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Methacrylates in polymer-resin based dental materials

Assessment of exposure and biological effects

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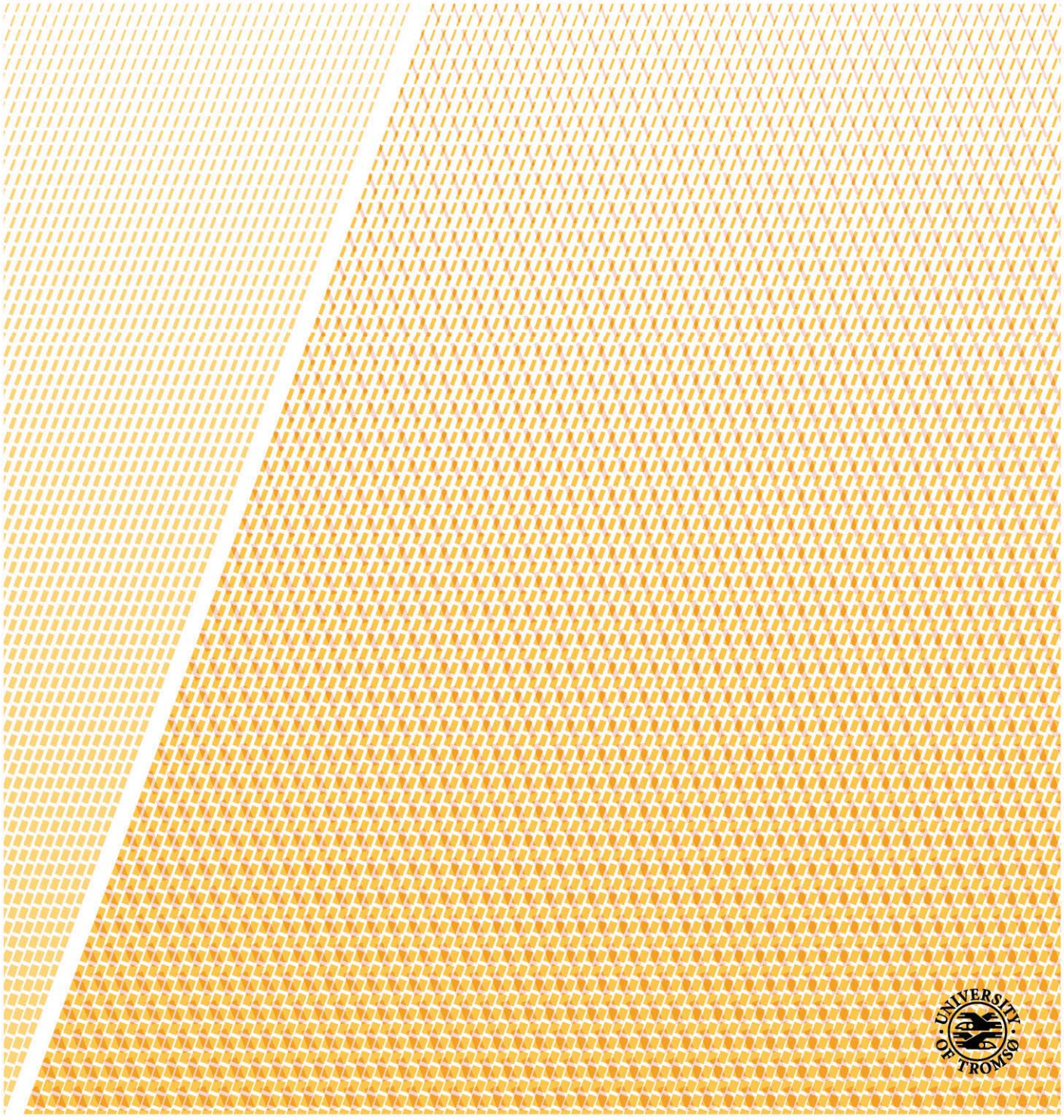
Bo Wold Nilsen

A dissertation for the degree of Philosophiae Doctor



“The difficulty lies, not in the new ideas, but in escaping from the old ones.”

John Maynard Keynes (1883 - 1946)

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Abbreviations and terms

Abbreviation/term	Explanation
(M)SDS	(Medical) safety data sheet
(UPH)LC-MS	(Ultra-High Performance) Liquid Chromatography
ADME	Absorption, Distribution, Metabolism and Excretion (Toxicokinetic terms)
Bis-EMA	Bisphenol A ethoxylate dimethacrylate
Bis-GMA	Bisphenol-A diglycidylmethacrylate
CLP	Labeling and Packaging of substances and mixtures Regulation (EU)
d_{ae}	Aerodynamic equivalent diameter
GC-MS	Gas chromatography–mass spectrometry
Genes names	All gene abbreviations are italicized and capitalized, *e.g.*, heme oxygenase gene (*HMOX1*)*
GSH	Globally Harmonized System of Classification and Labelling of Chemicals
HEMA	2-hydroxyethyl methacrylate
In silico	Performed on computer or via computer simulation
m/z	mass to charge (m/z) ratio
MDR/MDD	Medical Device Regulation/Medical Device Directive
NRF2	A transcription factor. Referred to the master regulator of antioxidant responses.
Omics	Omics, *e.g.*, proteomics, aims at the collective characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of an organism or organisms
PRM	Polymer resin-based dental material
Protein names	All protein abbreviations are capitalized version of the gene-name, *e.g.*, heme oxygenase protein (HMOX1)* Abbreviation used for regulated proteins are presented in appendix 1a, 1b
SILAC	Stable Isotope Labeling by Amino acids in Cell culture
TEGDMA	Triethylene glycol dimethacrylate
UDMA	Urethane dimethacrylate

* In line with the recommendations from the HUGO Gene Nomenclature Committee (1)

List of papers

This thesis is based on the following papers, which are referred to with their corresponding roman numerals in the text:

I. Methods and terminology used in cell-culture studies of low-dose effects of matrix constituents of polymer resin-based dental materials

Nilsen BW, Örtengren U, Simon-Santamaria J, Sørensen KK, Michelsen VB. European Journal of Oral Sciences 2016; Volume 124(6):p.511-525. doi: 10.1111/eos.12309.

II. Dose- and time-dependent effects of triethylene glycol dimethacrylate on the proteome of human THP-1 monocytes

Nilsen BW, Simon-Santamaria J, Örtengren U, Jensen E, Bruun JA, Michelsen VB, Sørensen KK

Submitted

III. Analysis of organic components in resin-modified pulp capping materials: critical considerations

Nilsen BW, Örtengren U, Jensen E, Michelsen VB.
European Journal of Oral Sciences 2017; Volume 125 (3): p.183 - 194. doi: 10.1111/eos.12347

IV. Exposure to gaseous and particle-associated organic substances in polymer-based dental materials during restorative procedures

Nilsen BW, Jensen E, Örtengren U, Bang B, Michelsen VB

Submitted

Summary

Materials used in restorative dentistry today are primarily polymer-resin based dental materials (PRMs). These materials contain methacrylates and other organic additives that may cause adverse effects in exposed patients and dental personnel. Despite that relative few adverse effects are reported for this type of materials, this does not rule out that adverse effects may occur. The purpose of this thesis was therefore to study biological effects of one of the most commonly used methacrylate in PRMs, and to investigate how patients and dental health personnel may be exposed to constituents in PRMs.

In paper I, methods and terminology used in cell culture studies on PRM constituents' toxicity were charted by systematic searches in several search engines. It was found that non-standardized nomenclature and methods are commonly used. In paper II, biological effects of triethylene glycol dimethacrylate (TEGDMA) were charted on the proteomic level in the human THP-1 monocyte cell line with the metabolic labeling strategy SILAC followed by liquid chromatography-mass spectrometry analysis. It was shown that TEGDMA induces time- and dose-dependent effects on cells, even at doses previously reported as non-toxic (as shown in Paper I).

In the studies in paper III and IV, liquid- and gas chromatography-mass spectrometry-based techniques were used to characterize and estimate exposure to organic substances in PRMs. More specifically, in paper III, the organic composition of, and eluates from, resin-modified pulp capping materials were examined. It was found that patients may be exposed to a range of organic substances, including methacrylates, if these materials are used for direct capping. In paper IV, release of particle-associated and gaseous organic substances in PRMs

during restorative procedures were examined in a simulated, clinical environment. The results of this study reinforced the notion that occupational exposure to particle-associated organic substances in PRMs may occur. However, it was also shown that this exposure was below the limit of detection during clinical circumstances.

In conclusion, this thesis add novel knowledge, and strengthen the current understanding, of how patients and dental personnel may be exposed to organic substances in PRMs. It also reinforce the notion that methacrylates are reactive compounds that can induce several biological effects in exposed cells.

Introduction

1 General background

1.1 Biomaterials in dentistry – and potential risks

Biomaterials can be defined as materials intended to be used inside or in contact with the human body (2). In dentistry, biomaterials are extensively used for a range of purposes. While many types of biomaterials exist, polymer resin-based dental materials (PRMs) are perhaps the most widely used materials today — in particular for direct restoration procedures with dental composites and adhesives. In fact, the use of these tooth colored restorations has soared in Scandinavian countries subsequent to the ban/limitations of amalgam (3). However, other reasons, such as patients demand for aesthetics, have also contributed to the increased use of these materials on a global scale. In 2011, it was calculated that more tooth surfaces were filled with composite than with amalgam (4). In light of the widespread use of PRMs, it may be questioned whether these materials, or rather their constituents, pose any risk for patients and/or dental health personnel.

Epidemiological research suggests that the frequency of adverse effects, in general, are low for PRMs compared to other dental materials, *e.g.*, alloys (5). However, direct contact with uncured PRMs (6) or repeated, low dose exposure to PRM constituents may induce adverse reactions (7–10). Several studies have shown that occupational effects of these substances could be of concern (7–10). For example, PIIRILÄÄ et al showed an increase in respiratory hypersensitivity for dental personnel after the transition to PRMs (9). Contact allergy to PRM constituents have also been documented (7,11,12). Still, limited numbers of

studies, absence of national reporting systems, and/or lack of symptomatic effects could imply that it is difficult to assess the total extent of adversities associated with substances in PRMs (5). As PRMs are among the most frequently used biomaterials in humans, exposure and effects of PRM constituents should be thoroughly examined to assess the risk associated with these materials. The purpose of this thesis was therefore to add novel knowledge about biological effects of one of the most commonly used methacrylates in PRMs, triethylene glycol dimethacrylate (TEGDMA), and to investigate how patients and dental health personnel may be exposed to TEGDMA and other PRM constituents.

1.2 Composition of PRMs

PRMs mainly consist of inorganic and/or organic filler particles embedded in a matrix consisting of monomers and additives (Figure 1). In PRMs for direct restorative treatment, the monomers are usually methacrylates; however, other monomers exists (*e.g.* ormocers and siloranes). Since this thesis will focus on the methacrylate-based PRMs, all further references to PRMs imply that these materials have a methacrylate matrix. The physical and biological properties of PRMs are influenced by the ratio and type of fillers and monomers. Thus, ideally, the composition of PRMs are tailored for their indication for use. Some PRMs contain other, sometimes therapeutic, ingredients that cannot readily be classified under either fillers or matrix constituents (discussed in section 1.2.3).

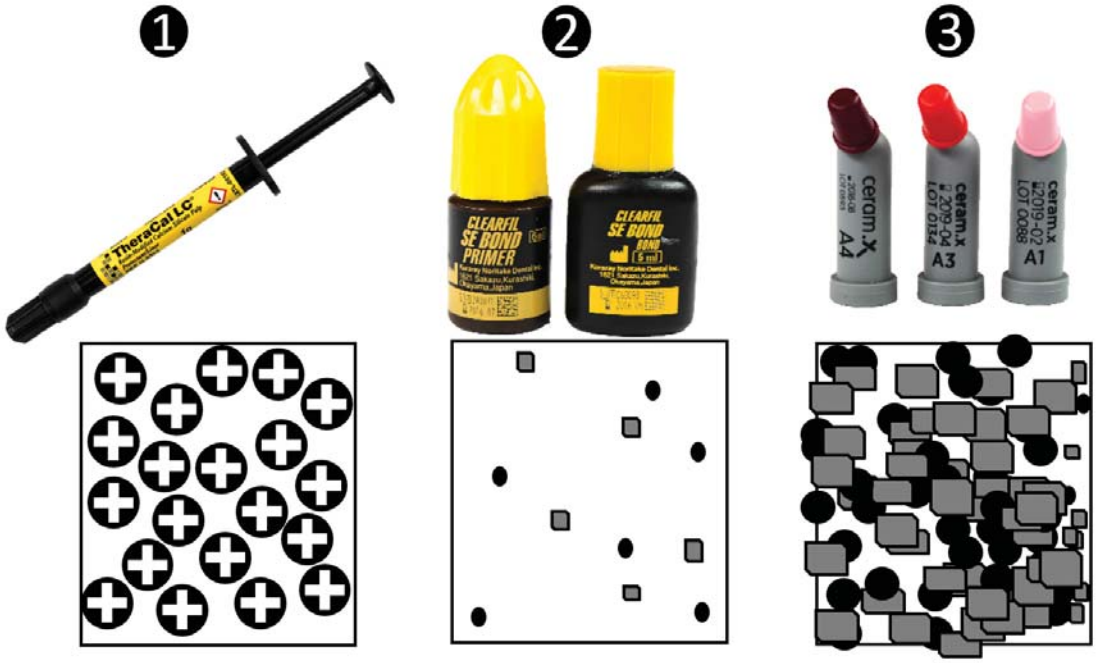

Figure 1: Schematic structures of three different PRMs with different applications. Filler particles (black and grey) are embedded in a continuous, polymer matrix-phase (white). Therapeutic agents are labeled with a cross. 1: Light curing capping materials (here exemplified by Theracal® LC (Bisco)) contain therapeutic agents as they are indicated for vital pulp therapy. 2: Adhesives (here exemplified by Clearfil SE Bond (Kuraray)) contain low amount of filler particles, to increase wettability. 3: Universal composites, (illustrated by ceram.x® universal (Densply)) contain large amount of filler particles to increase the strength of the material. Images: Bo Wold Nilsen

1.2.1 The filler phase

Filler particles make up the discontinued phase of PRMs and have many functions, *e.g.*, fillers reduce polymerization shrinkage, water sorption and thermal expansion and increase the strength, viscosity, wear-resistance, and stiffness of materials (13). Filler loading of materials usually reflects their intended application, *e.g.* hybrid, all-purpose composites usually contain between 75% to 80% by weight, and 60% to 65% by volume, of fillers (13). In comparison, adhesives contain slight amounts or no fillers (14). Fillers are in general relative biological inert compared to the constituents in the matrix phase of PRMs; however, concerns have been raised regarding the inhalation of nano-sized fillers generated during certain restorative procedures (15–18).

1.2.2 The matrix phase

The matrix represents the continuous, curable phase of PRMs, and consists of methacrylates and small amounts of additives (*e.g.* initiators, activators, inhibitors, and stabilizers). The proportion and type of methacrylates (and additives) will affect their biocompatibility, as well as their physical/chemical properties. For example, a high content of high molecular weight methacrylates such as bisphenol-A diglycidylmethacrylate (Bis-GMA) and urethane dimethacrylate (UDMA), will yield a viscous material (19). In comparison, low molecular weight monomers such as TEGDMA will dilute this effect, making the introduction of fillers during manufacturing easier and enhance the clinical handling of PRMs. TEGDMA is therefore a common ingredient in both high- and low-viscous PRMs (14).

For a majority of PRMs, polymerization of the matrix phase is initiated by light in the 370 – 470 nm spectrum range (20). Polymerization of monomers in PRMs is never fully completed as crosslinking of chains disables movement of monomers within the bulk of the material. Under ideal conditions, this results in a maximum double bond conversion of about 60 – 70 %, and 2-3 % unreacted monomers in the bulk of the material (21,22). Inadequately cured materials will have a poorer double bond conversion, and greater amount of unreacted monomers (and additives), that may be a source of exposure for PRM constituents (23,24).

In contrast to fillers, most matrix constituents are reactive by nature. Several studies have shown that additives cause adverse biological responses in cell cultures (25–28). Regarding monomers, methacrylates are electrophilic, α,β-unsaturated carbonyls with the ability to form polymer-networks through free radical polymerization. Importantly, this also enables them to react with

bionucleophiles such as DNA and proteins (29). However, due to different molecular structures, the biological effect and potency of methacrylates vary. For example, Bis-GMA cause cytotoxic effects *in vitro* at much lower concentrations than HEMA and TEGDMA (30).

1.2.2.1 TEGDMA

TEGDMA is present in many PRMs, and is the methacrylate being tested for biological effects in paper II. TEGDMA is a dimethacrylate with a molecular weight of 286.324 g/mol. The molecule consists of two methacrylate groups, in addition to three repeated units of ethylene glycol (Figure 2).

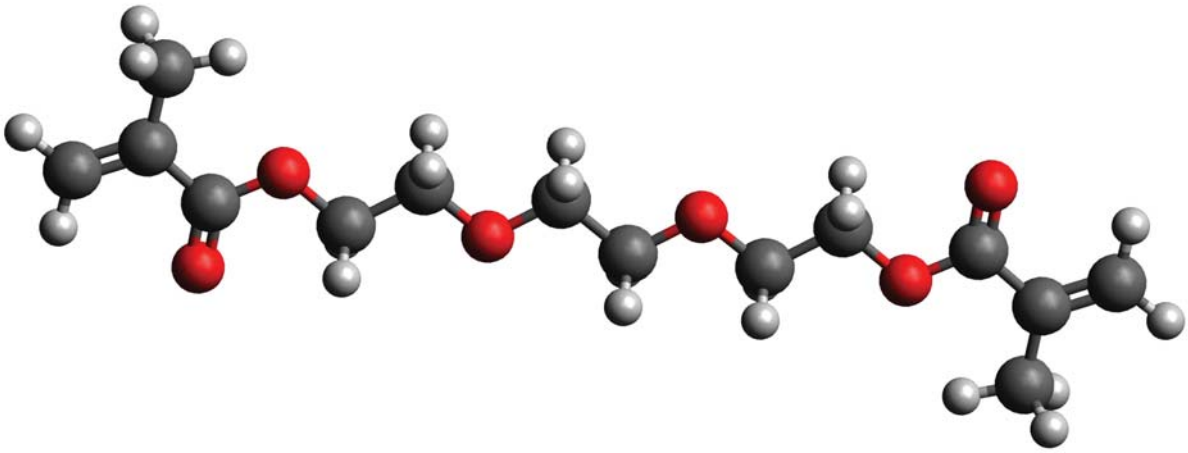

Figure 2: The 3d structure of TEGDMA. Model generated in Avogadro molecule editor and visualizer *(31)*.

The TEGDMA molecule has several rotatable bonds, and is highly flexible and mobile compared to other methacrylates, *e.g.*, Bis-GMA. This, combined with a slight water-solubility, makes it one of the major eluates from PRMs (23). TEGDMA is also one of two monomers — the other being HEMA — which have been shown to have the potential to cross the tooth dentin layer during restorative procedures (32–34). The ester bonds of methacrylates are sensitive to non-enzymatic and enzymatic hydrolysis. However, the extent of susceptibility seems to be methacrylate and enzyme-specific (35). For example, TEGDMA demonstrates

higher susceptibility than Bis-GMA to enzymatic hydrolysis (likely due to the availability of the ester bonds) (35). TEGDMA's susceptibility to enzymatic hydrolysis may also explain the rapid elimination of TEGDMA (~ 24 hours) observed in guinea pigs and mice models (36). When hydrolyzed, TEGDMA yields two methacrylic acid molecules and a tri-ethylene glycol chain - both less toxic than TEGDMA itself (37). However, *in vitro* studies suggest that other mechanisms may be involved in the elimination of TEGDMA in humans, as human lung-cells preferably form two epoxy-intermediates of 2,3-epoxymethacrylic acid when exposed to TEGDMA (38). The epoxy intermediate is reported to be as toxic as TEGDMA itself (39). TEGDMA has, owing to its amphiphilic nature, the ability to move in all compartments of a cell culture (cytosol, lipid fraction, culture medium), and can therefore cause a range of toxic effects in cells (40). Yet, the precise mechanisms of TEGDMA-induced toxicity is not yet fully understood. This was further explored in paper II.

1.2.3 Other components of PRMs

The light-curing ability of methacrylate-containing materials makes them easy to handle in the clinic. This have led to their introduction in groups of materials that traditionally did not contain methacrylates, *e.g.*, glass ionomer cement and pulp capping materials. In the latter group of materials, it may be speculated if the presence of PRM constituents will negatively influence the clinical efficacy of the material.

