studied. An additive inflammatory response was observed with particles and TEGDMA co-exposure.

Studies on the mechanisms of possible adverse biological responses to substances released from polymer-based dental materials could contribute to safer dental materials.

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1. INTRODUCTION

Polymer-based dental composite material is a class of biomaterials. A biomaterial is defined as any non-vital material intended to replace a part of or a function in the body (1). A biomaterial should be biocompatible, defined as the ability of a material to perform with an appropriate host response when applied as intended. The biocompatibility of a material is determined by its interaction with surrounding tissues and release of substances from the material (2).

The use of polymer-based dental composites is high and is still increasing due to restriction in use of dental amalgam filling. The dental composites were introduced in the 1960s and were intended for anterior restorations due to their esthetic properties. The products have continued to develop and are currently used for a variety of dental applications. However, their use as filling materials is the main application (3, 4). Thus, a considerable proportion of the population from children to the elderly receives composite restorations. The fillings have shortcomings, such as wear discolorations, fractures, loosening and at risk for development of secondary caries (5, 6). Consequently, there is a demand for improved materials.

Polymer-based dental composites mainly consist of inorganic filler particles embedded in an organic polymer matrix, hence the name composite (componere = to combine) (7). Both the polymer network and the inorganic part of the material represent an opportunity for improvements of the overall properties. To reduce shrinkage, development of methacrylate monomers systems have been introduced (5). However, it appears that the filler particles have been subject to the largest change over the years. The use of nanotechnology to improve properties of materials has been of great interest, and has also reached the field of dental biomaterials. Nanotechnology is known as the production, use and manipulation of materials and structures in the range of about 1–100 nanometers (nm) by various physical and chemical methods. This development, has led to use of smaller particles in dental materials (8, 9).

The focus of this study was to evaluate the biological effects of components used in polymer-based dental fillings and other related polymer-containing dental filling

materials such as compomers and glassionomers. For simplicity, these materials are referred to as composite fillings throughout this thesis.

1.1 Composition

The main constituents of composite filling materials comprise inorganic filler particles, a polymer matrix and a coupling agent that bind the fillers to the polymer matrix. In addition there are stabilizers and additives such as UV-stabilizers, inhibitors and coloring agents (figure 1) (10). Present-day composite are combined with an adhesive system that provides bonding to dentin and enamel. The adhesive can be considered as an integrated part of the composite system. The constituents of the adhesive systems are in principle similar to the composite filling materials (methacrylates, initiators, stabilizer, inhibitors and filler particles) (11), and are not discussed separately.

1.1.1 Filler particles

The purpose of the filler particles is to strengthen the composite and to reduce the matrix volume, thereby lowering the shrinkage that occurs during polymerization. Dental filler particles are coated with silane, which improve the binding between the polymer matrix and the particles (10).

Traditionally dental composite filling materials are classified according to the size of the filler particles; macrofilled, microfilled and hybrids (12). Today, most materials are so called hybrids, using a mixture of micro-sized particles and nanoparticles (5). There are also materials with nanoparticles only, termed nanocomposites (9). Commonly used particles are amorphous silica particles, quarts, barium and lithium aluminium silica glasses (borosilicate glass, or barium, strontium, or zinc glasses). Elements of high atomic weight, such as barium and strontium are also incorporated to provide x-ray opacity facilitating radiological monitoring *in vivo* (8, 10). Mechanical and esthetic properties of dental restorative composite materials depend on the chemical composition of the filler particles, the filler particle characteristics (filler surface

texture, filler shape, and size) and the filler loading (% by weight) (13, 14). Nanoparticles in composite fillings can be present as aggregates and agglomerates (nanocluster), non-agglomerated, non-aggregated nanoparticles (monodisperse nanoparticles) or as pre-polymerized resin filler (PPRF) (8, 14). Nanoparticles are added to increase the filler load which theoretically can be as high as 90-95%. High filler load will reduce the polymerization shrinkage, but could also adversely affect handling properties as well as mechanical and physical properties (8).

1.1.2 Polymer matrix

The polymer matrix consists predominantly of dimethacrylates. These monomers are able to crosslink via their vinyl groups and form a polymer network. The polymerization process is usually activated by an initiator system and blue light. BisGMA, Bis-EMA, TEGDMA, GDMA and UDMA are commonly methacrylates used in composite filling materials. The low viscosity monomers such as TEGDMA are used as diluent for high viscosity monomers such as BisGMA (15). In adhesives and polymer-containing glassionomer cements HEMA is also used (11, 16). The fraction of carbon double bonds that react during polymerization is referred to as the degree of conversion. However the polymerization process is never complete, and the degree of conversion normally does not exceed 70% (17, 18).

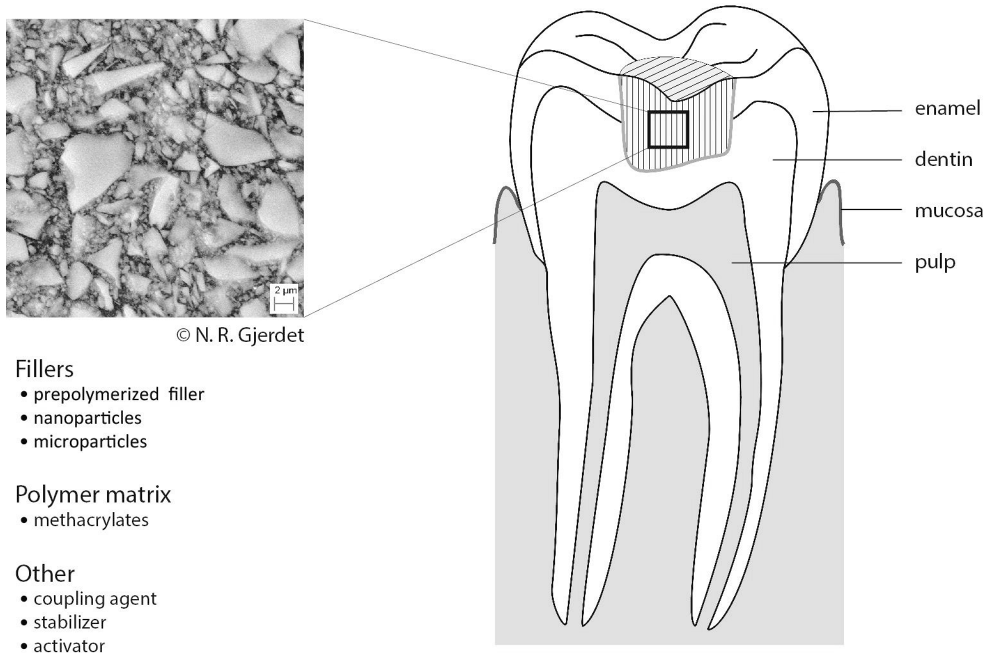

Figure 1: *Illustration of the composition of a composite filling. The SEM picture (left) is an example of the microstructure of a hybrid composite material consisting of both nanoparticles and microparticles.*

1.2 Exposure

Both dental personnel and dental patients are exposed to components of dental restorative composite materials (figure 2) (19-22). However, the extent is unknown due to limited exposure data. These materials are polymerized *in situ*, which can increase the risk of exposure to components during handling and placement. The exposure situations are different between dental personnel and patients. Dental personnel are occupationally exposed, most likely to higher concentrations for short intermittent periods. Dermal exposure and inhalation will be the main exposure routs (19, 20, 22, 23). Patients on the other hand are exposed during treatment and due to wear and degradation of the material over time. Leaching of components from composite fillings into the oral environment will occur. Thus, the oral cavity, the pulp and the gastrointestinal (GI) tract will be the main exposure routs (21, 24).

1.2.1 Exposure to particulates from composite materials

Occupational exposure

Dental personnel can be exposed to particulates from materials during trimming of models, material agitation, shaping of temporaries and hand mixing of materials (19). Hence the exposure is primarily due to inhalation. Recently, particulates from a controlled drilling procedure have been analyzed and the results revealed that the particles were irregularly shaped and had a very wide size distribution, from 7 nm to 10-15 µm. The particulates consisted of pieces of polymer matrix, particles partly embedded in the polymer matrix and single filler particles. Different commercial composite materials can give rise to different size distribution of the dust particles (22). In studies on indoor quality in dental clinics the concentrations of particulate matter (PM2.5 and PM10) was reported to exceed the air quality limits suggested by the European Directive 1999/30/EC and the US Environmental Protection Agency, 1997 (19).

Patient exposure

Patients are exposed to air-born particulates during dental treatments, but such exposure is infrequent and of short duration compared to the situation for dental personnel. In addition patients are exposed to particulates and ions that leach from the filler particles in the cured composite filling material (24-26). *In vivo* leaching has been reported to continue over longer periods (27). There is scarce information on the release of particulates during wear, degradation and leaching processes as well as the morphological, physical and chemical characteristics of the particulates released from composite filling materials.

Composite filling materials are used for restorations in all areas of the mouth and there are concerns regarding wear rate of restorations placed in high stress areas (28). Degradation of dental composite materials is influenced by the combined effect of mechanical and chemical forces, e.g. material stress and hydrolytic absorption. Absorption of water initiates a degradation process comprised of leaching of ions and residual unpolymerized monomers and disintegration of the polymer network (24, 29).

Salivary esterases can also degrade the surfaces of composite resins and increase the release of methacrylic substances (30). Particle pull-out is reported in composite filling materials. This is caused by breakdown of the silane bond between the resin and the filler particles (24). Material voids, filler aggregates of 20 µm in diameter can be completely removed from the surface of the materials due to mechanical stress and chemical degradation of the material (31).

1.2.2 Exposure to methacrylate

Occupational exposure

Methacrylates have been measured in the air of dental practices (19, 20). Exposure is prevalent during mixing of volatile methacrylic compounds, grinding and drilling of composite materials, removal of old composite fillings and application of bonding and composite materials in the prepared cavity (19, 32). Hence, dental personnel are exposed to methacrylates on a daily basis.

Patient exposure

Patients are primarily exposed to methacrylate during setting of dental composite materials, by release of residual monomers due to incomplete polymerization (21), and wear and degradation of the composite filling material. Release of methacrylates due to wear and degradation are previously described (section 1.2.1, *patient exposure*). Additionally, HEMA used in adhesives can diffuse through the dentin and lead to exposure of the pulp tissue (33).