Resin modified pulp-capping materials are indicated for either indirect or direct contact with pulp tissue. They contain methacrylates and organic additives, in addition to substances usually found in traditional, pulp capping agents, *i.e.* calcium hydroxide or calcium silicates (also referred to as Portland cement by the

manufactures). While the benefit of light curable materials is their easy handling, their use as direct pulp capping materials likely also implies patient-exposure to high concentrations of reactive matrix constituents (32). To the knowledge of the author, there are no published clinical trials with long term follow-ups (≥12 months) on these materials (41), and at present, only one published trail with 6 months follow-up exist (showing a non-significant, lower survival for Theracal® LC vs calcium hydroxide without laser therapy) (42). Yet, despite that there are pulp capping materials with ample data on clinical efficacy (43–45), light curing capping materials are advertised and sold to dentists all over the world. In fact, the light-curing resin modified pulp-capping material Theracal® LC was shown to be more widely used for capping procedures among Norwegian dentists than mineral trioxide aggregates (46) — a material that has demonstrated its clinical feasibility for this purposes in several clinical trials (43–45). The composition of resin-modified pulp capping materials, as well as their indications for use, have been investigated and critically discussed in paper III.

1.2.4 Regulation and labeling of PRMs

Dental materials are medical devices according to the European Medical Devices Directives (MDD) (47). Thus, these materials have to meet the general requirements of this directive to achieve the CE-marking that is required for permitting a material to be sold in the European Economic Area (including Norway). By CE-marking, the manufacturer demonstrates that a product complies with the applicable requirements of the regulation and other applicable harmonized Union legislation. As of 2017, the MDD will be replaced by the Medical device regulations (MDR), however with a transition period of three years (48). The aim of the new regulation is to address inherent weaknesses in the old directives, as

well as providing improvements such as the establishment of a comprehensive database on medical devices and strengthening of post-market surveillance (*e.g.* trend reporting) (48).

According to the MDD and MDR, medical devices should not compromise the clinical condition and/or safety of the patients or user (47,48). If risk is linked to the material, this must be weighed against the benefits of the device (47,48). Medical devices should not achieve its intended action by pharmacological, immunological, or metabolic means (47,48). Medical devices are placed in one of four categories based on the intended application and risk associated with the device (Table 1). Depending on the classification, different test regimes are required prior to approval. For example, Class IIa and Class IIb materials, do not demand the same extent of tests and documentation to fulfilling the criteria of the MDR/MDD as class III. However, any dental materials for long-term use (more than 30 days) classified as class II or higher (*e.g.* composite, resin-based pulp capping materials etc.), require that an independent part (*i.e.* notified body) has to control that the requirements are followed by the manufacturer.

Table 1: The European Union's Medical Devices Directive/regulative for classification of medical devices. A full description of rules that govern the classification of medical devices is available in the Annex VIII of the MDR *(48)*.

Class	Description (example)
I	Non-invasive products (*e.g.* bandages) Invasive products for transient contact (*e.g.* impression materials)
IIa	Surgically invasive products (*e.g.* PRMs)
IIb	Intraosseous dental implants
III	Products with drug-like effects (*e.g.* endodontic sealers with antibiotics)

As dental products sold in the EU may contain hazardous ingredients, they should be labeled and supplied with an information sheet in accordance with the Classification, Labeling and Packaging of substances and mixtures Regulation (CLP) – and of 2017, the MDR (49). The information sheets were previously available in many versions (implying that a material could have several differently labeled sheets). However, the EU has since January 2008 adopted the United Nations *Globally Harmonized System of Classification and Labelling of Chemicals* (GHS) through the CLP regulation(49). This implies that Medical Safety Data Sheets (MSDS) have been replaced by universal Safety Data Sheets (SDS). GSH has also been adopted by many countries outside EU, including Canada, United States, and Norway. As of today, GSH regulations are enforced in the EU, and in all the other countries mentioned.

With regard to the constituents of dental materials, Safety Data sheets (MSDS or SDS) can be a guidance for clinicians of which materials to avoid in case of sensitivity or toxicity concerns as SDS' should provide the necessary details to identify potential hazardous substances in a material. In addition to the name of substances, a CAS number (50) – an unique numerical identifier of a substance – should be provided. In previous studies, it has been shown that SDSs for PRMs, and other products, are incomplete (51–56). For example, Michelsen et al found that 25 – 85 % of quantifiable organic eluates from PRMS were not reported in the SDS of the material (53). One can hope that the new regulations will improve the situation, *i.e.*, that dentists will be better able to identify which substances they may expose patients or themselves towards when using dental materials. Problems associated with SDSs and CAS-numbers in relation to exposure assessment was discussed in Paper III and IV.

2 Exposure to organic constituents of PRMs

2.1 General

Exposure to a substance is a prerequisite of an adverse effect. Data on exposure is therefore also a prerequisite for conducting human health risk assessments. For constituents of PRMs, both occupational and non-occupational exposure occur (12,57). Exposure scenarios for patients and dental personnel are illustrated in Figure 3.

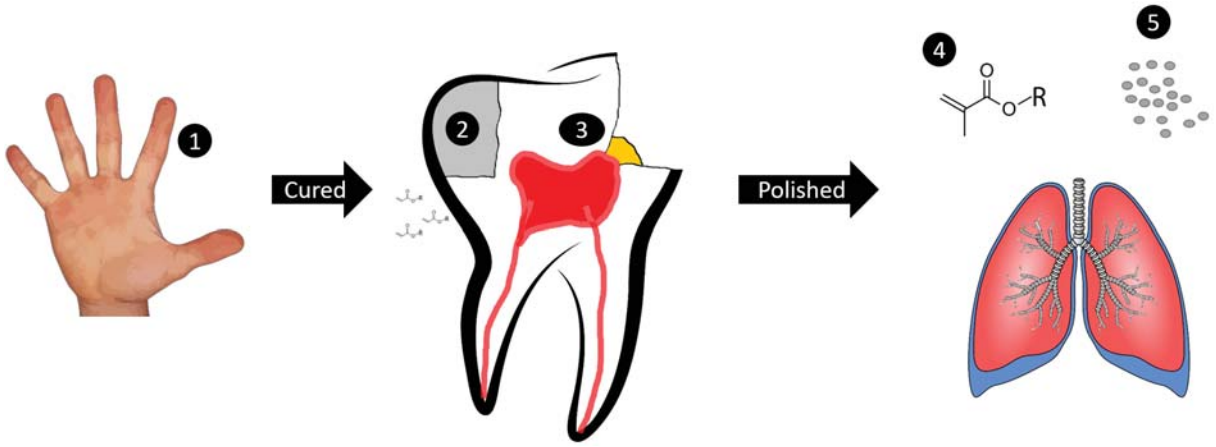

Figure 3: Illustration of exposure scenarios relevant for PRM constituents. 1: during direct contact with skin; 2: elution from cured materials into the oral cavity; 3: direct exposure to pulp-tissue; 4: inhalation of methacrylates in gas-phase: 5: elution of PRM-constituents from inhaled particles.
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2.2 Exposure, dose and route of exposure

Exposure and dose are closely linked, but separate entities. Exposure represent the opportunity for a substance to enter the body, and is a product of intensity, frequency and duration of exposures (58). Subsequently, the net exposure to PRM constituents during a lifetime is higher for dental personnel than for patients. Exposure to a substance can by definition be prevented. For PRM

constituents, relevant preventive measures include the use of gloves, high-vacuum suction, rubber dam, and the use of water during clinical procedures (59).

In toxicology, the dose is the fraction of the exposure that reach a particular site where it can exert an effect (60). This may be the local dose, the intracellular dose, or more commonly used, the internal dose. The internal dose is the amounts of substance that reach the circulation, and is influenced by the absorption, distribution, metabolism and excretion (ADME) characteristics of a particular substance. In addition, the exposure route of a substance can heavily influence the absorption of a substance. This is partly due to differences in the thickness and type of tissue that make up barriers against toxicants (61). For example, exposure to a toxicant via the lungs is in general regarded as more potent than skin or mucosal exposure, as the epithelial barrier of the pulmonary alveolus is only two cell layer thick (61). In a wound, *e.g.*, a pulp exposure, there is no barrier (implying a 100 % absorption of a toxicant).

The physiochemical properties of a substance also influences several important factors related to exposure and ADME, *e.g.*, lipophilicity of a substance influences all aspects of ADME (62). In general, water-soluble substances have a shorter half-life than that of fat-soluble substances, and are not as readily absorbed through the skin. Volatility of a substance, and thus the likelihood of inhaling the substance, is also influenced by its physiochemical properties. Important chemical characteristics relevant for exposure of three common PRM constituents are presented in Table 2.

Table 2: Summary of chemical characteristics which may influence exposure route and absorption of common methacrylates used in PRMs.

Parameter	Bis-GMA	TEGDMA	HEMA	MMA
CAS number*	1565-94-2	109-16-0	868-77-9	80-62-6
Predicted solubility (Log K_{OW}**) (63)	Log K_{OW} = 5.53	Log K_{OW} = 1.81	Log K_{OW} = 0.5	Log K_{OW} = 1.35
Vapor pressure (64)	-	9.4×10^{-4} mmHg	0.126 mmHg	38.5 mmHg

*CAS: Chemical Abstracts Service.

** Log K_{OW} is the partition coefficient of this substance for octanol and water. A positive number suggest that the material is more hydrophobic than hydrophilic. Substances with a Log K_{OW} above 4.5 have bio-accumulative abilities (in adipose tissues) due to low rate of elimination from the body.

With regard to exposure assessments and human health risk assessments, indirect measurements in the environment is more commonly used than direct measurements of exposure (*e.g.* measuring of toxicants in body fluids) (58). This is most likely because indirect measurements are less invasive and cheaper.

2.3 Patient exposure to PRM constituents

Patients may be exposed to leaching substances from all PRMs used in dentistry. This is due to unreacted and hydrolysis sensitive substances in PRMs that are exposed to humid conditions in the oral cavity. Evidence from laboratory studies on PRMs, suggest that the total amount of elutes are low (in the µg-range); however, variations have been reported between materials (23). Dissimilarities in eluates between materials can be explained by the degree of curing and by the composition of the PRMs (65,66). For example, the combined elution of TEGDMA and Bis-GMA from poorly cured composite samples have been demonstrated to be approximately 7-fold higher than that of a sufficiently cured material (67). With regard to monomers, some substances elutes readily, *i.e.*, low molecular weight,

water soluble, mobile molecules (TEGDMA, HEMA); while poorly soluble, less mobile, high-molecular weight substances (Bis-GMA) are released at a much lower rate (68). The release of unreacted monomers is in general highest the first day after a restoration is placed, and decreases rapidly thereafter (69).

While PRMs used for restoration purposes mainly expose patients to the constituents through elution of substances into the oral cavity, other PRMs may expose the dental pulp to PRM constituents indirectly or directly. In case of indirect exposure, this most likely occurs for flexible and low-viscous monomers, like HEMA and TEGDMA, that often are present in low-viscous bonding agents (14). Laboratory studies have suggested that HEMA may reach concentrations in the range of 0.2–3.6mM during application of bonding (34). However, these concentrations were reached in 30 min diffusion experiments ex vivo which are much longer than what would occur under clinical conditions (70). In another laboratory study, where the bonding agent was applied as recommended by the manufactures, it was shown that the amount of TEGDMA or HEMA entering the pulp chamber through 2 mm of dentin are in the range of 0.04–0.2 µg after three days (33). In case of a deep cavity (less than 0.5 mm between the pulp and the restoration), it has been calculated (based on the diluting effect of dentin) that TEGDMA concentrations can reach as high as 4mM in the pulp chamber (32,71). Still, concentrations of PRM constituents reaching the pulp during ordinary use are likely not causing acute toxicity, as PRMs for restorative procedures are in general used with clinical success. However, this does not imply that PRM constituents reaching the pulp do not adversely affect tissue homeostasis at a subclinical level.

Concerning exposure to PRM constituents, direct application of resin-modified pulp capping materials (Section 1.2.3) to pulp tissue may demonstrate a potent

route of exposure to PRM constituents. Few studies have looked in detail into the composition of these materials, and this is studied and discussed in paper III.

2.4 Occupational exposure to PRM constituents

Occupational exposure to PRM constituents can occur (12,57). Dental personnel are at risk of direct contact with unreacted monomers during handling of PRMs. Exposure to PRM constituents can even occur when gloves are used, as they do not readily protect against exposure (72,73). In addition, as an average worker inhales thousands of liters of air during a 8-hour work day, airborne exposure to PRM constituents is likely (74). Clinic measurements of gaseous exposure to methacrylates show that dental professionals may be exposed to HEMA and methyl methacrylate (57), *e.g.* HEMA levels of approximately 80 µg/m^3 have been measured during procedure-specific monitoring (57). Interestingly, ~50% of measurements were below the limit of quantification for both substances (57). Concerning TEGDMA, maximum air concentrations of 45 µg/m^3 and 81 µg/m^3 have been reported during adhesive procedures and removal of old PRM restorations, respectively (75,76). The latter suggests that there are latent, unreacted methacrylates in the bulk of PRM-materials. However, since there was no information in the articles about the brand/type of materials used during the procedures, it is difficult to assess material-specific contribution to the estimated exposure.

Composite particles may also be inhaled during polishing and removal of PRMs as a large portion of the particles generated during these procedures are respirable (15–17,77). In relation to PRM constituents, the large surface area of micro- and nano-sized particles (15,78), may enable unreacted constituents to elute from inhaled PRM particles in the humid environment of the lungs. In a recent

laboratory study, elution of both high and low-molecular substances from inhalable PRM dust was shown (24). In relation to exposure to PRM constituents, this finding signify that particles can act as vehicles for non-volatile substances that normally would not reach lung tissue (*e.g.* Bis-GMA) (24). However, this has not yet been confirmed to occur under clinical relevant conditions (where high-vacuum suction and water is used during the procedure), and was therefore investigated in paper IV.

2.5 Exposure to methacrylates in non-dental settings

Apart from exposure in dental practice, patients and dental personnel can be exposed and sensitized to methacrylates and organic substances found in PRMs in non-dental settings. Sensitization is especially relevant for cosmetic products containing methacrylates used in dentistry (*e.g.* HEMA and TEGDMA) (79–81). However, since cross-reactions between methacrylates and acrylates do occur (82,83), hypersensitivity can also occur as a results of exposure to "non-dental" methacrylates in products used during nail sculpturing or eyelash extension procedures (84,85). In analogy to occupation effects observed in dentistry (9,86), cases of asthma and dermal sensitization to methacrylates have also been reported to occur in staff performing nail sculpturing (79,82). Allergic reactions towards hearing aid materials that contains polyethylene glycol dimethacrylate – a substance present in the resin-modified capping material Theracal® LC – have also been reported (87).

2.6 Assessing exposure

An exposure assessment typically involves a collection and analysis of samples. The methods used in these steps will vary depending on the sample of interest, as well as the type of analysis required. Sampling and analysis of eluates from cured samples of resin modified pulp-capping materials and inhalable particles were performed in Paper III and IV, respectively. In paper IV, assessment of gaseous exposure was also performed.

2.6.1 Sample collection - Eluates

A common exposure assessment in dentistry is to evaluate leachables – or eluates - from composite restorations (18). This is usually performed under experimental conditions by immersing pre-cured samples of composite in an extraction solution for a period of time. A systematic review on this topic has shown pronounced variation in methodology in studies on this subject, including variation in surface-area of samples, volume of extraction solution, type of extraction solution (*e.g.* water, ethanol, methanol), immersion time and temperature, as well as the method used to analyze the collected samples (23).

The method used to assess leaching will most likely influence the obtained results. It has been shown that a protein-containing immersion medium (native saliva) yields a significantly lowered amount of detectable and quantifiable substances compared to immersion mediums without proteins (88). With regard to clinical relevance, cyclic stress, endogenous and bacterial esterases (35,89,90), and fluctuating pH and temperature may influence elution of unreacted substances from PRMs *in vivo*.

2.6.2 Sample collection – Gas

Release of gaseous methacrylates is not as commonly studied as eluates; however, some studies have been published on this subject (57,75,76). The collection of gaseous substances is usually performed by the use of sorbents used in conjunction with a personal carried sampling pump. Different sorbents may influence the obtained results as sorbent have dissimilar affinity for substances. In paper IV, a sorbent suitable to capture methacrylates – in particular TEGDMA – was used. After sampling, organic substances are typically extracted from the sorbents with solvents, prior to analysis.

2.6.3 Sample collection - Particles

Dust particles can be collected by different types of equipment attached to personnel carried pumps. The equipment used determines which type of particles that can be sampled. Particles relevant for health effects are all particles with an aerodynamic equivalent diameter (d_{ae}) below 100 µm (91,92). These particles are often referred to as inhalable particles, and is collected using a filter cassette. A sub-fraction of the inhalable particles is the respirable fraction, *i.e.*, particles with a d_{ae} between 0.01 and 10 µm, which can be deposited deep into the alveolar region of the lung. These particles may contribute to the pathogenesis of a range of chronic lung diseases (18,91,92), and are usually sampled using a cyclone with a filter. Prior to analysis, organic substances trapped on filters are extracted by the use of solvents. Particle-associated exposure to methacrylates has, to the knowledge of the author, so far only been investigated in the laboratory. The extent of exposure under clinically relevant conditions to particle-associated PRM constituents (and gaseous substances) was therefore assessed in paper IV.

2.6.4 Analysis - Chromatography

Chromatography is a powerful technique to separate analytes in a sample (Figure 4). Separation is achieved by the principle that substances may have different distribution coefficients between the stationary and the mobile phase in the chromatography column. This ultimately leads to different retention times of substances (*i.e.* the time the analyte reach the detector). The two major forms of chromatography are Gas Chromatography (GC) and Liquid Chromatography (LC). The distinction is based on the nature of the mobile phase. The combination of GC and mass spectrometry, MS, (GC-MS) is used to analyze volatile and semi-volatile substances. The combination of LC and MS (LC-MS) is used to analyze substances with low vapor pressure even at elevated temperatures (that cannot be analyzed on GC-MS). A disadvantage with GC is that the substances usually are subjected to high temperatures during injection (≈ 250 °C) that may cause thermal degradation of analytes. This may result in loss of signal and/or detection of products of the thermal degradation, *i.e.* detection of false positive signals (55). Concerning constituents of PRMs, the monomer UDMA has been described to decompose to HEMA in the GC-injector (55,93). Both LC (or more precisely, Ultra-high performance liquid chromatography (UHPLC)- and GC-based methods were used in paper III and IV for exposure assessment.

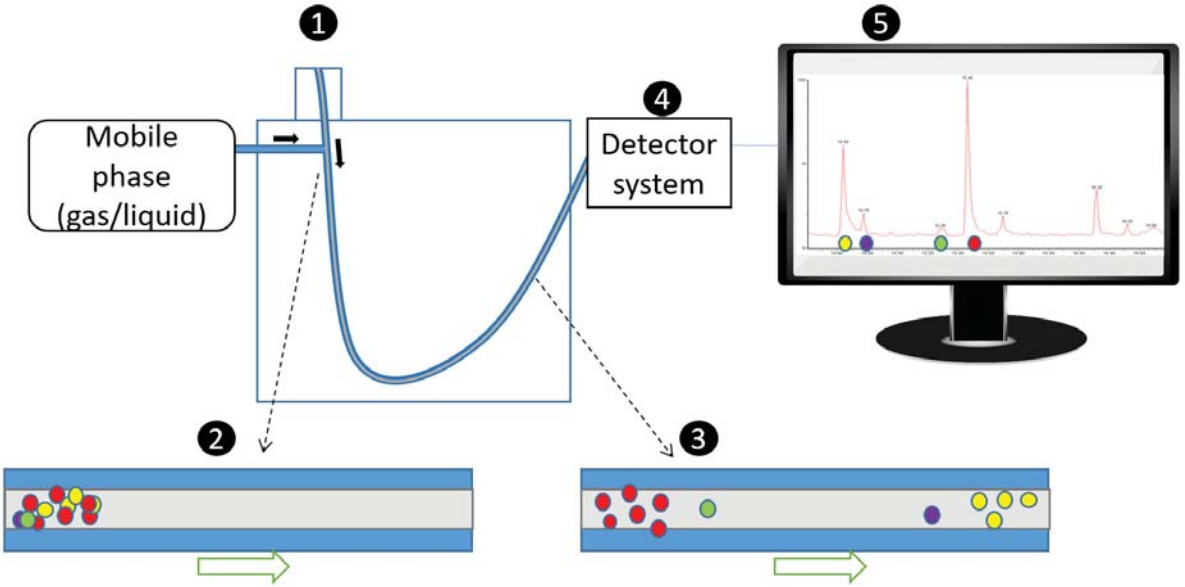

Figure 4: Principles of chromatography. 1: A sample is injected into the column (blue). 2: The analytes are clustered together in the beginning of the column. 3: At the end of the column, analytes may have been separated from each other. 4: Once the analytes reach a detector, they will generate a signal that are transferred to a computer. 5: Separated analytes will have different retention times (as observed in the computer software).

2.6.5 Analysis - Mass spectrometry (MS)

Mass spectrometry was the detector system used in paper II-IV. It has an unparalleled selectivity and sensitivity compared to other detector systems (*i.e.* UV-VIS light and flame ionization detectors), as substances can be identified not only by their retention time, but by their mass-spectrum as well (94). A mass spectrum is generated by ionization of analytes that are introduced from the column into the ion source of the MS instrument. In the ion source, the neutral molecules are converted to a number of ions with different mass, usually including both the molecular ion (the un-fragmented, ionized analyte) and a number of smaller fragment ions. The ions are further separated from each other according to their mass to charge (m/z) ratio and recorded, both with respect to mass and amounts. The result is often presented as a mass spectrum of the compound, a

plot of intensity of the ions versus the m/z ratio. The general principles of mass spectrometry is presented in Figure 5.

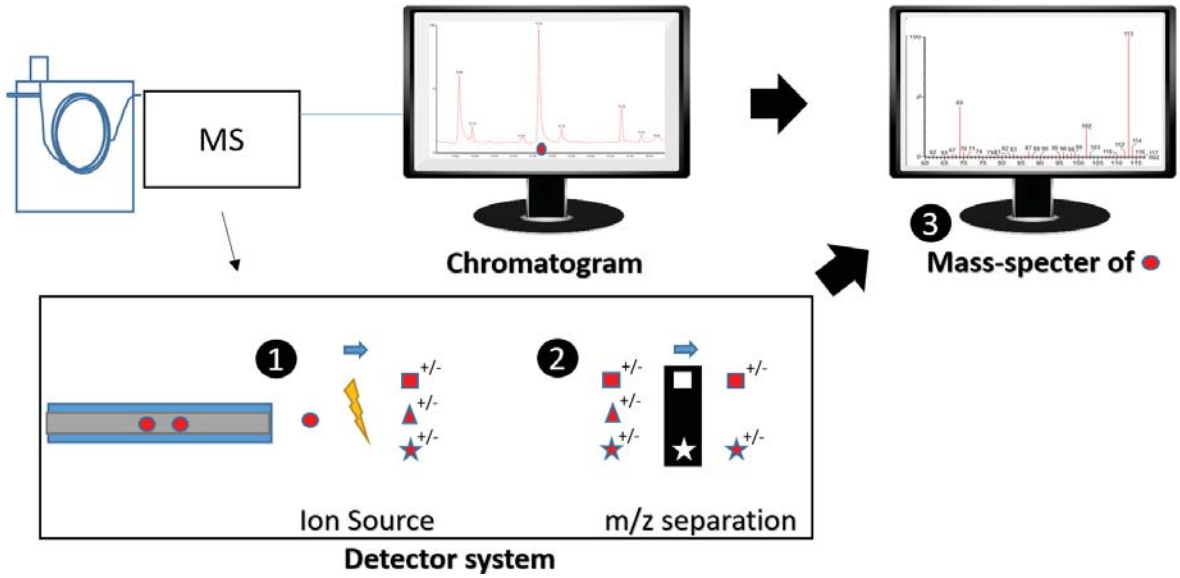

Figure 5: Principles of mass spectrometry. 1: A group of analytes reach the detector system. The ion source produce ions of the analyte(s). 2: The ions are filtrated depending on the instrument settings. 3: The mass spectrum of the red analyte is shown.

The MS can be used in either Selective Ion(s) Recording (SIR) or SCAN mode. When using the MS in the SCAN mode, all ions are detected, which provides both quantitative and structural information at the same time. In the SIR mode, the MS is set to detect only a limited type of ions (m/z) that characterize the compound of interest. Using the MS in the SIR mode increases the ability of the instrument to detect small amounts of the actual analyte, and also increases selectivity. Depending on the type of MS instrument and the analyte, the lower limit of detection is usually in the 10^{-9}–10^{-15} gram range (on column).

An advantage with GC-MS is that the results (*i.e.* the appearance of the mass spectra) are instrument independent and hence, extensive mass spectrum libraries are available, *e.g.* National Institute of Science and Technology library. In contrast, results from LC-MS depend on instrument settings, type of instrument,

and various other factors. The access to universal libraries is therefore much more restricted. By comparing the retention time, area under the peak and mass-spectra of a substance obtained in a LC-MS/MS or GC-MS analysis with the analysis results of reference substances, it is possible to identify and quantify the chemicals present in samples, and hence, estimate exposure.

3 Biological effects of methacrylates in PRMs

3.1 Risk

In toxicology, risk is the probability of harmful effects to human health after exposure to a toxicant (95). The risk associated with a particular substance encompasses the hazard, *i.e.*, the inherent health damaging properties of the substance, and the extent of exposure to that particular substance (95). Yet, while interpretable and reliable data on exposure can be achieved, it has proven challenging to collect reliable health-hazard data for humans — especially for chronic toxicants — thus making an accurate risk assessment very difficult (96,97).

3.2 The problem of identifying human health hazards

A hazardous substance is a substance that causes acute or chronic, local or systemic toxicity. Hazardous substances for humans can be difficult to identify and characterize as living organisms are immensely complex systems to study, with endless possibilities of direct and indirect effects to be examined. In addition, epigenetic and genetic variations between individuals imply that hazards may vary both within and between species (98,99).

The main challenge in identifying and characterizing human hazards is the lack of a proper gold standard (99). Hazard characterization of substances are mostly based on experiments conducted on short-lived animals, such as mouse or rat (96). However, animal experiments have limitations for this purpose, due to species-specific differences in physiology and anatomy (100), as well as methodological issues related to the testing (97).

Animal experiments are also time-consuming and costly in terms of money and animal lives. It is estimated that 54 million vertebrate animals and a 9.5 billion euro over the next ten years are needed to comply with the European Chemical

Regulation for Registration of Chemicals legislation on testing of chemical compounds imported or produced in quantities above 1 ton (101). The huge cost and uncertainty associated with the current methods for assessing human health hazards suggest that other alternatives should be sought (102). To quote a leading authority in toxicology, Dr. Thomas Hartung on the subject of toxicity testing: "To meet the challenges of the 21st century, revolution rather than evolution is required" (97).

3.3 Toxicity testing in the 21st century

In 2007, a new vision for toxicity testing was presented in the landmark National Research Council of USA report, *Toxicity testing in the 21st century: a vision and a strategy (21.tox)* (96). In this report, it is stated that human biology ought to be the basis for toxicity assessments in the future by using human cell lines, in conjunction with *in silico* methods (data simulation), to detect and map the molecular basis of adverse effects caused by chemicals. Within decades, such an approach is suggested to provide a less costly, faster and more accurate manner of predicting harmful effects of chemicals (96). It would also provide a more ethical way of testing chemicals; though, the 21.tox movement is not primarily motivated by animal welfare, but more by the limitations of current methods when it comes to ability of toxicity prediction. While the 21.tox approach is still in its infancy, the rapid development of *in silico* and *in vitro* tools is expected to catalyze the development, validation and acceptance of this approach in future human health risk assessments (99).

3.4 *In silico* approaches

The term *In silico* covers computer based tools that are used to generate or interpret data (103). In toxicology, it can be used to analyze, simulate, visualize or predict the toxicity of chemicals. In theory, *in silico* methods may generate non-testing data on new chemicals for a range of toxicological endpoints based on approaches such as 1) grouping (read-across between structural similar chemicals), 2) structure-activity relationship (predict biological effects of a chemical structure) and 3) expert systems (systems that mimic human reasoning and formalize existing knowledge) (103). Figure 6 illustrates some of the current possibilities of this approach in relation to hazard assessment. One controversy with the method is the risk of *trash in, trash out*; the quality of prediction is limited by the quality of the input. Thus, the easy interpretation of an *in silico* analysis may be compromised by its uncertainty.

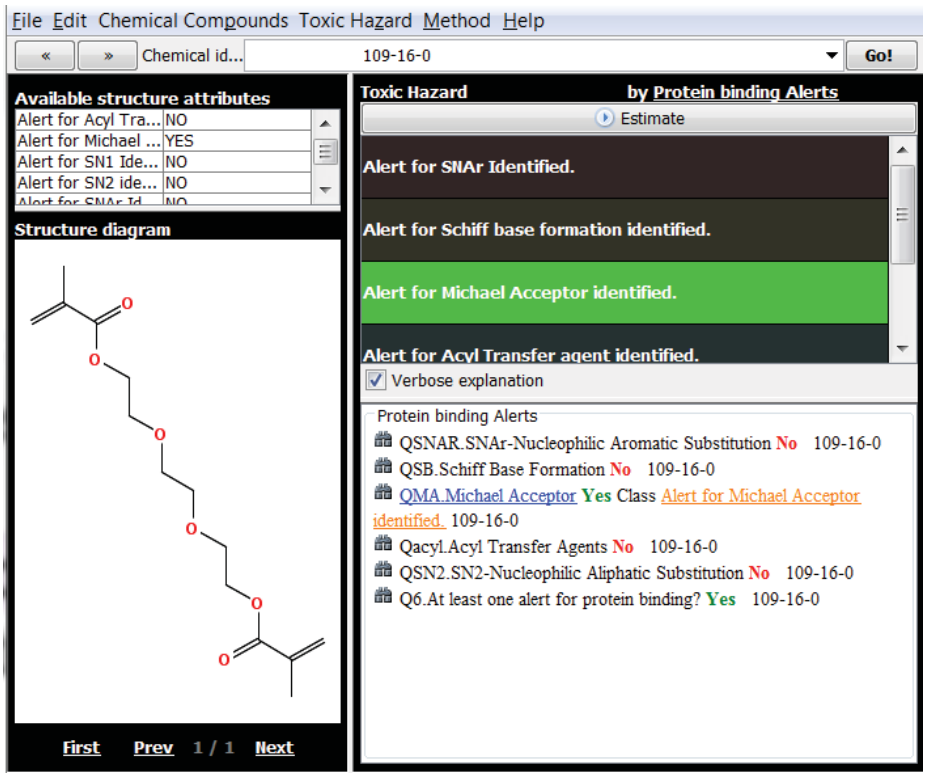

Figure 6: Toxtree (104). A free in silico tool that can be used to predict toxicity hazards of chemicals. The hazard assessment is based on different decision trees. The example shows an alert for protein binding of TEGDMA.

Regarding the data presented in the present thesis, *in silico* methods are used to analyze and visualize proteomic data. The STRING database used in paper II is a database of known and predicted protein-protein interactions, that derive data from other databases, automated text-mining and high-throughput lab experiments (105).

3.5 Mechanistic toxicology and omics

Toxicity is essentially a product of the initial interaction between a toxicant and one or more target molecules (the mechanism of action), and the molecular cascade – and late effects - following this interactions (the mode of action) (Figure 7 and 8) (106). The study of these events are called mechanistic toxicology. Vast advancements in biomedical methods and computation during the last decades have enabled scientists to study adverse effects at a higher fidelity than previously (56–60).

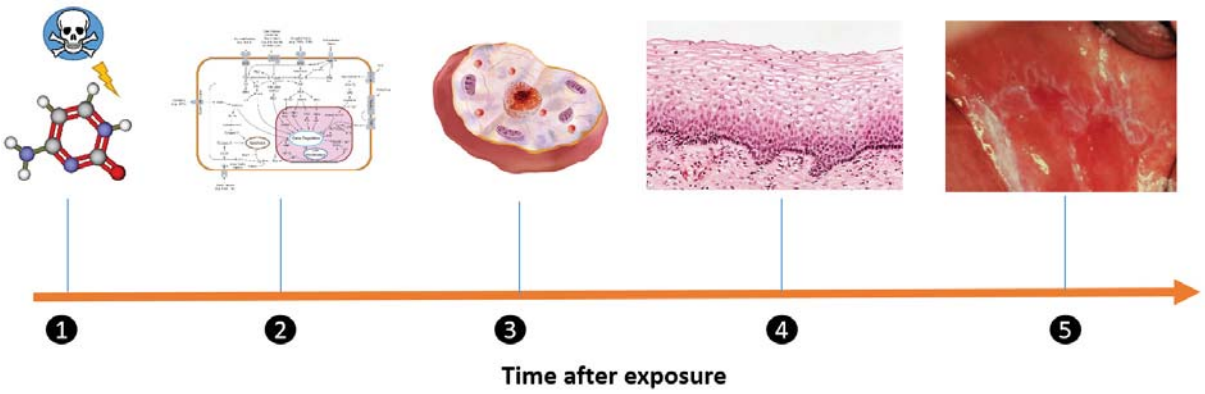

Figure 7: The chain of events following a toxic insult. 1: The initial interaction between the biomolecules and the toxicant, 2: The cellular signal transduction (pathways) initiated by the interaction, 3: Observable change at the cellular level, 4: Observable change at the tissue-level, 5: Clinical detectable effect.

The images used in bullets 1, 3, and 4 are a property of colourbox.com and used in agreement with their credit Attribution Guide. The image used in bullet 5 is of courtesy Ulf Thore Ørtengren. All other images are CC0 (from Wikipedia.commons).

Omics refer to the relatively new biological disciplines with a –omics suffix, *e.g.*, genomics, transcriptomics, proteomics, and metabolomics. Omics enable the collective characterization and quantification of biomolecules that are related to structure, function, and behavior of cells, tissues, and organisms. In toxicology, omics can provide data on up- and/or downregulated proteins, metabolites, and/or transcripts that can be used to comprehend how toxicants may cause harm, and potentially, predict the toxicity of substances (107–110).

In general, the results obtained with omics contain information of both the direct and indirect responses of a cell, tissue or organism to a toxicant (Figure 8). Differentiating these responses are difficult, as the indirect, homeostatic responses often are much wider and more pronounced than the direct responses. Yet, the indirect response is interesting as it can provide clues about pathways that are disturbed by the toxicant (111), as well as elucidate pathways that are important for cell survival/cell death. The latter can in theory be targeted by pharmaceuticals to modulate cell survival.

The change in omics parameters are time-dependent (106). The earliest changes will reflect the immediate interaction between the toxicant and the cells (*mechanism of action*), whereas intermediate changes reflect the functional and structural changes that occur due to the initial insult (*mode of action*) (Figure 8). In late phases of an exposure, detectable parameters will be unspecific alterations related to breakdown of homeostasis and cell death (106). Thus, from a mechanistic standpoint, omics analyses are best performed early in the exposure to readily identify the mechanisms of toxicity.

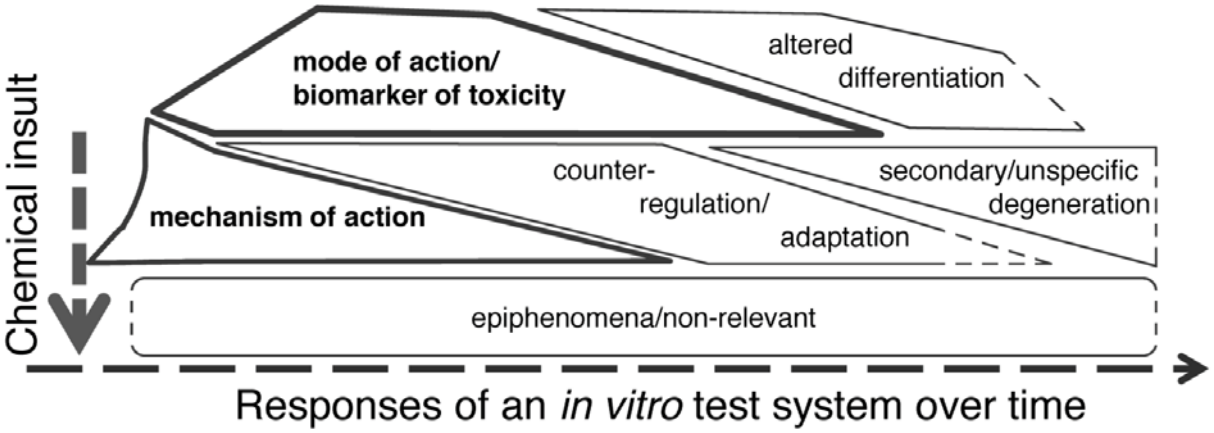

Figure 8: Groups of time-dependent responses of an in vitro test system after exposure to a toxicant. After a chemical insult (indicated by an arrow hitting the horizontal time axis) many parameters, e.g., metabolites, transcripts, or cell organelle functions will change in a time-dependent manner. Some of these parameters may be relevant for predicting/charting hazards of a chemical (mechanism of action, mode of action), while other may not be relevant (epiphenomena). Differentiating between groups of events may be difficult.

Unaltered figure from: Blaauboer BJ et al. t4 Workshop Report * The Use of Biomarkers of Toxicity for Integrating In vitro Hazard Estimates Into Risk Assessment for Humans. ALTEX. 2012 Jan;29(4):411–25. (published under Creative Commons Attribution 4.0 International license) (106).

The term early will be relative to the method used. For example, mRNA-levels are detectable prior to changes in levels of newly translated proteins. However, in contrast to transcripts, proteins are functional biomolecules that are directly related to the phenotype of an adverse effect. In addition, transcripts may not fully predict changes in protein levels, as it has been suggested that only ~30–40% of the variance in protein abundance is explained by mRNA abundance (112). Thus, charting global proteomes may offer a better insight into the mechanisms that orchestrate the biological effects induced by a toxicant.

3.5.1 Proteomics

Proteins are involved in nearly all cellular processes. However, biological functions are seldom initiated by a single protein, but a range of indirect and direct protein interactions. Thus, assessing the global expression of proteins, *i.e.* the proteome, offers many advantages compared to assessing the expression of individual proteins.

In this thesis, the metabolic labeling strategy SILAC (Stable Isotope Labeling by Amino acids in Cell culture) was used in conjunction with MS for proteomic analysis of TEGDMA effects on human THP-1 cells (Paper II). SILAC was first described by Ong et al. in 2002 (113), and is based on the metabolic incorporation of ^{13}C labelled amino acids into cellular proteomes. The essential and semi-essential amino acids lysine and arginine are commonly used for this purpose (114,115), and were used in the SILAC experiments in paper II. Prior to MS analysis, cells are grown in labeling medium for a number of cell doublings, before proteins are isolated/purified. Purified proteins are then separated by gel electrophoresis, followed by in-gel digestion of proteins by trypsin, before the MS analysis (referred to as bottom-up proteomics). Trypsin specifically cleaves the peptide bond C-terminal to lysine or arginine and produces peptides with an optimal size and charge distribution for MS (115).

As peptides from an isotope labelled proteome will be shifted in a predictable manner in the MS analysis (compared to peptides from the non-isotopic labeled cell culture), this enables the comparison of protein expression between untreated and treated cells (Figure 9). The sensitivity and output of a SILAC analysis imply that biological effects (that may be relevant from a health hazard perspective) can be detected and charted at concentrations that may not induce a cytotoxic response (as detected by a traditional viability assay).