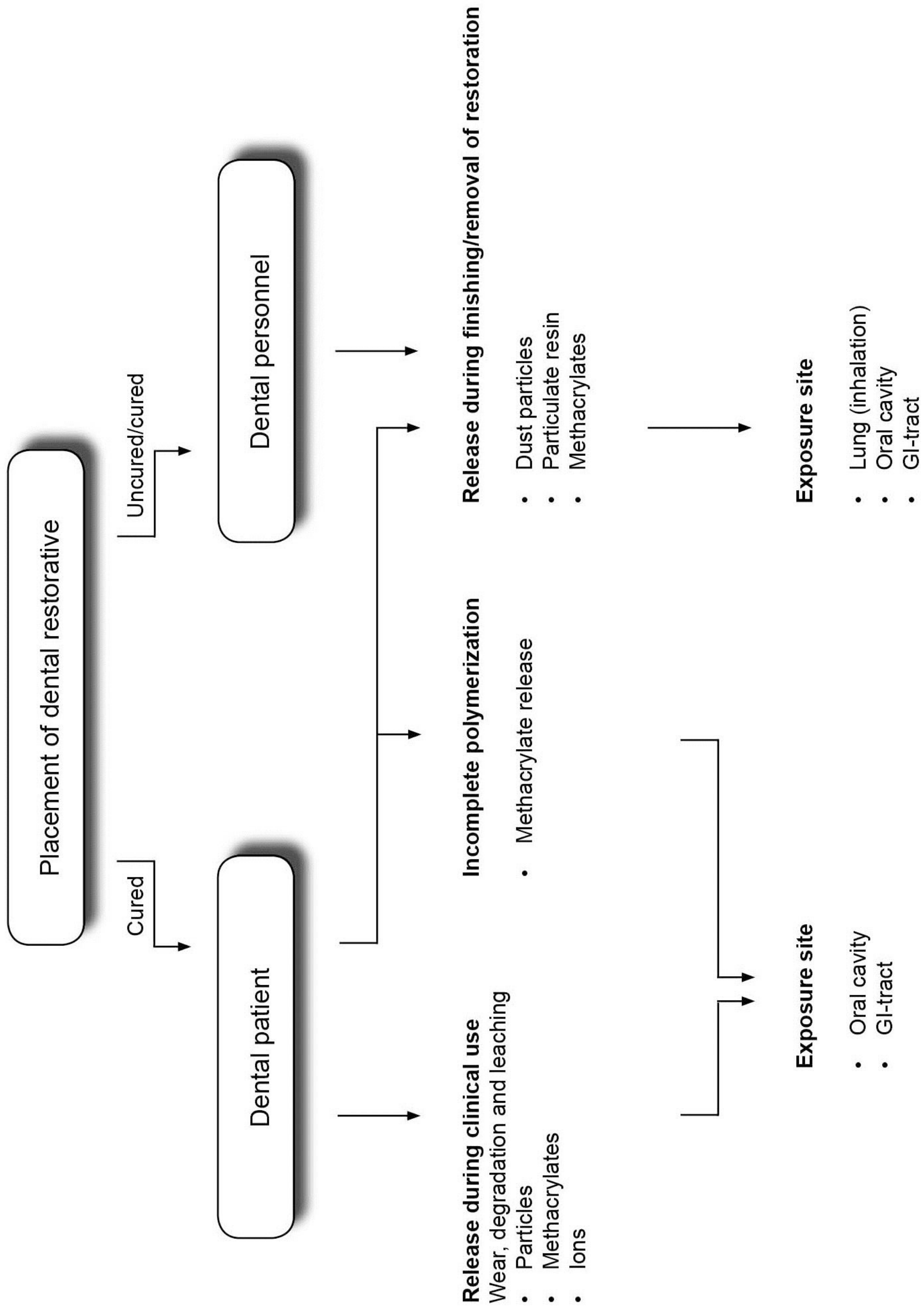

Figure 2: *Illustration of exposure, exposure sites and main routes of exposure during and after placement of dental composite filling materials.*

1.3 Toxic responses

Adverse reactions to dental composite filling materials do occur (34-39). Methacrylate-based materials are involved in about 30% of the reports submitted to the Norwegian Adverse Reaction Unit (40). However, relatively few adverse health effects are documented in the literature, and it is not established how cause and effect relationship could be evaluated in a clinical setting.

Components released from dental materials can exert a toxic effect on target tissues (41). A toxic reaction to a xenobiotic can manifest in numerous ways: it can be local reactions or systemic reactions occasionally involving an allergic mechanism. The response depends on the type, dose and duration of the exposure (42). Particles and methacrylates used in dental composite filling materials can be distributed to various cell compartments allowing for interaction with multiple cellular structures (33, 43).

1.3.1 Inflammatory responses

The innate immune system is the first line of defence in the human body, while the adaptive immune system is antigen dependent and specific. Allergic reactions are antigen-specific reactions of the adaptive immune system. Inflammation is a tissue response to a microbial pathogen infection, chemical toxins and irritation and/or wounding, and can involve both the innate and the adaptive immune system (44, 45).

An inflammatory response at the site of damage is characterized both by the local tissue response and infiltration of neutrophils, macrophages, dendritic cells and lymphocytes (immune cells) (44, 46). Cytokines are signalling molecules that are key mediators in both inflammatory and immune responses. They play an important role in initiation and maintenance of inflammatory response, and are produced by numerous cell types including macrophages, monocytes, fibroblasts, endothelial and epithelial cells. There are two main groups of cytokines, pro-inflammatory and anti-inflammatory. The latter are intended to modulate and inhibit excess inflammatory reactions. Important biological features of cytokines are their pleiotropic and

redundant properties allowing them to have multiple functions, in addition they can act synergistically and antagonistically. They can act in an endocrine, paracrine or autocrine manner through specific cell membrane receptors. Pro-inflammatory cytokines are produced early during an inflammatory response and are able to modulate the host response by recruitment of cells, initiate additional cytokine signalling, activating signaling cascades and induction of gene transcription (figure 3) (47, 48). Examples of pro-inflammatory cytokines are interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-8 and IL-6. IL-8 is chemotactic cytokine that stimulates recruitment of inflammatory cells, while IL-6 is a cytokine that has both pro-inflammatory and anti-inflammatory properties (48, 49). IL1-β is a cytokine that exists as inactive pro- IL-1β, and is activated by cleavage executed by caspase 1 and, regulated by the assembly of the inflammasome complex (50). General principles of a cytokine response are illustrated in figure 4.