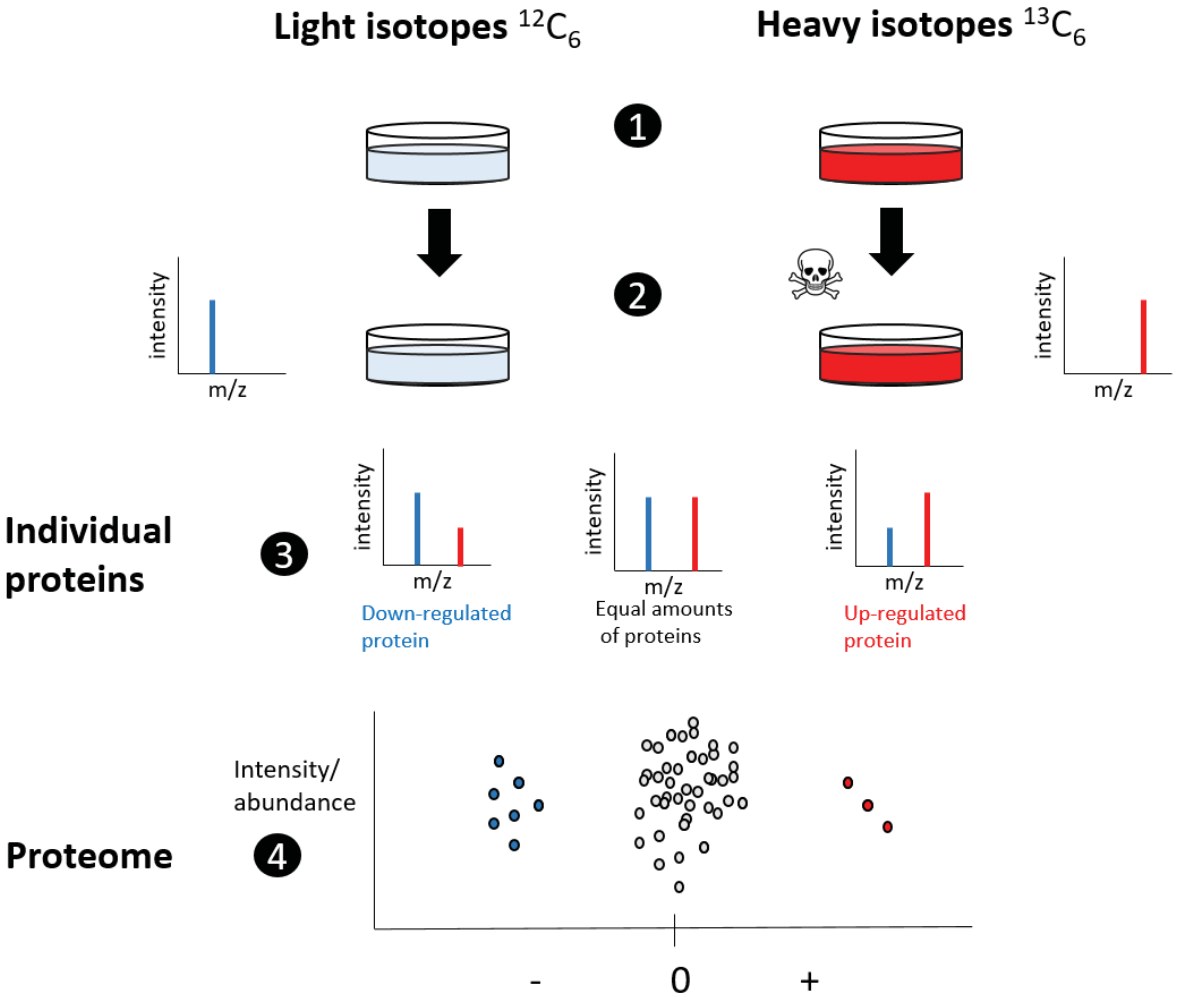

Figure 9: Principles of SILAC-based proteomics. 1: Cells are grown in SILAC-medium with heavy or light amino acids for 6-8 cell doublings to achieve high incorporation of isotope labeled amino acids into the cellular proteome. 2: One of the cell cultures are treated with a test substance for a period of time. The mass-spectrum exemplifies the relative abundance of a particular peptide in the cell prior to exposure. 3: Treated cells are mixed with control cells (equal numbers of cells). Based on the mass-spectra, up- or down-regulation of individual proteins as a consequence of the exposure can be determined as the shift in the mass-spectrum is predictable (6 dalton per peptide). 4: The proteome results can obtain data on negative, neutral or positive regulation of a wide range of proteins.

3.6 Toxicity of methacrylates

Methacrylates are a vast group of chemicals with different toxic potencies. For example, Bis-GMA has been shown to cause cytotoxic effects at lower concentrations than HEMA and TEGDMA (30). The variation in toxicity suggests that specific methacrylates act (partly) through dissimilar mechanisms. Yet, it is recognized that methacrylates share some common features in how they induce toxicity, *i.e.* oxidative stress. (116–119). As the present thesis has focused on the time- and dose dependent effects of TEGMA on human THP-1 cells (Paper II), the following sections on methacrylate-induced toxicity will mostly refer to studies on TEGDMA

3.6.1 Reactive oxygen species, antioxidants and toxicity

Reactive oxygen species (ROS) is an umbrella term that describes O_2-derived free radicals. Low levels of ROS are normally present at homeostatic conditions and are essential for cell survival as ROS directly modify redox-sensitive residues in molecules that participate in essential cellular pathways (120,121). However, excessive levels of ROS, for example induced by a methacrylate, can cause uncontrolled oxidative modification of essential macromolecules such as DNA, lipids and proteins/enzymes. Thus, a correct balance between ROS inducers and antioxidants is essential for cell survival (122).

The intracellular ROS balance is controlled by an interconnected system of enzymatic and non-enzymatic mechanisms. These mechanisms are supported by several proteins involved in the production, recovery and utilization of components central for the redox-equilibrium. The common function of non-enzymatic antioxidants is their ability to donate electrons to free radicals, and therefore stop free radical propagation (123). Some molecules, such as nicotinamide adenine

dinucleotide phosphate (NADPH) act both as direct and indirect antioxidants by participating as the substrate in the recovery of antioxidants such as glutathione and thioredoxins (124,125).

Among the antioxidants, the tripeptide glutathione is the most abundant free thiol and non-enzymatic antioxidant molecule in eukaryotic cells. It serves numerous functions (125). It is a co-factor for various antioxidant enzymes, regenerates the active form of other antioxidants (vitamin C and E), and is a direct scavenger of ROS (121). The balance between oxidized glutathione, glutathione disulfide (GSSG), and glutathione is tightly regulated. In a physiological environment, cells typically exhibit a high glutathione/GSSG ratio by performing glutathione synthesis, enzymatic reduction of GSSG (by glutathione peroxidase) and cellular uptake of glutathione (122). Low intracellular levels of glutathione are associated with increased intracellular ROS levels (126). In addition, low levels of glutathione have been shown to cause ROS-mediated apoptotic signaling (122). Methacrylates, including TEGDMA, cause depletion of cellular glutathione (117,127–130).

The fate of a cell following a ROS-injury is controlled by a complex interplay between several ROS sensitive pathways/molecules (Figure 10). Pathways can either be pro-survival, pro-apoptotic/necrotic, or both. The outcome for the cell will be determined by the balance between pro-survival and "death" proteins, succeeding the signaling and transcription of genes (121–123,125,131,132). The most studied antioxidant system with regard to methacrylate and TEGDMA-induced toxicity is the glutathione system. However, other antioxidant systems, as well as other mechanisms, may be important in modulating toxicity of methacrylates (121,122). In paper II, the use of proteomics to investigate

biological effects of TEGDMA, enabled an unbiased approach for the investigation of toxic mechanisms and highlighted new arenas to be studied.

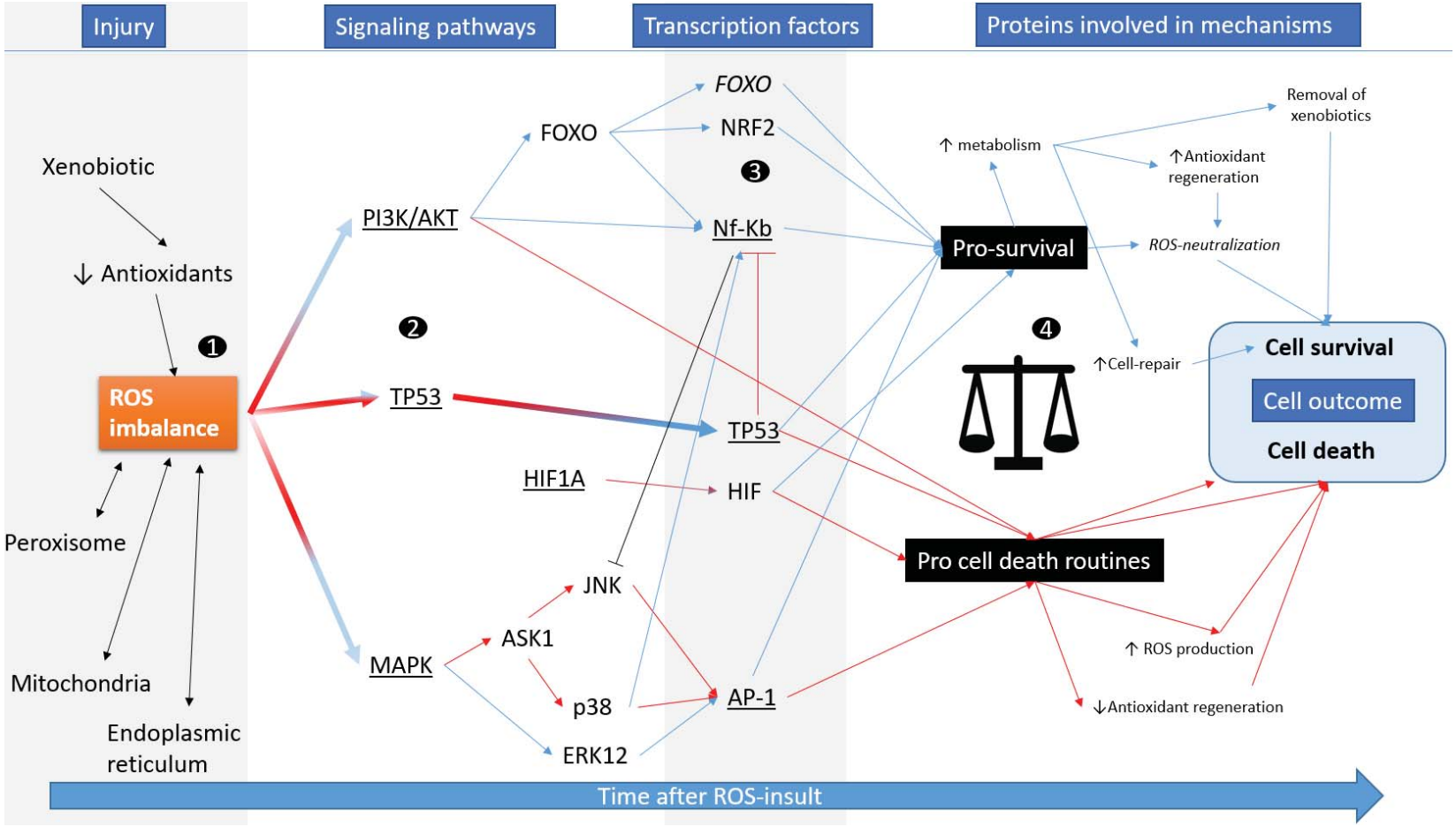

Figure 10: A simplified overview over pathways and mechanisms involved in resolving the fate of cells following a ROS-injury. 1: Antioxidant binding xenobiotics and endogenous ROS production cause a ROS imbalance. 2: Signaling pathways are activated in a ROS-dependent manner. 3: Several transcriptional factors are activated downstream of signaling or directly by ROS. 4: The balance between pro survival and pro-apoptotic/necrosis proteins (and their mechanisms) will determine the outcome of the cell. Underscored represent redox-sensitive pathways/proteins. Red arrows indicate pathways activated during severe ROS-insults, while blue arrows indicate pathways activated by slight/intermediate insults. Inspiration the for overview: (121–123,125,131,132)

3.6.2 Glutathione and TEGDMA

TEGDMA interacts with the glutathione redox system in several manners. For example, TEGDMA directly reduces the amount of intracellular glutathione in exposed cells due to its ability to make covalent bonds to nucleophilic regions of glutathione (29,129,133–135). Similar mechanisms also enable TEGDMA to interact with other crucial bionucleophiles, such as DNA, lipids and/or enzymes (29). TEGDMA can also cause depletion of glutathione without formation of GSSG. As TEGDMA-GSSG adducts prevent recovery of glutathione (136,137), and contribute to downregulation of enzymes important for recovery of glutathione (glutathione peroxidase), this shifts the cellular glutathione/GSSG balance towards increased ROS-production and ROS-associated damage (121). Antioxidants, such as n-acetylcysteine, ascorbate, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) have been reported to diminish some of the cytotoxic effects of TEGDMA (130). However, exogenous glutathione does not inhibit the toxic effects of TEGDMA (129). Shifts in the glutathione/GSSG balance, due to the formation of TEGDMA-glutathione adducts, may partly explain this phenomena, and further underlines the importance the glutathione/GSSG balance for cell survival.

3.6.3 Genotoxicity of TEGDMA

The lack of complete protection of antioxidants towards TEGDMA induced toxicity may partly be explained by the suggested direct DNA-damaging abilities of TEGDMA (in contrast to indirect genotoxic effects following excessive oxidative stress) (135,138,139). This is relevant for other methacrylates as well. HEMA has been shown to mediate cellular damage through DNA damage, and this effect was not counteracted by the antioxidant trolox (140). Regardless of mechanisms, TEGDMA has been demonstrated to induce formation of microneuclei in

mammalian cells, which is a sign of genotoxic events and chromosomal instability (37). Genotoxic effects (double-strand breaks) have also been observed after exposure of human lymphocytes, salivary gland cells, and gingival fibroblasts to TEGDMA (141,142). TEGDMA and HEMA have both demonstrated to induce DNA damage, assessed by multiple genotoxicological endpoints, in human lymphocytes at low doses (1μM–100μM) (143). Furthermore, TEGDMA also inhibits the cell-cycle through TP53 independent and dependent mechanisms in several cell lines (144).

3.6.4 Mitochondrial damage and TEGDMA

The mitochondria is a critical target for many ROS-generating toxicants, including TEGDMA (137,145); mitochondrial DNA (mtDNA) is particularly vulnerable. In contrast to nuclear DNA (which is protected by its helix structure, reparation machinery, nuclear glutathione, and histone sheets), mtDNA is circular, lacks a reparation machinery, and is located in close proximity to the main endogenous ROS source, the electron transport chain (146). Damage to mtDNA will compromise ATP-production and cause mitochondrial dysfunction, which may contribute to higher ROS production (146). In addition, following the inhibition of the citric cycle, recovery of crucial antioxidants will be prevented because of lack of NADPH substrates (which also are direct ROS scavengers) (124,125). Furthermore, TEGDMA is shown to induce a collapse in the mitochondrial membrane potential of human gingival fibroblasts at concentrations above 1mM (137)

Also lower concentrations of TEGDMA (<1mM) have been shown to affect metabolic functions of the mitochondria. For example, an increased ratio of nucleoside diphosphates to nucleoside triphosphates has been observed in Swiss mouse 3T3 fibroblast cells exposed to TEGDMA (0.5mM) (40). These effects

suggest that detoxification of TEGDMA requires high-energy phosphates which are not compensated for by higher metabolic turnover of ATP (40). It has been hypothesized that this increased energy-consumption could be due to induction of ATP-dependent, multidrug resistance associated proteins, which removes xenobiotics from the cell (137). If ATP levels drop below a certain threshold, for example after a high dose TEGDMA-exposure, vital cell activities will be compromised (147). Besides failure to remove xenobiotics, maintenance of ionic equilibrium across the plasma membrane would cease; thus compromising the cells ability to maintain structural integrity (147). Correlation between decreasing glutathione/GSSG ratio and cellular ATP levels, with increasing lipid peroxidation and lactate dehydrogenase leakage in studies on TEGDMA, supports the notion that the combined failure of several intracellular functions contribute to the cytotoxicity of this methacrylate (137).

3.6.5 Other effects of TEGDMA

On a molecular level, low concentrations of TEGDMA have demonstrated to influence the release of factors relevant for immune responses, *i.e.*, tumor necrosis factor-α (TNF-α) release (32,148). TEGDMA has also been reported to inhibit lipopolysaccharides-induced release of TNF-α, interleukin-6 and interleukin-10 and to decrease expression of cluster of differentiation proteins 40, 80 and 96 in murine RAW264,7 macrophages (149). In the same cell line, 0.4mM of TEGDMA increased expression of cyclooxygenase 2 and prostaglandin E2 (150). TEGDMA is further reported to induce expression of monocyte chemoattractant protein-1 in human monocyte-derived macrophages, and to increase hydrolase activity in human gingival and pulpal fibroblasts (151). In human pulpal cell cultures, TEGDMA has been shown to reduce odontoblast function by decreasing phosphatase activity, calcium deposition, and gene expression (152–155).

Taken together, this section suggests that TEGDMA is an environmental stressor that has the potential to modify cellular responses in different manners depending on the cell type, concentration and exposure interval.

3.7 Challenges and limitation of *in vitro* cell research

In vitro toxicology research currently has many limitations as a tool for health risk assessment in humans. The most obvious is perhaps the difficulty in interpreting results in context of *in vivo* conditions (97). Yet, novel developments in methods and *in silico* tools, as described in the 21.tox report (96), may address this in the future. Meanwhile, a more manageable topic for discussion is how *in vitro* toxicity research is conducted today. Publication standards of results from basic research in life sciences, including toxicology, have by some been described to be lower than the standards set for clinical experiments (97,156). For example, Guidance on Good Cell Culture Practice, which was published in 2002, is still not properly implemented in cell research (97,157). In fact, a wealth of factors may modify the toxic response observed *in vitro* (Figure 11). In the following paragraphs, some of these issues will be presented.

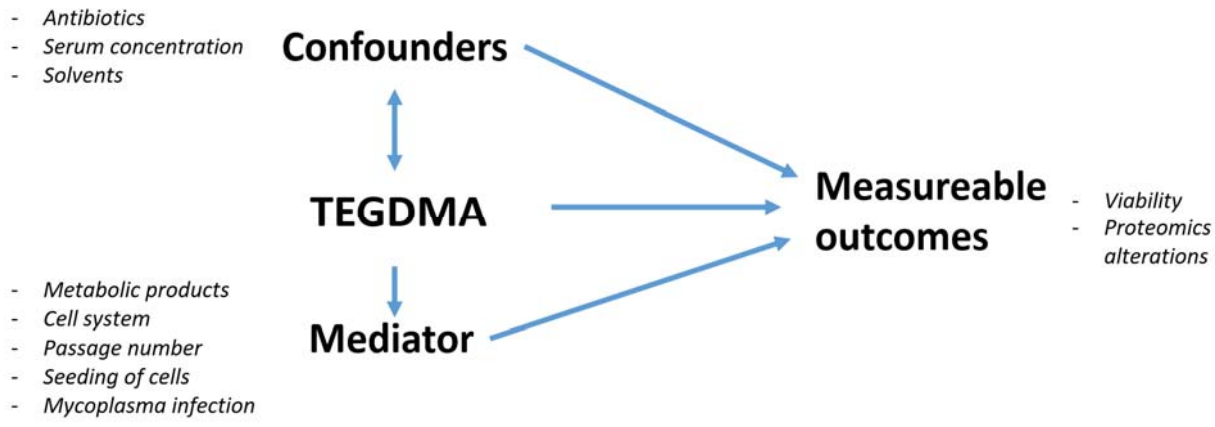

Figure 11: Examples of variables that may influence the measured response in vitro. Most of these parameters can be mapped and standardized in-between studies. Confounder: variables that can alter the TEGDMA concentration available for cells and the measurable outcome variable. Mediator: Variables that can influence the measureable outcome.

3.7.1 Terminology in cell biology research

Precise terminology is important for a proper understanding of research findings. In line with this, jargon and non-standardized nomenclature should be avoided (158). With regard to cell biology and toxicology research, misuse of words and concepts have been described to be detrimental for establishing a universal platform for understanding cellular events (158,159). When it comes to description of cell death, reported terms such as percent necrosis/apoptosis/cell death/cell survival cannot be measured directly, whereas terms like percent cells with condensed chromatin, percent propidium iodide positive cells, or percent activated caspase-3 positive cells are more precise terms of the measurable parameters (158,159). This notion is also relevant for terms such as non-toxic and sub-lethal, which may be ambiguous when used in the context of cell biology research (158). In paper I, terminology used in *in vitro* research on PRM constituents was investigated.

3.7.2 Exposure scenario – assessing toxicity

Live cells are a prerequisite for assessing biological changes *in vitro*. Thus, suitable concentrations and exposure intervals for experiments are usually determined prior to other tests (*e.g.* proteomic experiments). In practice, the cell status are determined by bioassays which indirectly detect gross cytotoxic events such as cytostasis, necrosis and/or apoptosis of cells (160). The result of an assay is only indicative of the state that is measured, as results might differ between assays (161). In fact, no single parameter can fully characterize cytotoxicity, and all facets of cytotoxicity are not understood as stated by the Nomenclature Committee on Cell Death (NCCD) (147). Assays measuring metabolically active cells (commonly referred to as a viability assay) cannot detect apoptotic or necrotic cells; thus, a zero reading with a viability assay should not be interpreted as

necrosis and/or apoptosis. In contrast, cells undergoing apoptosis may be detected as viable by a viability assay. A comprehensive discussion on what constitutes a viable cells, and the word viability, has therefore been warranted by some (162). Regardless, by using supplementary methods (to bioassays), such as assessment of cell proliferation, and microscopy techniques, as recommended by Nomenclature Committee on Cell Death, a greater certainty of the state of exposed cells can be achieved (162,163).

3.7.3 Dose metric

In vitro toxicity is not only related to the compound tested and the sensitivity of the cells, but also the availability of the substance in the *in vitro* system (164). Several factors can modify the concentration available for the cells in culture, *e.g.*, the physiochemical properties of the substance, exposure duration, metabolism, and cell vessel (Figure 12). Thus, the concentration of substance added to a cell culture — often referred to as the nominal concentration in literature on this topic (165) — may not reflect the effective concentration, *i.e.*, the free, unbound substances that cause a biological effect (164). Concerning matrix constituents of PRMs, differences between nominal and detectable concentrations of TEGDMA in cell cultures have been observed (166).