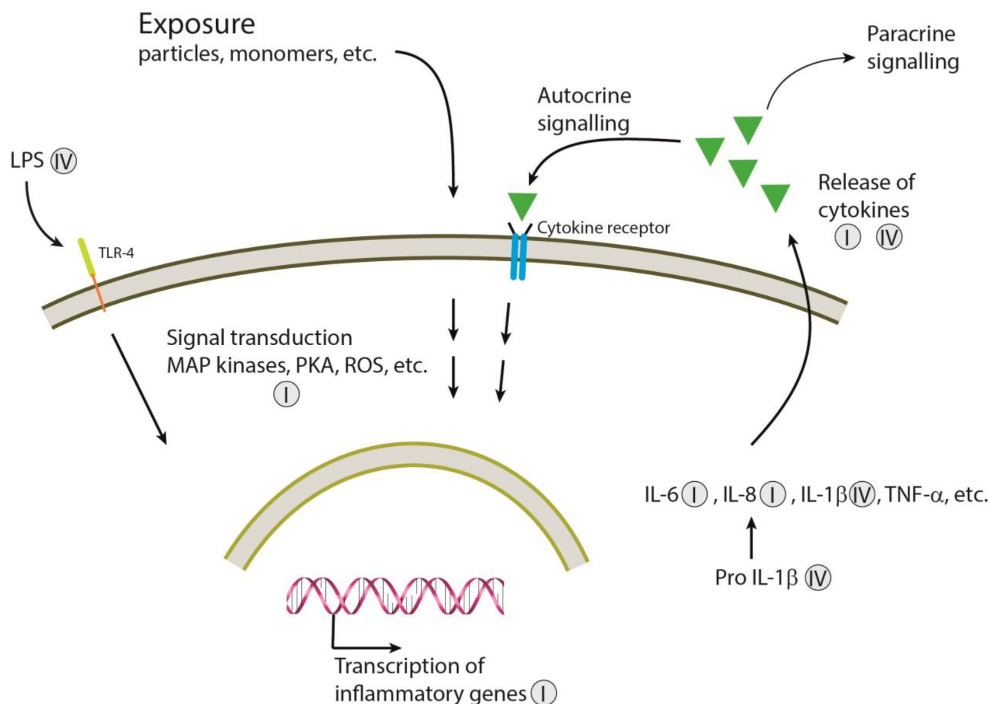

Figure 3: *Illustration of possible induction of cytokine gene transcription and release after exposure to methacrylate and particles. Induction of cytokine release through toll like receptor 4 (TLR-4) is also illustrated. The numbers in circles indicate in which study the specific biological endpoint is described.*

1.3.2 Cellular responses

Cellular oxidative stress

Reactive oxygen species (ROS) are chemically highly reactive molecules that contain oxygen ($O2^-$, OH^-) and their reactivity is mainly due to an unpaired electron. In addition, non-radical, but highly reactive molecules such as H_2O_2 is also called ROS. ROS is a product of metabolisms in the cell, but can also be initiated by exogenous and endogenous stimuli (51-53). Cells have defense mechanisms that maintain a controllable level of intracellular ROS. This includes non-enzymatic antioxidants such as glutathione (GSH), vitamin C and vitamin E, and antioxidant enzymes such as, thioredoxins, superoxid dismutase (SOD), catalase and gluthatione peroxidase. GSH is the most abundant non-protein thiol in the mammalian cell and is acting as reducing

agent. GSH has a cysteine moiety that includes a thiol group (SH-group). This group is a biological active group that is easy to oxidize and is susceptible to nucleophilic attack. Oxidized GSH termed GSSG is reduced back to GSH by the enzyme glutathione reductase (51). Depletion of GSH can cause change in the GSH/GSSG status leading to elevate levels of ROS in the cells. ROS can also react with nitrogen oxides and make reactive nitrogen species (RNS) (52, 54). Increased levels of ROS in the cells may initiate DNA (55), lipid (56) and protein damages (57) and thereby inhibit and active enzymes such as phosphatases and enzymes involved in stress responses. These ROS-induced responses may also trigger increased inflammation and initiate cell death (52, 56).

DNA damage

DNA damage occurs during replication of the cell, but can also be a result of toxic conditions. If the damage is not too extensive it will be repaired and the cell survives. When DNA damage is extensive and beyond repair, cell death is induced. However, if the DNA repair fails but the cell survives, the results could be mutation, genomic instability and cancer initiation (58).

Cellular response to DNA damage is mediated through a complex network of signaling. Mediators of this transduction can be categorized into sensors, transducers and effectors depending on their role in the DNA damage response. However, the mediators can also have multiple roles. DNA damage sensors such as the NBS1/MRE11/Rad50 (MRN) complex and members the phosphatidylinositol 3 kinase-like kinase (PIKK) family, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and RAD3 related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are engaged in monitoring the DNA. DNA damage sensors are activated upon DNA damage and the type of sensor activated is dependent upon the type of DNA damage. Generally ATM is activated upon double strand break (DSB) and ATR upon single strand break and DNA damage associated with replication (59-62). ATM can be a transducer of DNA damage signaling pathways and thereby activate and stimulate multiple DNA-damage effector proteins. For example ATM can

regulate effector proteins such as p53, mdm2 and chk2. ATM, ATR and DNA-PK also activates H2AX (denoted γH2AX when activated) early in the DNA-damage response. γH2AX have multiple functions and in additions to serve as a sensor for DNA damage it can also function as a docking site for DNA repair and cell cycle proteins (59). General principles of DNA-damage response is illustrated in figure 4.

Cell death

Cellular stress and extensive DNA damage can result in cell death (63, 64). Generally, cell death is divided into two main types; necrosis and apoptosis. In addition, autophagy is proposed as a third form of cell death (65). Apoptosis is an active ATP-requiring and controlled process morphologically defined by nuclear shrinkage and condensation, membrane blebbing, and release of apoptotic bodies. Apoptotic cell death is important to maintain normal cellular turnover (65-67). Autophagy involves digestion of the cells own organelles, often activated upon starvation In contrast to apoptosis and autophagy, necrosis is a passive process which is traditionally defined by swelling of the cell, rupture of the cell membrane followed by inflammation. Both accelerated and insufficient cell death is involved in many diseases (68, 69).

1.3.3 Toxic responses to methacrylate monomers

Methacrylate-induced cell death and underlying cellular mechanisms are studied in several *in vitro* cellular systems (70-74). Observed responses to methacrylates include increased apoptosis and cell cycle alterations (70, 75), induction of mitogen-activated protein kinases (MAPK) (76), micronuclei formation (77) and DNA damage (78-80) followed by DNA damage response (75). Methacrylate binding to GSH probably through a Michael-type addition has been pointed out as a key event in the observed toxic response to methacrylates. GSH-methacrylate adduct formation are reported to result in GSH depletion and increased ROS formation (81). Clinically, adverse reactions to methacrylates have been observed both in patients and dental personnel. Contact dermatitis among dental personnel is the most common type of adverse

reactions to methacrylates (23, 35). The allergic response is proposed to be initiated by HEMA binding to proteins such as serum albumin (82). Reactions to materials in patients are less frequent, but lichenoid reactions are observed in relation to composite filling materials (38).