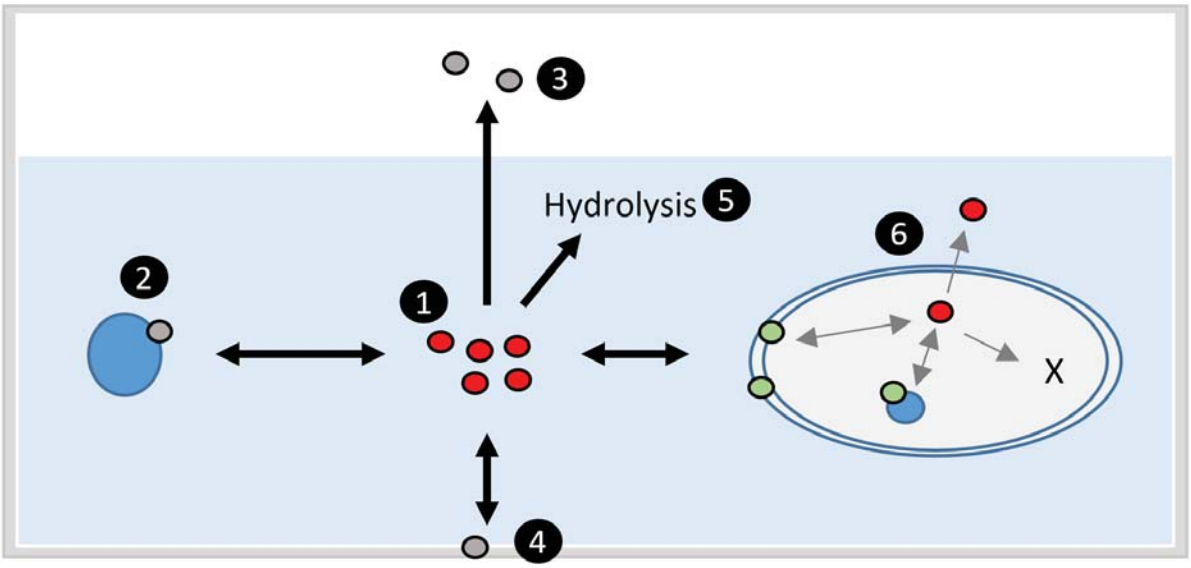

Figure 12: The bioavailability of chemicals in the cell culture may be influenced by many factors that make the use of nominal doses inaccurate. 1: Only the unbound chemical are available for interactions (red circles). The amount of chemical may be reduced if the chemical binds to serum constituents (2), if it evaporates (3), binds to the cell vessel (4), or is degraded or hydrolyzed (5, 6). In the cell, several things can affect the available intracellular concentration (6). The substance (green circles) may bind to molecules that may or may not be vital for the cell. The chemical may also be eliminated (marked as X) or pumped out of the cell. Figure inspired by GROOTHUIS et al (165)

The use of other dose metrics than nominal doses is in its infancy. This is most likely related to methodological challenges, and perhaps, the lack of knowledge of this topic among researchers. However, awareness of the phenomena is relevant to future cell research and risk assessments, *e.g.* the 21.tox report state that "*Chemical concentrations should be measured* ***directly*** *in the media used in the toxicity-pathway assays when administered concentrations might not represent the concentrations in vitro*" (106,165).

3.7.4 Solubility of toxicants

A topic closely related to dose metric is solubility. Hydrophobic substances will form dispersions when added to water-based mediums. Dispersions of a chemical will cause a heterogeneous distribution of the chemical in the *in vitro* system, and decrease the amount of active substance. In contrast, a solution is a homogenous mixture of the medium and substance. Thus, solvents are often used *in vitro* to achieve solutions. However, solvents such as dimethyl sulfoxide (DMSO), acetone, and ethanol may increase cytotoxicity of substances, including monomeric methacrylates (166). Solvents may increase the permeability of the plasma membrane; and thus, increase the intracellular levels of the substance (166,167). With regard to TEGDMA, some cell culture studies on this monomer use solvent, but not all (151,168). Thus, the use of solvents in cell culture studies on PRM constituents were reviewed in paper I.

3.7.5 Microbial contamination of cell cultures and antibiotics

Antibiotics are commonly used in cell cultures to prevent infection (169). However, guidance on Good Cell Culture Practice has since 2002 discouraged prophylactic and unnecessary use of antibiotics in cell culture mediums (157,170). Antibiotics may interfere with cellular functions of interest, and can mask otherwise evident infections (171,172). Antibiotics may also interact with the tested substance and serum proteins; thus, potentially influencing the biological effects observed (169,173).

Importantly, mycoplasma infections, which are common in cell cultures and often undiagnosed, are in most cases not prevented by prophylactic use of antibiotics, as 90 % of mycoplasma strains are resistance to commonly used antibiotics (174). Reviews on the topic have concluded that mycoplasma infections are a major problem in cell culture research (175,176). Screening for mycoplasma

infections in cell cultures should therefore ideally be performed and reported. Standardization of the use of antibiotics and contamination screening should be achievable – especially in immortalized cell lines.

3.7.6 Cell cultures for toxicology assessment

By using human cells in toxicology assessment, one may avoid the ethical, logistic and cost-related problems associated with animal experiments and animal-to-human extrapolation. Yet, the use of cell cultures introduces other issues related to cell culture conditions and cells that make *in vitro* to *in vivo* extrapolation challenging.

Several human and non-human cell lines exist for the purpose of *in vitro* toxicology research (Figure 13). However, as discussed in section 3.3, the use of human cells for toxicity testing will most likely become the norm in the future. A major problem with cultivated cells is that they often show a markedly different phenotype and behavior compared to their corresponding cell type *in vivo* (177,178). Furthermore, primary cells often rapidly de-differentiate ex vivo. Both of these events may partly be attributed to non-physiological culture conditions. For example, cell density in 2D cultures is often less than one percent of what is found in tissue (179). Additionally, a cell culture is a non-homeostatic environment, where there is a continuous buildup of waste products, and where medium conditions may change rapidly. Oxygen concentrations are also limited by diffusion in the medium (179). Altogether, these are all aspects that make it challenging to directly extrapolate results from cell culture experiments to the *in vivo* condition. However, being aware of these limitation, cell models are important tools for studies of cellular mechanisms.

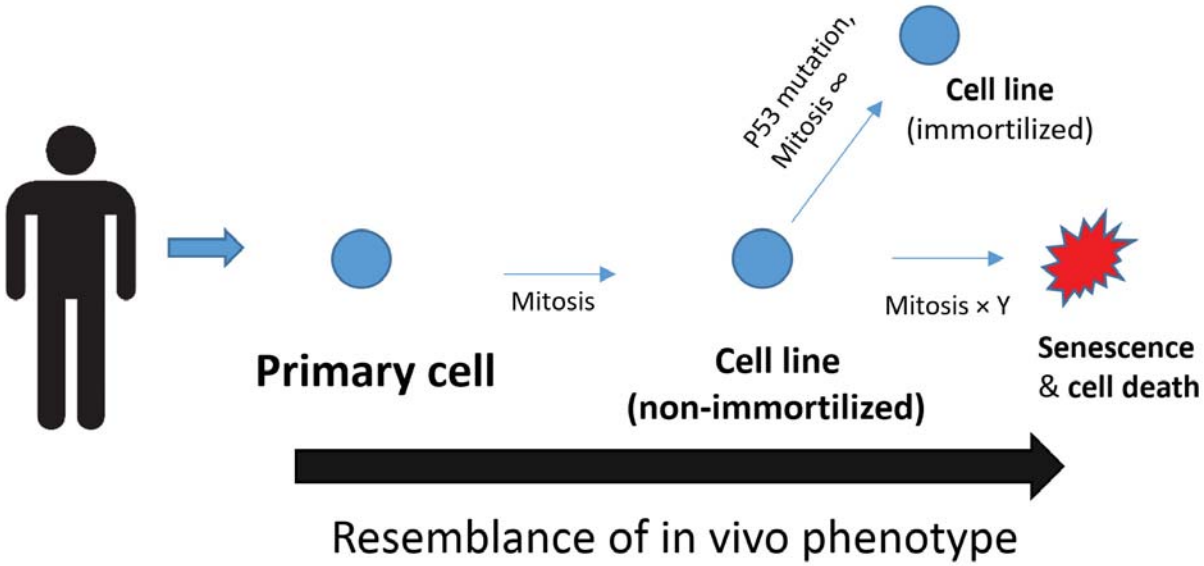

Figure 13: Overview of human cell types, and the resemblance of the in vivo phenotype. Primary cells and non-immortilized cells are finite and undergo cellular senescence after a number of mitosis. Immortilized cells are a result of selection and mutation that enables cells to undergo division indefinitely. Immortilized cells may also be sampled directly from cancerous tissue in vivo. For all cells, a loss of resemblance to the in vivo phenotype occurs during culturing.

Objectives

The main objectives of this thesis were to provide novel insight into the biological effects induced by one of the most commonly used methacrylate in PRMs, and to provide new knowledge on how patients and dental health personnel may be exposed to constituents in PRMs.

The specific objectives of this project were to:

1. Critically assess terminology and methods that have been used in *in vitro* toxicity testing of PRMs constituents (paper I)
2. Explore the mechanisms of TEGDMA-induced toxicity by studying proteomic alterations caused by a seemingly non-cytotoxic and cytotoxic dose of TEGDMA in a human monocytic cell line (paper II)
3. Examine and critically discuss the organic content and leachables from resin-modified pulp capping materials (paper III).
4. Examine exposure to gas-phase and particle-associated methacrylates released from PRMs during restorative procedures (paper IV).

Methods and results

Below follows a schematic summary of the methods and main results in paper I-IV.

Paper I – Methods and terminology used in cell-culture studies of low-dose effects of matrix constituents of polymer resin-based dental materials

Stated aim(s): This systematic review aimed to provide an overview of terms and methods used in studies on low-dose effects of matrix constituents in PRMs, as well as highlighting how these factors may influence comprehension and interpretation of results

Method	Rationale	Main results
Systematic searches in five search databases	Identify eligible articles for review	29 articles were identified
The articles were screened for the use of:	Assess terminology and methods used	
Cytotoxicity assay	in the eligible articles	Most studies used MTT cell viability assays to assess toxicity.
Solvent		Use of solvent was widespread and unaligned.
Antibiotics		Different types of antibiotics were prophylactically used.
Mycoplasma testing		Mycoplasma testing was not reported in any of the studies
Cell type		Human cell lines were the most utilized cells.
Passage number of cells		Passage number was generally reported for non-immortilized cells, while not reported for immortilized cell lines.
Dose metric		Nominal dose metric was used in all articles.
Terminology used for low-dose term		The terminology was not standardized. Definition of similar terms varied.

Paper II – Dose- and time-dependent effects of triethylene glycol dimethacrylate on the proteome of human THP-1 monocytes

Stated aim(s): The objective of the study was to explore time- and dose-dependent proteomic alterations caused by TEGDMA in human THP-1 monocytes

Method	Rationale	Main results
Photon correlation spectroscopy	Test solubility of TEGDMA	TEGDMA was soluble in cell culture medium
Real-time Glo™ MT Cell viability assay (reduction based viability assay)	Test effect on cell viability (0.07 – 5mM TEGMDA, 0-72h)	≤ 0.6mM TEGDMA yielded increased cell reduction potential. ≥1.25mM TEGDMA yielded decrease in reduction potential.
Automated cell counting by Sysmex XP-300™ Automated Hematology Analyzer	Test effect on cell proliferation (0.07 – 5mM TEGMDA, 0-72h)	≤ 0.6mM TEGDMA did not negatively influence proliferation. ≤1.25mM TEGDMA yielded a decrease in proliferation.
Transmission electron microscopy	Assess ultrastructural alterations caused by TEGDMA (0.3mM and 2.5mM; 5h , 16h, 72h)	Cells treated with 2.5mM TEGDMA showed signs of increased membrane blebbing compared to control cells and 0.3mM TEGDMA treated cells (6h and 16h), and massive cell death after 72h. No clear differences in ultrastructure could be seen between 0.3mM TEGDMA treated cells and non-treated control cells.
Metabolic labelling of cells with SILAC for 8-9 cell doublings prior to LC-MS/MS analysis, raw data processing with MaxQuant (180), and filtration of regulated proteins	Assess proteomic alterations in the THP-1 cell line after TEGDMA exposure (0.3mM and 2.5mM; 6h , 16h	26 proteins were upregulated and 12 proteins downregulated in cells exposed to 0.3mM TEGDMA. In cells exposed to 2.5mM TEGDMA, 18 proteins were upregulated and 38 proteins downregulated Full list of regulated proteins are enclosed in appendix 1a and b
STRING database analysis (105)	Provide information on possible biological relationship between regulated proteins	Cells exposed to 0.3mM TEGDMA showed upregulation of proteins associated with stress/oxidative stress, autophagy, and cytoprotective functions. Cells exposed to 2.5mM TEGDMA had changes in protein expression profile associated with oxidative stress, DNA-damage, mitochondrial dysfunction and cell-cycle inhibition. Altered expression of proteins associated with immune function was observed in both groups

Paper III – Analysis of organic components in resin-modified pulp capping materials: critical considerations

Stated aim(s): The objective of this study was two-fold: 1) To investigate the organic composition and the 7-day water leachables of three resin-modified pulp capping materials (Theracal® LC, Ultra-Blend® Plus and Calcimol LC) by GC-MS and UHPLC-MS analysis. 2) To evaluate the findings in light of the material composition, information provided in the material safety data sheets, and the indication for use.

Method	Rationale	Main results
Collect relevant SDS and instruction for use sheets from the suppliers of materials and online sources	Assess the information on hazardous substances available for dentists, and to compare with the GC-MS and UHPLC-MS analysis results	The safety data sheet provided by the suppliers of Theracal® LC was outdated The safety data sheet of Ultra-Blend® Plus and Calcimol LC listed different chemicals, despite similar composition The instruction for use in relation to capping, varied among the three materials
Prepare samples for investigation of leachables	Standardize specimens to investigate eluates	It was difficult to make adequately cured samples of Theracal® LC at 1 mm (= max recommended curing depth); 0.6 mm samples were therefore prepared. Visual pores were found in all samples
pH-measurements	Identify pH changes in the immersion medium of the samples	The pH-medium of the immersion medium of Ultra-Blend® Plus and Calcimol LC samples had a pH of ~8.4. The medium of Theracal® LC had a pH of ~10
GC-MS *HP6890 GC from Agilent (Santa Clara, CA, USA) connected to a QuattroMicro GC from Micromass (NC, USA)*	Identify organic substances in the uncured materials and quantify* leachables	The leachables quantified varied among the investigated materials. Theracal® LC eluted more substances associated with light-curing than the two other materials (CQ and DMABEE). The co-initiator DMABEE was the eluate with the highest amount detected (Theracal® LC). HEMA was detected and quantified in Ultra-Blend® Plus and Calcimol LC
UPHLC-MS *Acquity I-class UHPLC connected to a Xevo G2 quadrupole time-of-flight (Q-TOF) (both from Waters, Milford, MA, USA)*	Identify high molecular weight organic substances	UDMA was detected in Calcimol LC and Ultra-Blend® Plus. Theracal LC did not contain any Bis-GMA (as listed In the SDS). The standard used to identify polyethylene glycol dimethacrylate was not the same substances as found in Theracal® LC (despite similar CAS)
Micro-CT *BRUKER SKYSCAN 1272, Kontich, Belgium*	Investigate the extent of pores in the samples used to assess leachables	All the analyzed samples had internal pores; however, their distribution and size were heterogeneous between and within material groups

*Quantified substances: 2-Hydroxyethyl methacrylate (HEMA), 2-(Dimethylamino)ethyl methacrylate (DMAEMA), Camphorquinone (CQ), Ethylene-glycol dimethacrylate (EGDMA), Ethyl-4-(dimethylamino)benzoate (DMABEE), and Triethylene-glycol dimethacrylate (TEGDMA)

Paper IV – Exposure to gaseous and particle-associated organic substances in polymer-based dental materials during restorative procedures

Stated aim(s): The objective of the study was to assess exposure to gaseous and particle-associated organic constituents from PRMs used during restorative procedures in a simulated clinical environment. In addition, to assess patient exposure to the same substances by analyzing water collected in the patient mannequins.

Method	Rationale	Main results
Filter cassettes, gas-sorbents and cyclones* attached to personnel-borne pump	Collect inhalable particles, respirable particles and gaseous substances in order to investigate airborne exposure to PRM constituents during restorative procedures	See GC-MS/UHPLC-MS
Collection of water in mannequins	Collect water used during polishing, that patient may swallow in a real clinical situation, for GC-MS and UPLC-MS	See GC-MS/UHPLC-MS
GC-MS *HP6890 GC from Agilent connected to a QuattroMicro GC from Micromass*	Quantitative assessment of exposure to five ingredients in PRMs	No methacrylates were detected in the samples collected with the personnel-borne samplers. BHT was detected in 11 of 13 of the samples collected on the filter cassette.
	Assessment of composition of uncured PRMs used in the clinic and the positive control	The chromatograms (and respective mass spectra) of the samples from the positive control and the uncured materials were similar.
UPLC-MS *Acquity I-class UHPLC connected to a Xevo G2 quadrupole time-of-flight (Q-TOF)*	Qualitative and quantitative** assessment of exposure to high molecular weight ingredients in PRMs	The Bis-EMA*** present in the material used in the clinic (if any), did not match the reference standard used despite similar CAS-number.
	Assessment of the high-molecular substances in uncured PRMs used in the clinic and the positive control	The chromatogram of the positive control and uncured substances were similar.

*Used in the positive control,

**Substances quantified: 2-hydroxyethyl methacrylate (HEMA), Camphorquinone (CQ), Butylated hydroxytoluene (BHT), Ethyl 4-(dimethylamino)benzoate (DMABEE), and Triethylene glycol dimethacrylate (TEGDMA),

*** Bisphenol A ethoxylate dimethacrylate (Bis-EMA)

Discussion

Methological considerations

Systematic literature review (Paper I)

Articles published between 1996 and 2015 concerning *in vitro* effects of PRM constituents were identified by systematic searches with the PubMed, MEDLINE, Web of Science (Thomson Reuters), Scopus (Elsevier), and Embase (Elsevier) search engines. Keywords associated with "low-dose effects", polymer resin-based materials, *in vitro* parameters, and dental materials were used to identify eligible articles. The keywords associated with low-dose effects were used to limit the topic of the paper, but also because it was relevant to map nomenclature used to describe low-dose effects of PRM constituents for the reasons outlined in section 3.7.1 (Terminology in cell biology research).

The use of several search engines were employed to maximize the potential to find eligible articles. For all keywords, it was attempted to tailor the text to the indexing of the search engines. Interestingly, relative few duplicates were removed during the screening phase, supporting the importance of using several search engines to maximize the amount of eligible articles for the review. The timespan (1996-2015) was chosen to illustrate changes in handling of the reviewed parameters in the literature. The parameters assessed were chosen to give a broad overview of factors that may influence results and interpretation of results in cell culture experiments. It also helped identify methodological pitfalls that could be avoided in paper II.

In vitro toxicity of TEGDMA – experimental design of the studies in Paper II

The experimental design of paper II was influenced by the findings and discussion in paper I. For example, antibiotics were not used in the cell culture medium, and cell passage number, as well as mycoplasma-screening routines, were reported in the manuscript. The decision to determine solubility of TEGDMA was also inspired by the findings in paper I, which showed no consensus with regard to solvents (type and concentration) used in the reviewed cell culture studies (Figure 14). Experiments performed with the photon correlation spectroscopy indicated that solvents were not needed with TEGDMA concentrations up to 10 mM, and solvents were therefore not used in the present study. As solubility is a measurable parameter, standardization with regard to the use of solvents in cell culture studies should be possible. The latter may be important for other methacrylates, as variation in use of solvents similar the results displayed in Figure 14, was also seen for other PRM constituents (Paper I).

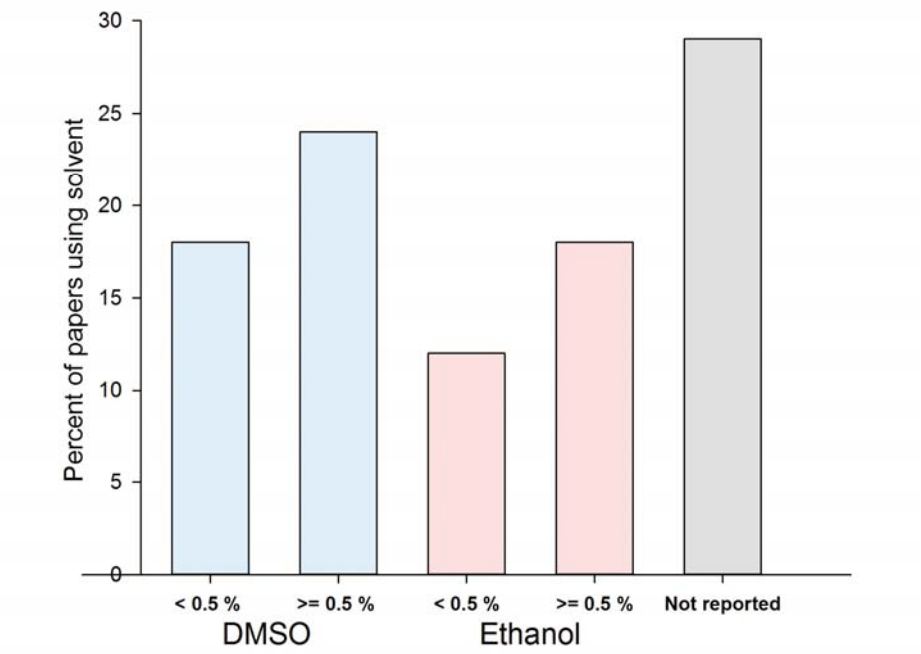

Figure 14: Solvents and concentrations used to dissolve TEGDMA in the papers reviewed in Paper I. No consensus between studies was seen. Number of studies using TEGDMA: n=17.

Cell model

The human monocytic THP-1 cell line (ATCC® TIB-202™) was used in all experiments in paper II. Monocytes and macrophages were in the 1980ies described to be critical in biological responses to biomaterials (181). Since then, monocyte and macrophage cell lines have been extensively used to study adverse effects of dental materials and their constituents. In Paper I, it was shown that THP-1 cells were the most commonly used cell model to study low-dose toxicity of methacrylates. The THP-1 cell line is derived from an acute monocytic leukemia patient (182), but show many important characteristics similar to human peripheral blood mononuclear cells such as seemingly similar lipopolysaccharide response, cytokine production, and morphology (183). The technical advantage of using the THP-1 cell line compared to peripheral blood monocytes harvested from humans, include a high doubling time (35 – 50 hours), similar genetic background (that minimizes variance in cell phenotype between research institutions), and a pure cell type (no contamination of other cells or viruses) (183).

However, as THP-1 is an immortalized cell line, obtained from a cancer patient, there are limitations to the extrapolation to healthy human monocytes. For example, it has been shown that THP-1 cells have alterations in TP53 mRNA which may influence their response to xenobiotics (184). Furthermore, while it has been found that THP-1 cells show similar response to some xenobiotics *in vitro* (183), this is most likely dependent on the mode of action of the tested substance. With regard to oxidative stress, research suggests that cancerous cells are more susceptible to increased oxidative stress as they have a higher metabolic baseline than healthy cells (125). In a paper which compared the toxicity of methacrylates in human peripheral blood monocytes and THP-1 cells, an increased sensitivity to PRM constituents was observed in THP-1 monocytes (185).

Determination of cell viability

Several approaches were used to assess the effect of TEGDMA on the viability of THP-1 cells prior to the proteomic experiment, namely viability bioassay(s), cell counting, phase contrast microscopy and transmission electron microscopy.

As bioassay, a newly developed, real-time viability assay (Realtime-Glo MT cell viability assay (Promega Corporation, Madison, WI, USA)) was used. This assay measures the cell reduction potential (186). A luciferase and a substrate is added to the cell medium prior to experiments. Metabolically active cells will reduce the substrate, which will produce a luminescent signal that correlates with the number of viable cells upon plate reading. Note, upon contacting Promega, the company consultant was unable to tell us which proteins that are involved in the reduction of the assay substrate.

There are several advantages with using a real-time bioassay. In a real time assay, the readable signal is dependent on the continuous substrate-reduction by the cells (186). This enabled the determination of *when* the treatment groups start to diverge from the control. This is not possible with traditional, accumulative-based viability assays like the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which was the most utilized bioassay in articles reviewed in paper I (20/29 articles used the assay). The Realtime-Glo assay enabled multiple measurements of viability from the same plate, in contrast to end-point measurements, which often require a post-treatment accumulation of converted substrates. For MTT, this post-treatment is associated with exacerbated cell injury, which may make measurements of viability inaccurate (187).

The Realtime-Glo assay may also, according to the manufacturer, be used in multiplexing with other cytotoxic assays. In the pilot phase, we tried to multiplex

this assay with the Celltox-Green fluorescence assay (Promega) which binds to DNA exposed after cell membrane lysis. However, the latter assay did not yield stable results. Furthermore, time lapse confocal microscopy of TEGDMA treated cells, which was also employed during the pilot phase, did not indicate membrane lysis in the doses and time points tested.

The optimal use of real-time bioassays is achieved by using a plate-reader coupled to a gas-exchange system. However, in lack of this equipment, it was shown that reliable results can be obtained with this assay by transporting the cells between the plate reader and the incubator, using a transportation device insulated with pre-warmed lead to keep the loss of heat to a minimum (< 0.4°C per minute). The loss of heat during transport was evaluated by a thermal camera (Figure 15). The thermal effect of ethanol, which was used to sterilize the plate during the transportation, was also assessed by the camera, and was shown to be negligible. Negative effects on cell viability were only seen when the cells were transported too often, *i.e.* once per hour over a longer period of time.

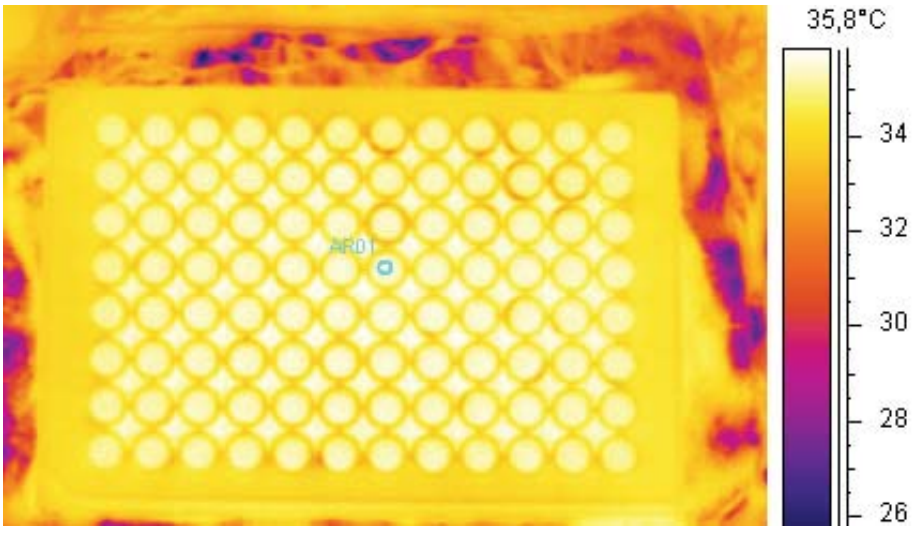

Figure 15: Loss of heat from plates during transportation was evaluated by ThermaCam S65 HS (FLIR systems, Wilsonville, Oregon, USA) kindly provided by Professor James Mercer, University of Tromsø. Loss of heat was kept at a minimum in the central wells when a preheated lead box was used for transportation of cells between the incubator and plate reader.

Concerning the Realtime-Glo assay data in Paper II, it was decided to focus on trends between independent experiments, as it was not easy to get similar nominal values during plate reading. In addition, the timing of plate-readings were difficult to standardize as they had to be manually transported and read for a period of up to 72 hours.

In addition to the real-time viability assay, automated cell counting and light and electron microscopy was used to assess TEGDMA effects on cell viability. This showed that exposure to ≥ 1.25 mM TEGDMA inhibited cell proliferation, while lower concentrations did not influence cell numbers compared to non-treated control cells. Thus, the increased reduction potential observed at ≤ 0.6mM TEGDMA with the Realtime-Glo was not due to increased proliferation. Electron microscopy was used to assess the ultrastructure of TEGDMA treated cells. Together, the methods used to assess cell viability enabled a broad characterization of the state of the cells at the concentrations and time points used in the proteomic experiments (Figure 16).

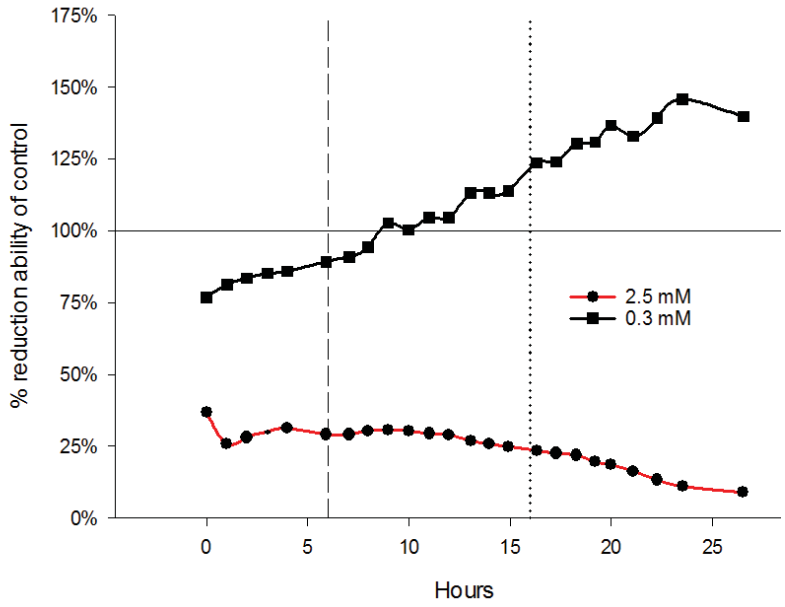

Figure 16: Real-time viability results and time points and concentrations used in the proteomic experiments. The horizontal line represents the positive control. The vertical lines show the time points used in the proteomic experiments.

Proteomic analysis

Based on the viability assessments, 2.5mM and 0.3mM TEGDMA were decided as appropriate concentrations for the proteomic experiment. These concentrations would shed light on the early effects on the THP-1 cell proteome of an apparently non-cytotoxic and a cytotoxic dose of TEGDMA. The proteomic analyses were conducted after 6h and 16h exposure of the cells to TEGDMA, which is different from other toxicoproteomic studies that used one concentration and one exposure time of either 24h or 48h (188–190). The use of two concentrations at two early time points, was anticipated to better elucidate the mechanisms involved in the cellular response to TEGDMA (106).

In the present proteomic experiment, SILAC was used. SILAC is a powerful and versatile metabolic-labelling strategy to study differential expression with mass spectrometry (MS)-based quantitative proteomics (114,191). With SILAC, it is important with a high level of incorporation of labelled amino acids in the cell proteins for accurate quantification (114). In a pilot study, we found that six cell doublings were not enough to achieve this goal; thus, in the SILAC experiments reported in paper II, 8-9 cell doublings were used. Furthermore, when differentially SILAC- labeled cell extracts (with either ^{12}C or ^{13}C-labeled amino acids) were mixed, this was performed at the earliest possible time-point to reduce experimental variations that could influence protein abundance ratios (as described in reference (114)).

SILAC is a relative quantification method, as it yields ratios of protein expression between treated and non-treated cells (in contrast to absolute quantification methods) (192). This may influence how results should be interpreted. For example, a small change in ratio for an already abundant protein

may represent a major effort for a cell. In contrast, a small change in protein levels of a scarcely expressed protein will lead to a large change in ratio (111). Increased or reduced ratios of individual proteins should therefore be evaluated in a one-to-one basis, as changes in protein ratio is not necessarily correlated with the biological importance of the protein.

Regulation of important proteins like primary transcription factors (*e.g.* NF-κB, NRF2, ATM) are not easily detected by SILAC, as they may be present in cells in an inactive state and do not require new protein synthesis in order to become activated (193). In addition, if regulated, the half-life of transcription factors are short, and may not be active when the harvesting of proteins are performed. Furthermore, protein activity that is dependent on phosphorylation, *i.e.*, proteins in signaling pathways such as PI3K/AKT/mTOR and MAPK pathway, were not investigable by the SILAC-method employed in this study.

An advantage with the omics approach in toxicology is that it is an unbiased methodology for discovering new information on toxic effects of a substance(111). However, discussion of proteome findings is often restricted by a precise knowledge of protein function and relationships. Thus, regulated proteins may be biologically linked, despite no indicated relationship. As a result, several of the regulated proteins were not discussed in paper II. Similarly, biological processes suggested by a Gene Ontology enrichment analysis may not give a complete picture of the biological processes that proteins are involved in, as an enrichment analysis is based on the current evidence that suggests a relationship between a gene (and its products) and a biological process (194,195).

Resin-modified pulp capping materials – experimental design in paper III

Pulp capping materials represent a high-dose exposure scenario for patients to a range of organic substances in PRMs – especially methacrylates. In paper III,

the organic composition as well as leachables of these materials were studied since previous publications on the subject are scarce. Some methodological considerations of paper III are presented in the following paragraphs.

Cured samples were made in a Teflon mould that allowed the production of samples with varying thickness. Initially, 1 mm thick samples were prepared. However, it was visually detectable that 1 mm samples of TheraCal LC® were insufficiently cured. The reason for this could be related to the material itself or the curing light, as mould materials have demonstrated not to influence degree of conversion of PRMs (196). It was attempted to switch curing device from Bluephase Style to Bluephase G2 (Ivoclar Vivadent, Liechtenstein), as the former device has been reported to have a more uniform light distribution (197,198). However, the problem persisted, suggesting that this was a limitation with light curing capabilities of TheraCal LC®. It was therefore decided to use a sample thickness of 0.6 mm in the final experiments.

Another problem encountered during sample preparation, was that all the prepared samples had visible pores. It appeared to be caused by air in the material syringe, as air bubbles kept raising from the uncured material during sample preparation. The extent of the pores were later assessed by micro-CT analyses.

Concerning leaching medium, several types of mediums have been used in the literature, including water and ethanol (23). Water has sometimes been described as a "clinical relevant" leaching medium, while ethanol has been used as a "worst-case" estimate, as it more readily extracts available organic substances from the materials (23). In a 24h pilot with pure ethanol, large amount of eluates were detected from the resin-modified pulp capping materials. However, it was decided to use water as the leaching-medium, as one of the main component in TheraCal LC® — Portland cement — sets through a series of water-dependent

steps (199). Ethanol would likely negatively influence the setting of TheraCal LC® and cause an overestimate of eluates.

Another immersion medium parameter, which briefly was assessed during pilots, was temperature. In contrast to what may be expected, increasing temperatures appeared to increase the presence of particles and eluates after 7-days for the calcium hydroxide containing material Calcimol LC (appendix 2a and b). This was in contrast to what was observed with samples of a flowable composite (Tetric EvoFlow, Ivoclar Vivadent) stored under similar condition. While this was not investigated further, it underscores that medium conditions, as well as the composition of the PRM material, may influence the behavior of immersed samples. This latter may be especially important for materials with a less studied composition (*e.g.* resin-modified pulp capping materials). In case of the observed phenomena for Calcimol LC, this was conceivably partly attributed to the fact that calcium hydroxide in Calcimol LC is less soluble at high temperatures. It was decided to use 37 °C in the experiments presented in Paper III as it represent a clinical relevant temperature, and is commonly used in other elution studies (23)

Airborne exposure to constituents in PRMs – experimental design in Paper IV

In contrast to the possible exposure to methacrylates from resin-modified pulp capping materials, airborne exposure to methacrylates seems to represent a low-dose exposure scenario (75). As respirable particles recently have been described as a source of methacrylate exposure under laboratory condition (24), we wanted to sample gaseous and particle-associated PRM constituents – including TEGDMA, during restorative procedures at the simulation clinic at the Department of Clinical Dentistry, UiT- The Arctic University of Norway.

The reason for selecting the simulation clinic as the place for sampling was partly due to practical concerns, *e.g.*, no patients, and easy access to operators.

However, it also enabled collection of data on hypothetical patient exposure through sampling of water collected in the mannequins, a precise determination on the amount of dust generated per procedures, as well as control over all the materials used during the clinical procedures. Another important aspect was that airborne exposure to methacrylates have not yet been studied in this subpopulation of dental personnel.

As the dental units used in the simulation clinic are similar to those used in the student clinic (and elsewhere), and the work done in the simulation clinic represent exposure to airborne methacrylates that can occur in dental offices during restoration procedures, the results obtained in the experiments should be possible to extrapolate to real-life conditions.

Concerning the sampling equipment, conductive polypropylene filter cassettes were used to account for electrostatically-related loss of particles during sampling. Extraction of organic substances was done immediately after sampling to avoid loss of methacrylates due to diffusion of volatile substances.

Initially, it was planned to conduct sampling of inhalable and respirable particles from all the restoration procedures; however, as pilot experiments indicated that both the filter cassette and cyclone sampling yielded non-detectable results, it was decided to only sample inhalable particles (which per definition also include respirable particles).

The BHT quantified in the majority of the sampled particles originated most likely not from the restorative procedures as there was no correlation between the amount of dust generated and the BHT signal in the samples. Furthermore, and perhaps more importantly, if BHT originated from the particles sampled, a signal for TEGDMA should also have been detected (as supported by analysis of the positive control and the uncured restorative material). Since BHT was not detected

in the blank samples of equipment and in the carry-over blanks, it was speculated that the source of the BHT was from cosmetic products from the participants (200).

Mass-spectrometry based analyses in paper III and IV

In paper III and IV, MS based methods were used to analyze organic substances in PRMs. Both UHPLC-QTOF-MS/MS and GC-MS were used for sample analysis. This approach enabled us to utilize the strengths of both methods. For example, GC-MS was a viable tool to screen the investigated materials for their constituents in paper III and IV. The use of UHPLC-MS, enabled us to investigate the presence of high-molecular weight, non-volatile substances that were present in the investigated materials. In paper III, it was found that Bis-GMA was not present in Theracal® LC, despite it being listed in the MSDS/SDS. In paper IV, the use of UHPLC enabled us to demonstrate that non-volatile, high-molecular substances can be transported with particles from PRMs to enter deep into the lung (as shown in the positive control).

Concerning identification and quantification of high-molecular weight substances, it was discovered that the CAS-numbers of substances listed in the SDSs of the investigated materials were not enough to be able to perform proper analysis of some substances, *i.e.*, polyethylene glycol dimethacrylate (paper III) and Bis-EMA (paper IV). Confusion regarding CAS-numbers have previously been described for other substances in PRMs, e.g., UDMA (201). The consequence of CAS-confusion may prevent proper identification and quantification of substances, as well as misinform scientists studying biological effects of substances that they think is present in PRMs.

The use of UHPLC-MS showed that the HEMA quantification performed in paper III on GC-MS may not be representative of the HEMA present in the materials investigated, as HEMA was not detected in the UHPLC-analysis. This

indicates that the detected HEMA may (partly) have originated in the GC-injector from decomposition of UDMA into HEMA and isocyanates — as previously hypothesized by others (55,93). Additional experiments to clarify this issue are currently being conducted. In case HEMA was not present in Calcimol LC or Ultra-Blend® Plus, the detected eluates of HEMA would likely reflect elution of UDMA, a substance that has been shown to be ten-folds more cytotoxic than HEMA *in vitro* (142,202)

To ensure the validity of the mass-spectrometry analysis, and to identify potential contaminants, blank samples from equipment and chemicals were analyzed. In addition, carry-over was assessed by analyzing blanks in between samples. Precision measurements (intra- and inter-day) were performed in both paper III and IV for the quantitative analysis. Of note, the inter-day precision related to TEGDMA quantification in paper IV was high (> 80 % relative standard deviation). The reason for these results were most likely a fluctuation in the ionization of substances, and indicates that there is an uncertainty in the quantification performed in paper IV for TEGDMA.

Related to term and use of internal standard, some thoughts are warrant. Per definition, the internal standard should undergo the same sample preparation procedure as the analytes being analyzed to be able to account for loss of analyte (203). In paper IV, the internal standard was added to the solvent used to extract organic substances from the dust and sorbent. However, in paper III, the internal standard was added during the extraction of organic substances from the elution medium. Ideally, it should have been added to the elution medium at the same time as the precured samples were immersed to be able to take into account loss of analyte due to evaporation/hydrolysis during the 7d immersion. However, this would have required an isotope-labelled standard to make sense. In the dental

literature, isotope labeled analogs of the analytes are seldom used as internal standards (23).

Concerning negative findings in the quantitative analysis, it is important to be aware that substances may still be present below the lower limit of detection. For example, eluates from composite restoration has been shown to be able to cause *in vitro* cytotoxicity, despite the fact that no substances were detected in the eluates (204).