1.3.4 Toxic responses to particles

The toxicology of particulates has emerged as a field in toxicology involving exposure, distribution and elimination of particulate matter. The first studies on toxicology of particulates were associated with exposure to urban air particles. During the last decade toxicological studies on particles used in engineered materials, particularly nanomaterials (nanotoxicology) have emerged as a separate discipline in toxicology (83). Nanoparticles exist in a domain between quantum physics and bulk chemistry. The nanometer size of particles results in increased surface area pr mass. These properties are associated with altered physicochemical properties as well as increased biological reactivity (84).

A wide variety of different particles has been investigated both *in vitro* and *in vivo*. The biological responses to particles are largely governed by the physicochemical characteristics. As a consequence it is problematic to generalize responses to particles therefore, only examples are mentioned here. Silica particles are among the most studied particles (SiO_2) and occur in crystalline (quartz) and amorphous forms (glass). Occupational exposure to crystalline silica is associated with silicosis, fibrotic lung diseases, chronic obstructive lung disease (COPD), and lung cancer. In general, exposure to silica particles induces severe inflammation which seems to be a common step for silica-induced diseases. *In vitro* SiO_2 exposure is associated with cell death, increased cytokine release and ROS formation (85). Particulate wear debris (metals, ceramics, polymers) from orthopedic joint implants with articulating surfaces can initiate a local inflammatory response through release of proinflammatory proteins such as IL-6, IL-8, IL-1β and TNF-α. Such immune responses can initiate bone resorption by osteoclasts (46, 86, 87) and have been shown to disturb differentiation of

mesenchyme stem cells (88). In addition wear debris from implants are associated with altered collagen synthesis (87), and apoptosis (89).

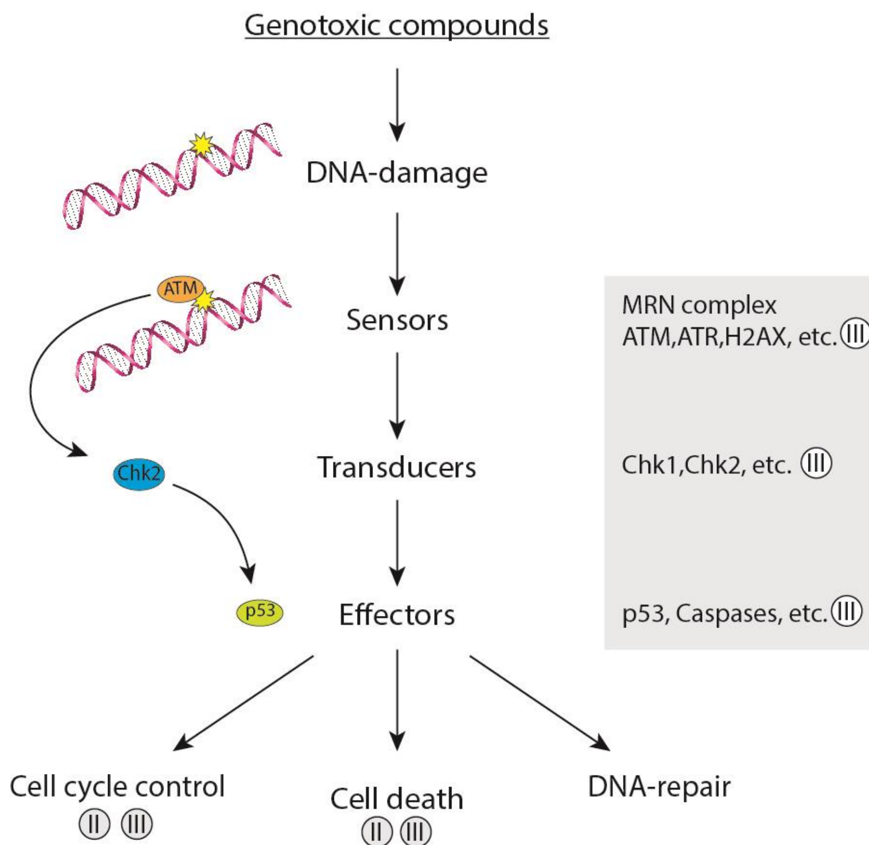

Figure 4: *Simplified illustration of cellular DNA damage response to a genotoxic compounds or ROS generation. P53 (effector) activation through ATM (sensor) and chk2 (transducer) are exemplified in the figure. Circles indicate in which study the specific biological endpoint is described.*

2. AIMS OF THE STUDY

The overall hypothesis of the present work was that organic and inorganic components of polymer-based dental materials, alone and in combination, can induce biological responses.

The toxic potential of selected inorganic filler particles and methacrylates commonly used in polymer-based dental materials were to be characterized. The specific aims were to:

1) Study the *in vitro* inflammatory potential of filler particles and monomers alone and in combination
2) Study the *in vitro* potential of methacrylates to induce cell death and to elucidate underlying mechanisms.

3. SUMMARY OF RESULTS

Paper I: Filler particles used in dental biomaterials induce production and release of inflammatory mediators *in vitro*

In paper I the potential of particles to induce cytokines IL-6 and IL-8 was studied in BEAS 2B cell after 24h of exposure (2.5-40 µg/cm^2). Two types of ceramic particles with a mean particle size of 1µM were used (BaAlSi and BaAlSiF particles). Both types of particle increased the release of IL-6 and IL-8 in a dose-dependent manner. BaAlFSi particles induced a more marked IL-8 response compared to BaAlSi particles. Mechanistic studies using specific inhibitors and activators indicated that cyclic AMP-dependent protein kinase A is partly involved in the observed IL-8 response; this inhibitory effect was not observed for IL-6. An increase in the transcription of the IL-6 and IL-8 gene was also observed. We concluded that these particles which are used in composite filling materials have the potential to induce adverse inflammatory response in BEAS 2B cells.

Paper II: Cell toxicity of methacrylate monomers – the role of glutathione adduct formation.