General discussion of results

In vitro testing and the comprehension of toxicity

The toxic actions of a substance on an organism is ultimately exerted at the cellular level. In this respect, *in vitro* methods have a great potential in toxicology as they enable a molecular classification of the events that triggers adversity in human cells (96). In the future, such mechanistic information — obtained *in vitro* — may be used to predict hazards and risks of chemicals (96).

In relation to risks posed by methacrylates, relative few adverse effects are reported in patients despite the widespread use of PRMs (5). For dental personnel, the prevalence of adverse reactions is a little higher (approximately 1 % of dental personnel show signs of allergy towards methacrylates) (11). Yet, these are reported and symptomic adversities. As there are several scenarios where patients and dental health personnel may be exposed to low doses of these substances (23,57,75), the concepts of low-dose effects is an interesting theme to discuss.

One of the topics discussed in paper I, was the use of terminology related to low-dose effects of methacrylates. While this discussion was not related to the quality of the studies of the reviewed papers, the different definitions of the terms used may cause confusion. For example, it was not always clear whether the term non-toxic was referring to a risk assessment of the chemical (for human health),

or related to the effects observed with a particular assay. To avoid confusion, results should therefore be reported in accordance with the parameter/end-point measured by a specific assay (163). A term like non-toxic may also act as anchor for which concentration of a substances that are interesting to study. However, toxicity is also a question about definition. A seemingly non-toxic response *in vitro*, like increased cell survival, may be an unwanted response in the whole organisms. In the 21.tox vision (96), *in vitro* methods are envisioned to be able to identify non-toxic chemicals and concentrations; however, this would require mechanistic knowledge and a framework for understanding toxicity that currently is not yet fully developed (109,205).

In paper I, methods used in cell culture studies on PRM constituents, which may influence the interpretation and/or biological response of substances, were examined. Despite the existence of Good Cell Culture Practice guidelines since 2002 (157,170), these recommendations seem not yet to have been implemented to a great extent in research on *in vitro* effects of PRM constituents (as seen in the results in paper I). This problem is, however, not only related to investigation of PRM constituents (156).

The effects of a high and low concentration of TEGDMA on human cells

In paper II, the human monocytic cell line, THP-1, was exposed to an apparently cytotoxic (2.5mM), or non-toxic (0.3mM) dose of TEGDMA for 6h and 16h to assess early proteomic alterations in the exposed cells. The doses and time points were chosen after thorough testing of the effects of different concentrations and exposure times on cell viability, cell proliferation, and morphology, as discussed under Methods, in order to capture early changes in the cell proteomic profile. Several proteins were regulated in the cells after exposure to both TEGDMA doses (Appendix 1a and b); many of these proteins have not been previously reported to be induced by TEGDMA, and are involved in events described in section 3.6 (Toxicity of methacrylates). More specifically, oxidative stress and/or stress responses were upregulated in both dose groups. Some of the proteins involved in these pathways were regulated in a dose-independent manner. Of these, heme-oxygenase 1 (HMOX1), which is reported to be regulated by the transcription factor NRF2 (206), showed the highest up-regulation of all regulated protein – independent of both the treatment dose and exposure time.

Proteins associated with severe cytotoxic effects, *e.g.* DNA-damage, cell cycle disruptions and apoptotic signaling were only regulated in cells exposed to 2.5mM TEGDMA. In contrast, several cytoprotective proteins NAD(P)H dehydrogenase [quinone] (NQO1), multidrug resistance-associated protein 1 (ABCC1), thioredoxin reductase 1 (TXNRD1) were only overexpressed in the cells exposed to 0.3mM TEGDMA.

In summary, a non-cytotoxic (0.3mM) concentration of TEGDMA, as defined by the results of the real-time viability assay, cell counting, and morphology studies, caused changes at the proteomic level in THP-1 monocytes that may alter the cell phenotype, and increase cell survival. In contrast, the high dose tested,

caused early changes in proteins associated with cytotoxic reactions. This signifies that materials containing TEGDMA may negatively influence cells beyond certain doses.

Resin-modified pulp capping materials – a source of methacrylate exposure

Despite their easy-to-use nature, the direct application of an unpolymerised PRM to a pulpal wound surface will signify an acute, high-dose exposure to methacrylates and organic additives for the patient. Furthermore, the moist pulp can prevent a complete cure of the applied material at the site of application, signifying a risk for long-term exposure to unreacted constituents for the patient (6). Despite seemingly similar composition, Calcimol LC and Ultra-Blend® Plus (both containing TEGDMA) did not have the same indication for use. Ultra-Blend® Plus are indicated for direct pulp capping, while Calcimol LC is indicated for indirect capping (implying the presence of a dentin-bridge between the pulp and the material). The more cautious indication of Calcimol LC may be due to cytotoxicity concerns – however, this is only a speculation, since indication for use is determine by the manufacturers.

The adverse effects of resin constituents on pulp hemostasis has been demonstrated in cell experiments, as well as under clinical conditions (6,154). Heat development during light curing may also contribute to pulp injury: When used for indirect capping, Theracal® LC and Ultra-Blend® Plus have demonstrated to increase the pulp temperature more than 7.5 °C (207). In a human tooth culture model, it was shown that Theracal® LC was more toxic to cells, produced higher inflammatory effects and had a lower bioactive potential than a calcium silicate material without resins (208). It was concluded that "*… adding resins to tricalcium silicates alters their bioactive potential and leads to pulp toxicity*" (208). The availability of therapeutic agents from resin-modified pulp capping materials is

another issue. Several studies suggest that matrix constituents may inhibit the release of therapeutic agents under physiological conditions compared to conventional capping materials (209–211). This may further affect their clinical efficacy and feasibility, and question the clinical relevance of the pH-measurements in Paper III.

Sensitization is a concern for both patients and dental personnel. It was demonstrated that both Calcimol LC and Ultra-Blend® Plus contained TEGDMA, UDMA (and potentially HEMA) which are latent sensitizers, whereas Theracal® LC — a material indicated for all types of pulp-capping — contained mainly polyethylene glycol dimethacrylate (Paper III). Polyethylene glycol dimethacrylate is described as a moderate sensitizer (212). Bis-GMA – a compound listed in the SDS supplied with the purchased Theracal® LC in paper III, is also described as a sensitizer (83). Yet, the UPLC analysis of Theracal® LC (Paper III) revealed that it did not contain Bis-GMA. In the newer SDS of Theracal® LC, Bis-GMA is not listed (213). Thus, Bis-GMA seems to have been removed from the material. The reason for the removal can only be speculated on, but it could be related to cytotoxicity issues, as Bis-GMA have been shown to have a higher cytotoxic potential compared to other methacrylates (30). Yet, it could also be related to release of therapeutic agents, *i.e.*, the resin matrix of Theracal® LC (presumingly containing bis-GMA) have shown to modify the setting mechanisms and hydration characteristics of calcium silicate, resulting in lower release of therapeutic calcium ions *in vivo* (209,210). Interestingly, in an article published in 2016 by the manufacturer of Theracal® LC, it was advocated that Bis-GMA was considered too hydrophobic to achieve the hydration characteristics needed (214). Whatever the reason, the transparency associated with changes in material composition is worrisome for patients, dental personnel and researchers.

In accordance with the European Medical Devices Regulation (which also govern Norwegian legislations) (48), dental materials should not compromise the clinical condition and/or safety of the patients or user. With regard to resin-modified pulp capping materials, it would be difficult to see how these materials should be considered anything else then a compromise for patients – compared to methacrylate-free alternatives, which have clinical data that support their clinical efficacy (45). Furthermore, dental materials in general are classified as IIa-products, and should not achieve its intended action by any pharmacological means, *i.e.* it is stated in the MDR and MDD that "*All devices incorporating, as an integral part, a substance which, if used separately, can be considered to be a medicinal product … are in Class III*" (47,48). With regard to resin-modified capping materials, one may argue that the calcium hydroxide or calcium silicates present in these materials are a prerequisite for their advertised purpose (and interaction with the host). Other, bioactive dental materials – like adhesives that contain bioactive, antimicrobial monomers, have in the past been relabeled as class III materials for this reason (215).

Airborne exposure to methacrylates – a case of low-dose exposure

In paper IV, it was demonstrated that airborne exposure to methacrylates did not occur above the detection limit under clinical circumstances. This can be explained by several factor, *e.g.*, high-vacuum suction and water was mostly used during all aspects of the monitored polishing procedure (and always after the initial oxygen inhibition layer had been removed). In addition, the material used during a procedure may also influence the amount and type of unreacted constituents that may leech from particles. For examples, differences in quantifiable eluates has been shown between composite materials (216). With regard to the composite used in in Paper IV (ceram.x® universal), it has been demonstrated that it leech

considerable less TEGDMA (and other organic substances) in water during 24h compared to other materials (Figure 3, Paper IV). Yet, the positive control revealed that particles may be a potent source of exposure to airborne methacrylates even for this material, reinforcing the laboratory findings by COKIC et al (24). Furthermore, there are other clinical procedures that involve PRMs and particle generation that may be of concern. For example, in orthodontic practices and during aesthetic build-ups or treatment of severely worn teeth, a higher amount of PRM material is likely polished/grinded compared to the procedures monitored in paper IV. Thus, these are all scenarios that should be further studied.

Effects of low-dose exposure of methacrylates in humans

The applicability of *in vitro* research results in human health risk assessment is today limited. However, results from *in vitro* experiments may be used to generate hypotheses. From a health perspective, occasional, low-dose exposure for PRM constituents to patients — *e.g.* from leaching — may not pose a significant risk because of the low doses and frequencies of exposure involved. The low quantities of reported adverse effects in patients also underlines this notion (5). However, based on the present findings and literature, it may be speculated whether everyday exposure to low doses of airborne methacrylates, and other organic additives, in an occupational setting may be of concern as it is theorized that (repeated) exposure to chemicals may have long-lasting effects in cells thorough epigenetic mechanisms (217). That is, following a toxic insult, epigenetic changes can contribute to altered expression of proteins that may cause an delayed manifestation of disease or increased susceptibility to other chemicals (217) (Figure 17). There are a few real-life examples that support this mechanisms, *e.g.*, the lung expression of metabolic enzymes that influences the susceptibility to various chemicals vary between smokers, non-smokers and ex-

smokers (218). Furthermore, in monozygotic twins, epigenetic differences is evident – suggesting that susceptibility may even vary between genetically identical individuals (98).

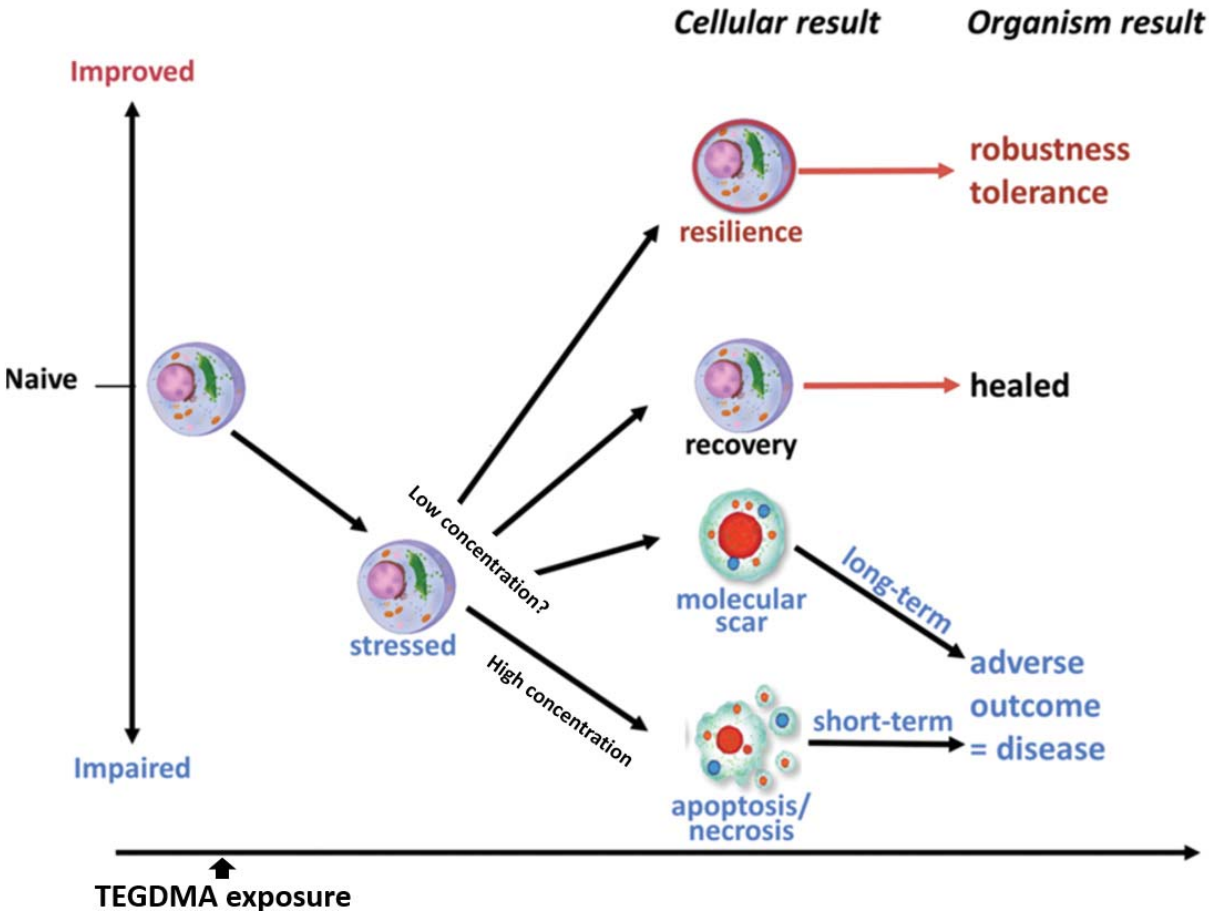

Figure 17: Cells subjected to any challenge to the intracellular homeostasis will mount a response aimed at removing the stimuli, repairing the damage induced by the challenge and restoring homeostasis. The cell may recover from damage, or go into a cell death routine. However, the chemical may also induce molecular scars, such as epigenetic changes, that potentially can lead to adverse outcomes over time.

Figure modified from: Smirnova L, Harris G, Leist M, Hartung T. Food for thought... Cellular resilience. ALTEX. 2015;32:247–60 (published under Creative Commons Attribution 4.0 International license) (217).

From the results presented in paper II, it seems that THP-1 cells in the 0.3mM TEGDMA treatment group were able to negate TEGDMA toxicity through upregulation of several proteins involved in antioxidant responses. Although not examined in the present study, these proteins are typically regulated by the NRF2 transcription factor, which is regarded as the master regulator of antioxidant

responses (125). As 0.07mM TEGDMA had nearly the same effect as 0.3mM TEGDMA on the THP-1 cell reduction potential in the real-time viability assay (both doses increased the cell reduction potential compared to control), it is likely that exposure of cells to even lower concentrations of TEGDMA than 0.3mM may affect the cell proteome in a similar fashion. With regard to other methacrylates, it has been shown that some perturbate similar pathways as TEGDMA, *i.e.* NRF2-regulated antioxidant cell responses inhibited HEMA-induced oxidative stress and supported cell viability in a similar fashion as observed in paper II (219). This underline that synergetic interactions may be important when characterizing risks of chemicals.

While increased antioxidant activity following exposure to a xenobiotic may be beneficial for the cell for a period, one may speculate if this can cause unwanted cellular effects with time as antioxidants indiscriminately increase cell survival (220). This may be particular relevant to malignant cells, which in general are more susceptible to ROS-induced damage (125). NRF2-regulated antioxidant responses were previously identified as a tumor suppressor; however, recent publications on the NRF2-pathway discusses its oncogenic potential (220,221).

For example, chronic exposure to low levels of arsenite — a ROS-inducing chemical — has been reported to cause transformation of cells *in vitro* by mechanisms that involves accumulation of NRF2 due to epigenetic dysregulation of Keap1 (the factor responsible for normal NRF2 degradation) and increased resistance to oxidative stress (222). Similar mechanisms may be relevant for long-term, low-dose exposure to other ROS-inducing chemicals, including methacrylates, and should therefore be addressed in future studies.

Conclusions

In line with the specific objectives specified for the thesis, the following main conclusions were drawn:

1. Non-standardized nomenclature and methods are commonly used in in-vitro cell studies on biological effects of PRM matrix constituents.
2. TEGDMA caused several dose- and time-dependent proteomic alterations in human THP-1 monocytes, even at low concentrations, that may be relevant from a health perspective.
3. Light-curing, resin-modified pulp-capping materials contain and elute several reactive organic substances that may influence their clinical feasibility. Despite seemingly similar composition, manufacturers have different indications for use for their materials.
4. Dust particles generated during restorative procedures may contribute to exposure to organic substances in PRMs. However, neither particle-associated nor gaseous exposure to airborne methacrylates were detected in samples collected on dental students.

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Appendixes

Appendix 1a: Upregulated proteins (after filtration of raw data) – Paper II

TEGDMA up																		
	4				6			22				12						
6h0_3_1	6h0_3_2	6h0_3_3	6h0_3_4	6h2_5_2	6h2_5_3	6h2_5_4	16h0_3_1	16h0_3_2	16h0_3_3	16h0_3_4	16h2_5_1	16h2_5_2	16h2_5_3	16h2_5_4	T: Protein names	T: Gene names
6H - 0,3mM				6h - 2,5mM			16h - 0,3mM				16h - 2,5mM					
c12	c13	c12	c13	c12	c13	c12	c12	c13	c12	c13	c12	c13	c12	c13		
0,44	0,39	0,01	0,30	0,03	0,00	0,28	0,59	0,65	0,31	0,53	0,80	0,78	0,56	0,59	Ferritin light chain	FTL
3,67	2,37	NaN	NaN	2,18	2,22	2,72	3,53	3,77	2,79	3,36	4,45	3,84	3,39	NaN	Heme oxygenase 1	HMOX1
0,47	0,00	0,29	-0,06	1,32	0,83	0,70	0,56	0,36	0,39	0,15	2,48	2,93	1,92	1,78	Heat shock 70 kDa protein 1B;Heat shock 70 kDa protein 1A	HSPA1B;HSPA1A
0,85	0,06	0,38	0,14	0,13	0,67	0,47	0,37	-0,11	0,26	-0,05	0,89	0,12	0,57	0,91	Squalene synthase	FDFT1
0,51	0,11	0,21	0,00	0,13	0,46	0,28	0,22	-0,11	0,15	-0,01	0,49	0,19	0,33	0,11	Isopentenyl-diphosphate Delta-isomerase 1	IDI1
NaN	0,36	0,37	0,21	-0,34	-0,13	-0,17	1,35	1,25	1,16	1,12	0,96	0,31	0,79	0,75	Glutamate--cysteine ligase regulatory subunit	GCLM
0,06	0,16	-0,03	0,10	0,31	0,04	0,18	0,16	0,28	0,06	0,23	0,62	0,87	0,26	0,51	Heat shock protein 105 kDa	HSPH1
NaN	0,19	0,42	0,27	NaN	-0,16	NaN	1,10	0,99	NaN	0,81	0,45	0,16	NaN	NaN	Perilipin-2	PLIN2
0,10	-0,02	0,27	0,09	-0,03	0,04	-0,10	0,53	0,41	0,52	0,33	-0,25	-0,47	-0,05	-0,17	Glutathione reductase, mitochondrial	GSR
0,19	-0,08	0,26	-0,14	-0,10	0,18	-0,12	0,55	0,27	0,44	0,19	0,06	-0,28	0,09	-0,16	6-phosphogluconate dehydrogenase, decarboxylating	PGD
0,05	0,13	0,09	0,11	0,76	0,43	0,53	0,10	0,18	0,08	0,16	1,57	2,32	1,10	1,32	DnaJ homolog subfamily B member 1	DNAJB1
NaN	0,09	-0,33	-0,17	NaN	1,74	2,25	NaN	0,54	-0,51	-0,28	0,77	2,84	NaN	1,91	NLR family member X1	NLRX1
NaN	NaN	0,39	0,23	NaN	-0,28	-0,45	1,22	1,06	1,13	1,27	0,56	0,84	-0,52	0,22	Sequestosome-1	SQSTM1
0,10	-0,03	-0,06	0,06	-0,73	-0,49	-0,20	0,41	0,39	0,41	0,37	-0,60	-0,64	-0,68	-0,61	Thioredoxin reductase 1, cytoplasmic	TXNRD1
0,39	-0,15	0,10	NaN	-0,16	0,25	-0,11	1,02	0,66	0,75	0,56	0,33	-0,16	0,10	-0,14	NAD(P)H dehydrogenase [quinone] 1	NQO1
NaN	0,04	0,11	0,05	-0,35	-0,03	0,02	0,52	0,51	0,46	0,52	-0,02	0,07	0,04	0,11	Histone chaperone ASF1A	ASF1A
0,25	-0,05	0,14	0,06	-0,22	-0,12	-0,40	0,79	0,48	0,47	0,31	-0,24	-0,70	-0,30	NaN	Pirin	PIR
NaN	NaN	0,31	NaN	NaN	NaN	NaN	0,70	0,62	0,46	NaN	NaN	-0,80	0,18	NaN	Protein CREG1	CREG1
NaN	NaN	NaN	NaN	NaN	NaN	NaN	1,08	1,01	NaN	NaN	1,00	0,92	1,83	1,21	Ferritin heavy chain;Ferritin heavy chain, N-terminally processed;Ferritin	FTH1
NaN	NaN	-0,05	NaN	NaN	NaN	NaN	0,64	0,39	NaN	0,44	-0,56	-0,57	NaN	NaN	Multidrug resistance-associated protein 1	ABCC1
NaN	NaN	NaN	NaN	NaN	0,17	-0,41	0,74	0,77	NaN	0,43	NaN	0,03	NaN	0,01	Microtubule-associated protein 1B;MAP1B heavy chain;MAP1 light chain LC1	MAP1B
NaN	NaN	0,27	-0,02	NaN	0,10	-0,17	0,56	0,32	0,29	0,21	0,09	-0,06	0,02	NaN	Oxidoreductase HTATIP2	HTATIP2
NaN	0,14	0,24	0,28	NaN	-0,09	-0,23	0,67	NaN	0,66	0,59	0,42	0,38	NaN	0,24	Transcription factor MafG	MAFG
0,11	-0,15	0,12	-0,13	-0,41	0,00	-0,27	0,35	0,14	0,36	0,20	-0,26	-0,60	-0,12	-0,31	Fermitin family homolog 3	FERMT3
0,15	NaN	NaN	NaN	0,07	NaN	NaN	1,23	NaN	NaN	1,98	NaN	1,37	NaN	2,09	Sulfiredoxin-1	SRXN1
NaN	NaN	NaN	NaN	NaN	NaN	NaN	0,86	0,47	NaN	NaN	1,94	2,57	NaN	NaN	Glycogenin-1	GYG1
0,04	0,08	0,05	0,03	0,06	-0,04	-0,02	0,28	0,35	0,30	0,37	0,58	0,55	0,60	0,50	4F2 cell-surface antigen heavy chain	SLC3A2
0,06	0,04	0,07	0,04	0,01	0,00	-0,02	0,25	0,25	0,37	0,31	0,33	0,25	0,33	0,41	Phosphoserine aminotransferase	PSAT1
-0,01	0,11	0,14	0,01	0,16	-0,02	0,13	0,13	0,19	0,05	0,06	0,47	0,61	0,21	0,37	DnaJ homolog subfamily A member 1	DNAJA1
NaN	0,26	-0,23	0,01	NaN	-0,49	-0,43	0,24	-0,48	-0,27	-0,33	-0,13	1,36	0,95	-0,39	Protein PML	PML
-0,10	0,14	NaN	0,03	0,16	NaN	NaN	NaN	-0,01	0,03	0,13	0,41	0,45	0,62	0,49	Urotensin-2	UTS2