The aim of this study was to explore the mechanism of toxicity of five commonly used methacrylates monomers. The methacrylate monomers studied were HEMA, TEGDMA, BisGMA, GDMA and MMA. Methacrylate-GSH adducts formation and GSH depletion were studied and related to the toxicity of the methacrylates. All methacrylates were found able to deplete GSH levels in cells and/or cell-free environment, but the methacrylates differed in the potency to deplete GSH. Moreover, BisGMA was found not spontaneously form adducts with GSH. The toxicity of the methacrylates did not fully correspond to their ability to deplete GSH. A similar lack of correlation was also observed in the cell cycle analyses where only HEMA induced significant alteration. Together, the results indicate that mechanisms other than a GSH-dependent mechanism are involved in the toxicity of the investigated methacrylates.

Paper III: DNA-damage, cell-cycle arrest and apoptosis induced in BEAS-2B cells by 2-hydroxyethyl methacrylate (HEMA)

The aim of study III was to investigate mechanisms of HEMA-induced toxicity. Cells from BEAS-2B human lung epithelial cell line was exposed to HEMA (1-8 mM) for up to 48 h. Depletion of cellular glutathione (GSH) and an increased level of reactive oxygen species (ROS) were seen after 2 h of exposure, but the levels were restored to control levels after 12 h. 24 h of exposure to HEMA inhibited cell proliferation. At 24 h apoptotic cell death were also found. The result of the Comet assay indicates that HEMA induced DNA damage. In addition, phosphorylation of DNA-damage-associated signaling proteins including Chk2, H2AX, and p53 were observed. HEMA also induced an accumulation in the s-phase of the cell cycle. The antioxidant trolox did not counteract this HEMA-induced cell-cycle arrest, which indicates that the DNA damage is of non-oxidative origin.

Paper IV: TEGDMA and filler particles used in dental composites additively attenuate LPS-induced cytokine release from the monocyte-macrophage cell line RAW 264.

This study investigated if combined exposures to (methacrylate) monomers and particles from composite fillings caused additive, synergistic, or antagonistic effects on LPS-induced cytokine release in RAW264.7 cell line. The cells were exposed to The TEGDMA, silica (SiO_2) nanoparticles (12 nm) or quartz microparticles (1 µm) either alone or in combination. Both nanosilica and quarz particles reduced LPS induced IL-1β release in RAW264.7. However, only for nanosilica this reduction was significant (4 $\mu g/cm^2$). The cellular viability and the TNF-α release were not significantly affected by the exposures. To evaluate possible additive and synergistic response the induced effect of the combined exposure was compared to the sum of the individual exposure (calculated additive effect). Co-exposure to nanosilica and quartz resulted in an additive attenuation of the LPS-induced IL-1β release. The effect of co-exposure to TEGDMA and to the filler particles was also additive.

4. DISCUSSION

In recent years the amount and number of different dental composite filling materials on the market have increased. Short-term and long-term implications of exposure to particles and monomers from dental materials are not established. In line with the precautionary principle, biological mechanisms that could be involved in clinical effects should be addressed.

4.1 Methodological considerations

In vitro toxicity assays do not replace an *in vivo* system for exposure and toxicity. However, *in vitro* toxicity assays provide information which can accompany risk assessment (90). Choosing appropriate *in vitro* methods could be challenging and methods are not always accessible. As all *in vitro* methods have strengths and limitations, ideally more than one method should be applied (91).

4.1.1 Methods applied

Table 1 present the use of different *in vitro* model systems, biological endpoints and the methods used in the current studies.

4.1.2 Biological model system

In the current studies, *in vitro* cell line models were used to study effects of constituents in dental restorative materials. In paper I, II and III a human epithelial bronchial cell line (BEAS-2B) were used. BEAS-2B is an adenovirus transfected cell line and is chosen as a model system for an inhalation exposure and exposure of the GI-tract. The mice macrophage cell line (RAW246.7) was used as a model system in paper IV. Exposure to material constituents may lead to a local tissue response followed by infiltration of macrophages.

Cell lines are useful biological tool for studying cellular mechanisms, and results can be used as an indicator for important mechanisms involved in adverse health effects. However, cell lines can have altered properties compared with primary cells and the complexity of an *in vivo* situation, where multiple cells and factors constantly interact are absent. Consequently, results from studies on cell lines cannot be extrapolated directly to a clinical setting. On the other hand, cell lines are cost effective, easy to work with and have high reproducibility compared with primary cells and *in vivo* systems. In addition to the relevancy of the origin of the cell line a, stable model system that produces reproducible results is also of importance. In the current studies stable and well characterized cellular model systems were used.

Table 1: Overview of used methods, *in vitro* model systems and measured endpoints.

	Paper I	Paper II	Paper III	Paper IV
Biological system	Cell line (BEAS-2B)	Cell line (BEAS-2B)	Cell line (BEAS-2B)	Cell line (RAW 246.7)
Exposure	Particles (µm, nm)	Methacrylates	Methacrylates	Particles (µm,nm)/ methacrylates
Biological endpoint	Cytokines	Viability, DNA damage	Viability, GSH	Cytokines
Particle characterization				
SEM	X			X
TEM				
DLS	X			X
Protein level/ gene transcription				
Western		X	X	X
ELISA	X			X
PCR	X			
Flow cytometry		X	X	
Cell viability/ Growth				
Flow cytometry		X	X	
MTT		X		X
Cell growth assay			X	
Other				
LC/MS		X		

4.1.3 Cellular exposure

Particles

The particles used in the current studies are commonly used particles in commercial composite filling materials. To avoid interference from silane with the biological system the particles in the present studies were not silane treated. Due to water

absorption which could be followed by hydrolysis of silane bond, released particles *in vivo* could also lack the silane coating (92).

In vitro particle exposure presents several methodical challenges. Cell culture media, agglomeration state and sedimentation rate are of importance when evaluating particle toxicity *in vitro* (66). Physicochemical characterization (e.g. shape, aggregation, surface properties) of particles is essential for understanding interactions with biological environments (84, 93). For the particles used in the current studies, information on particle size and chemistry was provided by the manufacturer. Additional analysis of the size in solution as well as analysis of surface charge (zeta-potential) and morphology was performed (table 1) (paper I, IV).

A protein corona forms on the particle surface when suspended in protein containing media (94**.** This will influence the biological interactions with cells (94, 95). The corona formed is dependent on the particle surface characteristics and the proteins available in the media. Hence, the activity of the particles may differ with type and concentration of protein and serum in the cell culture media, and is also likely to differ from particle exposure *in vivo* (96). In the current studies low serum media was used in paper I while media with higher serum content was used in paper IV. It has been demonstrated a lower toxicity of SiO_2 in high serum media compared with serum free media, possibly due to flocculation and therby increased particle size. Results from the dynamic light scattering (DLS) revealed probable agglomeration of SiO_2 nanoparticles (paper IV). Agglomeration of particles could interfere with cellular interaction and uptake (95). However, this is not the case for particles in general, for example, addition of bovine serum albumin is also used to inhibit aggregation of nanoparticles (97).

To disperse agglomerates and aggregates before exposure the particle solutions used were subjected to ultrasound (sonicated). This is a commonly used procedure in *in vitro* nanotoxicology studies, but relevance can be questioned as it might not be likely that single particle exposure represents a realistic *in vivo* scenario.

Particle mass per surface area or volume is the most used dose measure exposing cells in vitro. However, the use of mass as an estimate for dose in toxicity studies of nanoparticles are currently debated (98). Number of particles or total particle surface area has been proposed as more appropriate measure for assessing the dose. In the present studies nano- and microparticles were used. It is problematic to compare the toxicity of nano- and microparticles based on a mass as a dose measure, as nanoparticles have much higher surface area pr mass compared to microparticles. However, accurate use of these metrics are difficult to accomplish technically (99).

Methacrylates

Concentrations of methacrylate monomers used in the present studies are in line with those used in other published works (63, 71, 75). HEMA, TEGDMA, GDMA, BisGMA and MMA were studied presently. All but MMA are used in both the dental filling composite and the adhesive systems. MMA is included in this study as a reference material. MMA are used in a number of medical, dental and industrial applications and has been extensively studied (100).

The solubility of methacrylate monomers in cell culture media varies. In the present studies HEMA, TEGDMA, MMA and GDMA were dissolved in medium, while BisGMA was dissolved in dimethyl sulfoxide (DMSO). BisGMA is highly viscous and has relatively low solubility in water, making it necessary to use a stock solution in DMSO. Concentration of DMSO used (<0.01 %) did not influence the endpoints studied. In line with this, other studies have reported similar results (101, 102).

4.2 Discussion of results

Composite fillings can release multiple components and create a complex exposure scenario. The microbial environment in the mouth will also add to the complexity of the system (103). Whether released monomers and particles can induce cell death, tissue inflammation or other adverse effects are important to clarify. Although, results

in an *in vitro* study cannot be directly extrapolated to an *in vivo* situation it provides useful insight into possible mechanisms involved in the toxicity of these materials.

4.2.1 Inflammatory potential of filler particles and methacrylates

An increased incidence of silicosis and respiratory conditions such as asthma, allergic alveolitis and rhinitis have been found among dental personnel, and are reported to be increasing (37, 104, 105). Local Inflammation, immunological effects (35, 37, 106), long term pulpal inflammation and irritation (107) and lichenoid reactions (38, 39) are clinically observed in relation to composite fillings. However, the cause and effect is difficult to establish. It has also been proposed that methacrylates used in composite filling materials can facilitate bacterial growth (41, 103).

An important topic in the present studies is the pro-inflammatory proteins, IL-6, IL-8, IL1β and TNF-alpha (paper I, IV). Altered levels of these cytokines have been implicated in many diseases including oral chronic inflammatory conditions such as periodontal diseases and oral lichen planus (108-111). In the recent years increased and chronic inflammation has also been associated with the onset and progression of cancer (112). Elevated levels of IL-6 are found in the saliva of patients with oral cancer and cancer of the head and neck (113). Cytokine signaling consists of complex networks where cytokines exert both pleiotropic and redundant effects. Studying release of only one cytokine *in vitro* will give an incomplete picture of the complex *in vivo* situation. Nonetheless, *in vitro* studies are important to study the potential involvement of single cytokines in biological responses to toxic components in a controlled environment.

In BEAS 2B cells particles increased release of cytokines (IL-6 and IL-8) (paper I) while in LPS-stimulated RAW 246.7 cells particles and monomers reduced the cytokine response (IL-1 β) (paper IV). The reason for this difference in response could be the use of different cells and exposure protocols. Relatively high exposure concentrations were used in paper I compared to paper IV. The concentrations chosen in paper IV aimed to use clinically relevant exposure, whereas the aim of paper I was

to address the potential to induce inflammatory mediators. However, the relevance of high dose acute exposure is a debated subject, as the real life setting will probably be chronic exposure to low concentrations over an extended time period.

The size of the dust particles released from composite filling materials due to wear and degradation can be influenced by the material composition (22). Also, particle size, size distribution and shape could potentially influence the degree of conversion and wear of the materials (13). Particle characteristics such as, chemical composition, size and morphology are also determinants for the biological interactions (93, 97). The specific properties of nanoparticles that are desirable and unique may also induce adverse biological effects. For example, higher cytotoxicity is observed for nanoparticles compared to micro particles of the same material (114, 115). The influence of particle composition and size is exemplified in paper I, where the potential to induce IL-8 differed significantly between two particles that differed slightly in their chemical composition. Moreover, SiO_2 nanoparticles showed higher toxicity at the same mass compared to the microparticles (paper 1). The mechanisms by which particles induce cytokine induction and transcription are in the current study not fully elucidated, but signaling pathways involving activation of MAP-kinase (116), NFκB (117), GSH/ROS (115, 117) and activation of the inflammasome (118) may take part in the inflammatory response to particles in general. Necrotic cell death can also initiate an inflammatory response due to release of intracellular substances to the surroundings (119). Cell death was therefore monitored to ensure that the observed response was not due to necrotic cell death.