Red text = significant value (Benjamini–Hochberg procedure with a false discovery rate (FDR) of 0.05). Green color: protein regulated in at least one of the treatment groups.

Values are Log2 (a number of 1 represent a two-fold change in expression compared to control). NaN = protein not detected in sample.

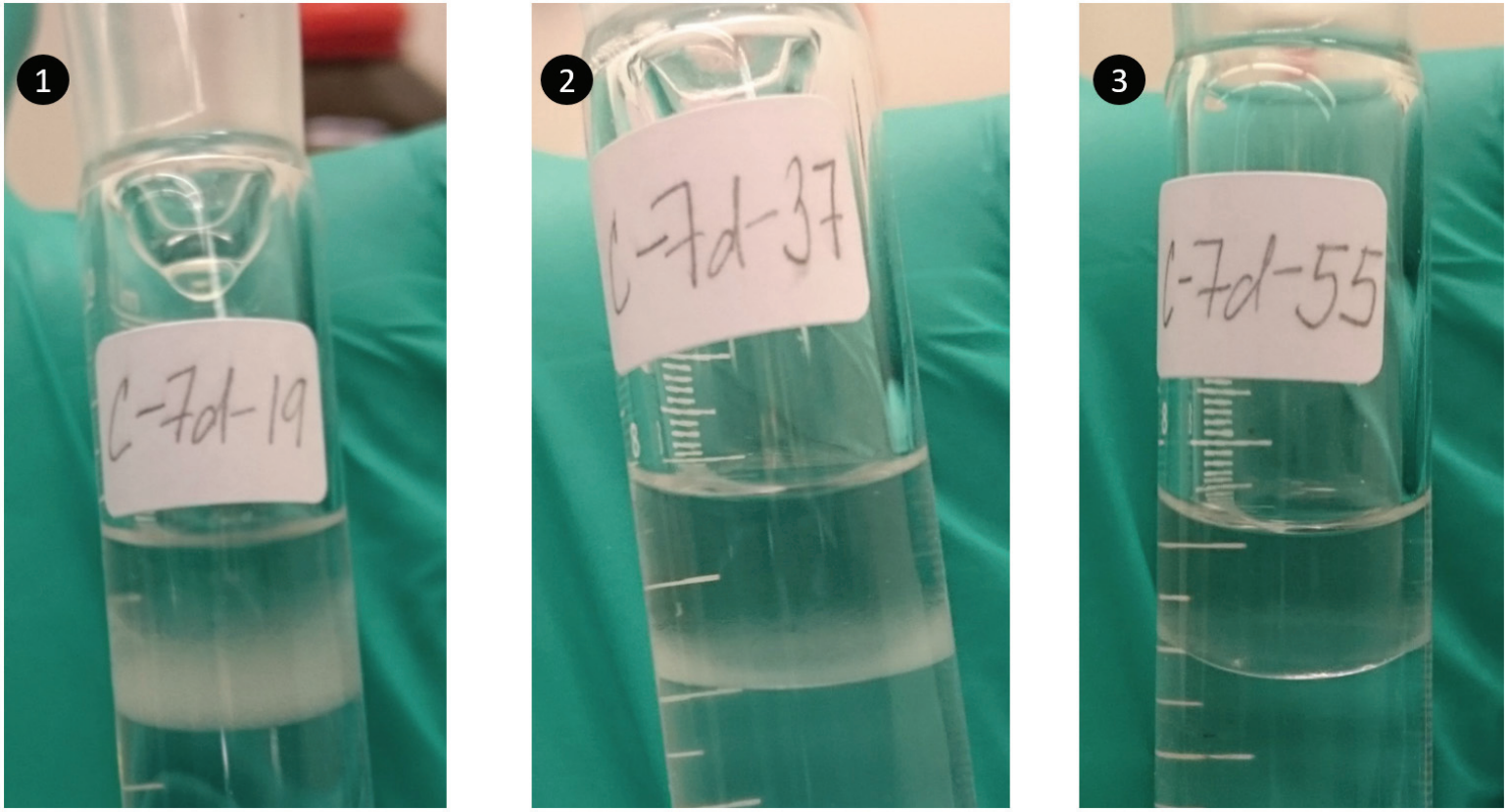
Appendix 1b: Downregulated proteins (after filtration of raw data) – Paper II

6H - 0,3mM				6h - 2,5mM			16h - 0,3mM				16h - 2,5mM					
TEGDMA down																
0				18			12				20					
c12	c13	c12	c13	c12	c13	c12	c12	c13	c12	c13	c12	c13	c12	c13	T: Protein names	T: Gene names
-0,73	NaN	NaN	NaN	NaN	NaN	NaN	-0,52	-0,53	-0,52	NaN	NaN	NaN	-2,30	NaN	Reticulon	RTN3
0,21	-0,22	0,30	-0,35	-0,60	-0,07	-0,70	-0,45	-1,13	-0,16	-0,92	-0,92	-1,22	-0,37	-1,10	Cathepsin G	CTSG
-0,20	-0,31	-0,08	-0,20	-0,74	-0,61	-0,80	-0,29	-0,42	-0,35	-0,48	-0,53	-0,49	-0,05	-0,15	Phosphoglycolate phosphatase	PGP
-0,11	-0,02	-0,25	0,08	-0,62	-0,52	-0,33	-0,02	-0,06	-0,20	0,01	-0,63	-0,98	-0,70	-0,61	Vimentin	VIM
-0,38	-0,18	-0,39	-0,15	-0,52	-1,71	-1,25	-0,45	-0,34	NaN	-0,42	-1,34	-1,77	-2,45	-2,39		PCM1
0,03	0,04	0,17	-0,22	-0,04	0,03	-0,12	-0,68	-0,90	-0,48	-0,71	-0,68	-0,76	-0,40	-0,64	Lysozyme C;Lysozyme	LYZ
-0,07	-0,14	-0,18	-0,07	-0,50	-0,47	-0,49	-0,37	-0,50	-0,41	-0,38	-0,15	-0,14	-0,40	-0,32	Chitinase-3-like protein 1	CHI3L1
-0,10	-0,09	NaN	NaN	-0,77	-1,45	-1,07	-0,80	-0,76	NaN	NaN	NaN	-1,02	NaN	NaN	CD70 antigen	CD70
-0,19	-0,03	-0,15	-0,02	-0,52	-0,63	-0,47	-0,24	-0,16	-0,11	-0,07	-0,67	-0,47	-0,53	0,18	Proteasome activator complex subunit 2	PSME2
-0,11	-0,10	-0,06	-0,05	-0,67	-0,55	-0,55	-0,14	-0,20	-0,07	-0,13	-0,71	-0,44	0,16	0,43	Proteasome activator complex subunit 1	PSME1
-0,27	-0,07	-0,10	-0,04	-0,62	-0,45	-0,56	-0,04	-0,06	0,04	0,03	-0,76	-0,59	-0,88	-0,82	Dihydrolipoyl dehydrogenase, mitochondrial;Dihydrolipoyl dehydrogenase	DLD
NaN	-0,07	-0,20	-0,32	-1,45	-0,63	-0,90	-0,13	-0,08	-0,16	-0,25	-0,38	-0,48	-0,54	-1,85	Thymidine kinase, cytosolic;Thymidine kinase	TK1
0,10	-0,03	-0,06	0,06	-0,73	-0,49	-0,20	0,41	0,39	0,41	0,37	-0,60	-0,64	-0,68	-0,61	Thioredoxin reductase 1, cytoplasmic	TXNRD1
0,02	-0,02	0,15	-0,11	-0,94	-0,33	-0,67	-0,35	-0,86	-0,35	-0,63	-0,72	-1,13	-0,39	-1,20	Neutrophil elastase	ELANE
-0,18	-0,02	-0,04	-0,02	-0,49	-0,22	-0,35	0,00	-0,06	-0,11	0,01	-1,05	-1,07	-1,05	-0,05	Dynamin-1-like protein	DNM1L
0,08	0,00	0,16	0,09	-0,62	-0,27	-0,30	-0,36	-0,49	-0,46	-0,40	-0,51	-0,78	-0,69	NaN	Azurocidin	AZU1
0,14	-0,05	0,09	-0,13	NaN	-1,06	-0,66	NaN	-0,29	-0,09	-0,03	-0,03	NaN	-1,85	0,54	Mitochondrial 10-formyltetrahydrofolate dehydrogenase	ALDH1L2
-0,14	0,04	-0,08	-0,06	-0,28	-0,69	-0,62	-0,08	-0,09	-0,18	-0,13	-0,37	-0,56	-0,69	-0,81	Antigen KI-67	MKI67
NaN	NaN	-0,14	-0,09	NaN	-1,14	-1,03	0,02	-0,15	-0,17	-0,07	-0,01	-0,15	NaN	-1,03	Centromere protein F	CENPF
NaN	NaN	-0,16	-0,15	NaN	-0,80	-0,87	NaN	-0,46	-0,36	NaN	NaN	NaN	NaN	-1,35	BRCA1-associated ATM activator 1	BRAT1
NaN	0,01	0,07	-0,07	NaN	-0,51	-0,65	0,19	0,12	0,27	-0,03	0,01	0,02	0,00	0,39	Golgin subfamily A member 2	GOLGA2
NaN	-0,08	-0,05	0,01	NaN	-0,74	-0,62	0,18	0,11	0,02	0,04	0,15	-0,19	-0,13	-0,05	Cell division cycle protein 20 homolog	CDC20
NaN	0,36	-0,08	-0,29	NaN	-0,89	-1,13	-0,03	-0,16	NaN	-0,21	-0,47	-0,38	NaN	-0,72	Golgin subfamily B member 1	GOLGB1
NaN	NaN	0,10	0,01	NaN	-0,85	-0,86	0,02	0,00	NaN	-0,08	-0,64	NaN	NaN	-0,84	Centrosome-associated protein 350	CEP350
NaN	NaN	-0,31	0,21	NaN	-0,80	-0,55	NaN	NaN	NaN	-0,10	-0,90	NaN	NaN	-0,40	Nesprin-3	SYNE3
NaN	NaN	-0,13	0,05	NaN	-0,67	-0,42	-0,24	-0,06	-0,13	-0,13	-1,73	-1,84	NaN	-1,87	Fanconi anemia group I protein	FANCI
NaN	-0,02	-0,12	-0,02	NaN	-0,53	-0,44	-0,22	-0,10	-0,14	-0,19	-0,61	-0,41	-1,49	-0,93	Telomere-associated protein RIF1	RIF1
NaN	NaN	-0,11	0,14	NaN	-0,93	-0,55	-0,27	-0,27	NaN	-0,26	NaN	-1,24	-1,26	-1,76	Tonsoku-like protein	TONSL
NaN	-0,12	-0,07	-0,16	NaN	NaN	-1,14	-0,76	-0,83	-0,77	-0,74	-2,58	-1,93	NaN	NaN	Interferon regulatory factor 8	IRF8
-0,10	0,07	-0,20	-0,05	-0,70	-0,73	-0,54	-0,24	-0,27	NaN	-0,07	-1,34	-1,22	NaN	-1,19	Thymidylate synthase	TYMS
-0,26	-0,05	-0,10	-0,14	-0,37	-0,28	-0,30	-0,80	-0,59	-0,63	-0,79	-0,97	-0,87	-0,67	-0,96	Myeloblastin	PRTN3
-0,17	-0,07	-0,11	-0,05	-0,17	-0,26	-0,20	-0,31	-0,23	-0,24	-0,25	-0,45	-0,39	-0,47	-0,37	Fatty acid-binding protein, epidermal	FABP5
NaN	0,04	-0,05	0,07	NaN	-0,16	-0,09	NaN	-0,41	-0,29	-0,17	-0,75	-0,93	-0,92	-0,82	Dynein assembly factor 5, axonemal	DNAAF5
NaN	NaN	-0,11	0,05	NaN	0,08	NaN	NaN	-1,26	-0,43	NaN	NaN	NaN	NaN	NaN	tRNA (guanine(26)-N(2))-dimethyltransferase	TRMT1
-0,02	-0,08	0,02	-0,17	-0,53	-0,08	-0,47	-0,18	-0,45	-0,06	-0,36	-0,90	-1,86	NaN	NaN	Stimulator of interferon genes protein	TMEM173
NaN	0,33	-0,12	-0,01	NaN	-0,62	-0,50	-0,22	-0,11	NaN	-0,06	-1,11	-1,23	-1,34	-1,35	Condensin-2 complex subunit D3	NCAPD3
-0,13	-0,03	-0,14	0,06	-0,29	-0,25	-0,12	-0,19	-0,01	-0,16	-0,04	-0,73	-0,88	-0,36	-0,65	Proteasome activator complex subunit 3	PSME3
-0,11	0,02	-0,07	-0,04	-0,28	-0,06	-0,07	-0,06	-0,09	-0,09	-0,03	-0,59	-0,64	-0,31	0,43	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	SAMHD1
NaN	NaN	-0,04	-0,07	NaN	NaN	-0,57	-0,32	-0,34	NaN	-0,17	NaN	-1,64	-1,50	-0,82	Zinc finger MYM-type protein 3	ZMYM3
NaN	NaN	-0,02	-0,18	NaN	-0,48	NaN	NaN	-0,13	-0,31	NaN	NaN	NaN	-1,62	-1,17	Coilin	COIL
-0,07	0,06	-0,06	0,01	-0,16	-0,18	-0,10	-0,03	-0,12	-0,27	-0,13	-0,52	0,03	-1,21	-0,71	Importin-4	IPO4

Red text = significant value (Benjamini–Hochberg procedure with a FDR of 0.05). Green color: protein regulated in at least one of the treatment groups.

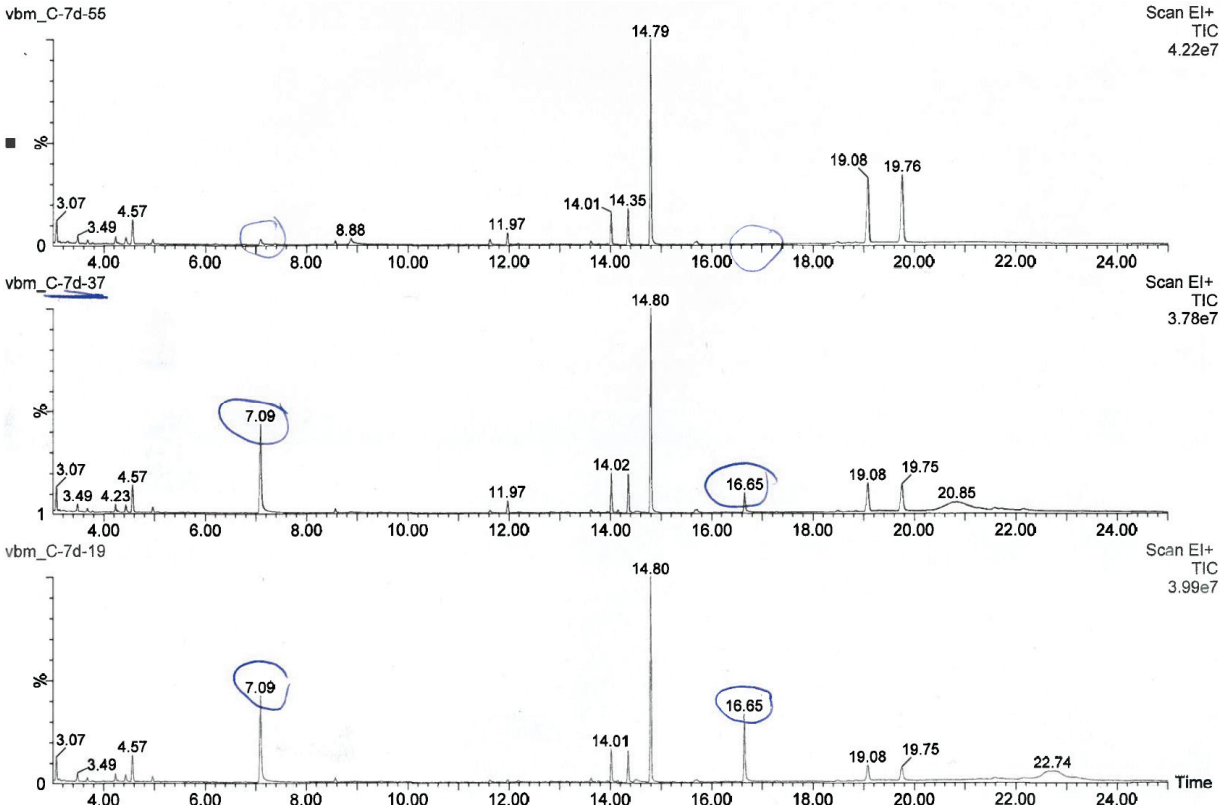
Values are Log2 (a number of 1 represent a two-fold change in expression compared to control). NaN = protein not detected in sample.

Appendix 2a: Visual changes in the immersion medium of samples of Calcimol LC kept at different temperature for 7 days – Pilots performed in relation to Paper III



The temperature of the immersion mediums appears to influence the appearance of the mediums after 7 days. The samples store at lower temperatures (1,2) seem to have more particles than the sample stored at 55 °C (3). This might partly be explained by the fact that calcium hydroxide inside Calcimol LC is more soluble at lower temperatures.

Appendix 2b: GCMS-analysis of the influence of immersion temperature on 7-day Calcimol LC leachables – Pilots performed in relation to Paper III



The temperature of the immersion medium appears to influence the amount of leachables in the mediums after 7 days. In the samples stored at lower temperatures (the two lower chromatograms), higher amounts of TEGDMA (16.65) and HEMA (7.09) in relation to internal standard (14.80) were detected than in the sample stored at 55 °C (uppermost chromatogram). This observation might partly be explained by the release of calcium hydroxide from Calcimol LC samples (appendix 2a), and the subsequent increased mobility of leachables.