The cytokine response in BEAS 2B cells was not investigated after exposure to monomers. However, in RAW 246.7 cells (paper IV) and in several other studies the inflammatory potential of methacrylate monomers has been explored. Methacrylates have been shown to both reduce (paper IV) (118, 120) and increase (121) cytokine release. Methacrylate-induced cytokine release could be due to increased cellular ROS, cellular stress responses and activation of NFκB (120). Mechanisms involved in the reduction of cytokines are unclear, but it has been suggested that it can be regulated posttranscriptional (118).

4.2.2 Methacrylate-induced cell death

Increased intracellular ROS due to GSH-methacrylate adduct formation has been suggested as the main mechanism involved in methacrylate-induced cell death. The GSH-methacrylate adduct is formed when the electrophilic β-carbon of the methacrylate undergoes a michael-type addition to nucleophiles (122). Altered ROS homeostasis in the cell leads to activation of numerous cellular stress responses and cell death (76, 123). In addition to DNA damage caused by ROS (52, 123), methacrylates have been proposed to directly interact with DNA which could lead to apoptotic cell death if the damage is extensive (124, 125).

Methacrylates induce cell death in a variety of both cell lines and primary cells although the potency to do so may vary (paper II) (77, 101). MTT/XTT assays are the most commonly used methods to evaluate methacrylate-induced cytotoxicity, recording SDH-activity as a measure of cytotoxicity and cell proliferation. Methacrylates are reported to induce both growth inhibition and cell death (70). However, studies have shown accordance between MTT/XTT data and microscopic evaluation of cell death (63, 126).

GDMA, TEGDMA and BisGMA induced dose-dependent cell death in BEAS 2B cells (paper II). In contrast HEMA and MMA did not induce measurable cell death at the concentrations used. Moreover, HEMA, UDMA, BisGMA and TEGDMA have induced significant dose-dependent cell death in other cell lines (64, 126, 127). In general, apoptotic cell death seems to be more prevalent in the lower concentration range whereas necrosis seems to dominate when cells are exposed to higher concentrations (76, 126). Results from paper II revealed that other mechanisms other than GSH-dependent are likely to be involved in the toxicity of these methacrylates. Cell death induced by BisGMA was prevalent at low concentration where GSH depletion was not found. Hence, BisGMA seem to be a methacrylate causing toxicity independent of GSH binding.

DNA damage can be initiated by direct interaction with DNA. Possible mechanisms are nucleophilic attack on DNA leading to formation of methacrylate-DNA adduct

(125) and DNA interactions through methacrylate intermediate epoxide formation (128, 129). In paper II it was observed that the potential to induce alterations in the cell cycle differed between the methacrylates, and that the ability to deplete GSH did not appear to be the main factor involved, as HEMA was the only methacrylate that induced alterations in the cell cycle. In paper III the mechanism of HEMA induced toxicity with emphasis on the potential to induce DNA damage was investigated. Activation of signaling proteins involved in the DNA damage sensor, transducer and effector signaling was observed. In cells exposed to HEMA H2AX and Chk2, but not Chk1 were activated. The absence of Chk1 activation indicates that HEMA induces double strand breaks (58). Genotoxic potential of MMA, BisGMA, GDMA and TEGDMA in BEAS2B cells cannot be ruled out. DNA damage can be present without inducing cell cycle arrest or cell death due to rapid repair of the damage. Induction of DNA damage response, repair and cell death if the damage is too extensive is a crucial response due to the importance of eliminating damaged cells. Overall, the present studies demonstrate that methacrylate monomers can initiate DNA damages. However, DNA damages may not always be recognized and repaired, advancing the damaged cells as a possible outcome.

4.2.3 Combined exposure

Particles from wear and handling consist of mixture of both particles and particle embedded in matrix (22). Although it would be comparable to the clinical situation, such an exposure system would be very complex and could produce data that are difficult to interpret due to the multiple and uncontrolled variables.

In the present study we used a “bottom up” approach for combined exposure was applied (paper IV). Meaning that cells are exposed to individual components to elucidate separate mechanism and secondary investigate possible interactions. A different approach would be to expose to a complex mixture (“top down”) (130). Effect of compounds in a mixture that have the similar mode of action would probably be different compared to compounds that have a dissimilar mode of action (131). This

emphasizes the importance of elucidating biological mechanisms for the individual compounds. Synergism and antagonism have previously been demonstrated for methacrylates *in vitro* (132). In paper IV co-exposure to particles and monomer (TEGDMA) had an additive effect on the IL-1β release in RAW 246.7 cells. Thus, interactions between components in composite filling materials could result in a higher total biological burden than from the individual exposures.

5. CONCLUSIONS

The present study has provides new information about the toxicity of particles and methacrylates used in polymer-based dental materials. The overall hypothesis is supported as components in polymer-based dental materials are shown to induce toxic responses.

1. Micro- and nanoparticles show inflammatory potential *in vitro*. However, there is a difference in the inflammatory potential between the particles studied. Combined exposure to monomers and particles induce an inflammatory response, this response is additive.

2. The methacrylate monomers in this study induce cell death, but the potential differs. GSH adduct formation appears not be the main mechanism of toxicity of methacrylates. In contrast to the other methacrylates studied HEMA induces cell cycle alterations. HEMA also induces DNA damage and DNA damage associated responses.

FUTURE PERSPECTIVES

The present work presents new results regarding the potential of components in dental filling materials to induce adverse biological responses, but also opened new questions that should be addressed in future studies.

In the current studies pure particles were used. In the future clinically relevant wear particulates should both be characterized and studied biologically. In paper IV the interesting aspect of co-exposure to methacrylates and particulates was raised. This is clinically relevant for both personnel and dental patients and further studies on co-exposure are needed to elucidate the toxicity of composite filling materials.

Several new research questions emerged regarding the mechanism of toxicity of methacrylates. Differences in the mechanisms were found between methacrylates, especially BisGMA seemed to have a mechanism of toxicity that was not dependent on adduct formations with GSH, and induced toxicity at low exposure concentrations. Further studies that enlighten the relation between the chemical structures and toxicity should be undertaken.

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