Mastergradsoppgave

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Toxicity of the dental monomer BisGMA using cultured THP-1 cells as a model system

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Sammendrag

Bisphenol A-glycidyl dimethacrylate (BisGMA) er en av de vanligste monomerene brukt i polymerbastere tannfyllingsmaterialer. Studier har påvist utlekkinger fra tannfyllingsmaterialer, og dermed eksponering av både tannhelsepersonell og pasienter. I denne studien ble det benyttet en *in vitro* cellekultur, THP-1, for å undersøke cellulære effekter av BisGMA-eksponering. Resultatene viser at BisGMA reduserer celleviabiliteten, og ved mikroskopi er den bekreftet å stamme fra økt celledød (ved apoptose og nekrose). Andre metakrylater har vist toksisk effekt ved å binde til antioksidanten glutation (GSH) og å føre til dannelsen av reaktive oksygenforbindelser (ROS). BisGMA førte tilsynelatende ikke til økt ROS-nivå, og man så heller ikke GSH-nedgang som beskrevet ved eksponering for andre metakrylater. Tilførsel av noen antioksidanter så ut til å ha en effekt på celleviabilitet (MTT), men denne effekten kunne ikke observeres på celledøds- eller celleveksstmønster.

I andre BisGMA-eksponeringsstudier har man observert endrede cellevekstmønstre og DNA-skader. Dette kunne ikke denne studien verifisere, verken ved undersøkelse av cellevekstmønster, eller ved å analysere proteiner relatert til DNA-skade-respons. Det er godt beskrevet at andre metakrylater binder GSH, gir økt ROS og kan medføre DNA-skader.

Resultatene fra denne studien tyder således på at BisGMA induserer celleskader ved mekanismer forskjellig fra andre metakrylatmonomerer.

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Tord Lind-Hansen

List of abbreviations

BisGMA	bisophenol A-diglycidylmethacrylate
BPA	bisphenol-A
BSA	bovine serum albumin
DCFH-DA	2,7-dichlorodihydrofluorescein diacetate
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DSB	double strand break
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GDMA	glycerol dimethacrylate
GSH	glutathione
HEMA	2-hydroxyethyl methacrylate
HGF	human gingival fibroblasts
HRP	horseradish peroxidase
IL	interleukin
mBrB	monobromobimane
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
NIM-DAPI	4’,6-diamidino-2-phenylindole-dihyrochloride
PBS	phosphate buffered saline
PI	propidium iodide
Rb	retina blastoma
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SH	sulfhydryl
TBS	tris buffered saline

Abstract

Bisophenol A-diglycidylmethacrylate (BisGMA) is the most frequently used monomer in polymer-based dental composites. Studies have shown that leaching from these materials can occur, and both dental personnel and patients are likely to be exposed to methacrylate monomers.

In this study, an *in vitro* cell culture, THP-1, was employed to investigate cellular effects of BisGMA exposure. The results show that BisGMA will reduce cell viability, and fluorescence microscopy suggests the reduced cell viability is owed to increased cell death (apoptosis and necrosis). Other methacrylates induce toxic effect by binding to the antioxidant glutathione (GSH) and to cause an increase in the generation of reactive oxygen species (ROS). BisGMA exposure did not appear to affect the level of both GSH and ROS. The addition of some antioxidants produced an effect on cell viability, but this effect could not be identified as change in cell death or cell cycle distribution.

Other BisGMA exposure studies have observed altered cell growth patterns and DNA damage. This was not verified by the current study, by investigating cell growth or by analysing DNA damage response proteins. Many studies show that other methacrylates will bind to GSH, increase ROS and induce DNA damages.

The results from the current study suggest that BisGMA has an alternative mechanism of toxicity compared to other methacrylate monomers.

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1 Introduction

Humans have always tried to restore and improve the functions of the human body, particularly in the case of disease or trauma. Since the dawn of civilization, people have for that reason resorted to the invention and use of biomaterials to improve their conditions of health (Migonney, 2014). Although they were not labelled biomaterials until about 60 years ago, simple biomaterials have been an important part of human medical history, and in the present day, biomaterials are extensively used throughout medicine, biotechnology and dentistry (Ratner, 2013).

1.1 Biomaterials

Biomaterials are synthetic materials that are placed in contact with the human body to restore functions of natural living tissues. Biomaterials used in modern medicine include artificial heart valves, artificial ligaments and tendons, cochlear replacements and dental restorations and implants. Most implants prior to the 1950s suffered from low understanding of biocompatibility and lack of sterilization (Ratner, 2013). According to Van Noort (2013) "a biomaterial may be defined as a non-living material designed to interact with a biological system". As the biomaterial is in direct contact with living tissue, the interactions between the biomaterial and, in this case, the human body is of special importance, and is associated with the term *biocompatibility*: "Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimising the clinically relevant performance of that therapy." (Williams, 2008). Or, as defined by Ratner (2013): Biocompatibility is the "stable healing without significant ongoing inflammation or irritation".

1.2 Dental biomaterials

Teeth are disposed to both decay and wear, and attempts to repair tooth structure dates historically back to the early Neolithic (Coppa et al., 2006). Mayan people used sea shells to restore tooth enamel thousands of years ago, and wrought iron dental implants have been discovered in skeletons dating back to 200 AD (Ratner, 2013). In fact, the various dental applications are the most frequently encountered use of biomaterials discovered archaeologically (Migonney, 2014). Porcelain, ivory and dental amalgams are materials used in dental restorative procedures traditionally, and particularly the use of dental amalgam consisting of mercury has been prominent in the last 150 years (Van Landuyt et al., 2011). The fact that mercury is toxic to humans has been an increasing concern regarding its use in biomaterials, even if the presumed adverse effects of dental amalgam have yet to be documented scientifically (SCENIHR, 2015), and some researchers believe that the public opinion has been misled by non-scientific views driven and supported by media sensationalism (Jones, 2008).

Nonetheless, in Norway, these concerns lead to a ban on the use of mercury in 2007 (Miljøverndepartementet, 2007), ending the use of amalgam as a dental restorative material. Moreover, a polymer-based composite material offers a more aesthetical and mercury-free alternative, and the use of these materials in dental restorative procedures is increasing, both as a consequence of its aesthetic properties and the ban of dental amalgam.

1.2.1 Polymer-based dental composites – methacrylate monomers

Resin based (polymer based) composites is the most common used alternative to dental amalgam. A composite is composed of two or more components, typically to form a product superior to the individual components. A polymer is a large molecule consisting of repeating units called monomers, which are linked together by covalent chemical bonds (fig. 1-1). The methacrylate monomers are the most commonly used resin component of the composite (Van Landuyt et al., 2011), which easily will form polymers due to the reactivity of its double bonds.

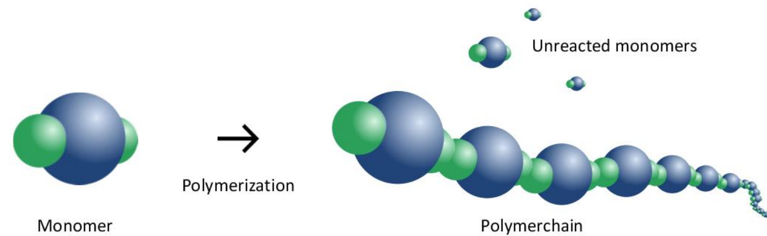


Figure 1-1 The polymerization reaction; the monomers are joined together by chemical bonds, forming a strong polymer. The accumulation of cross links between polymer chains further improves polymer strength.

The resin based composite consists of the following components with separate properties: the polymerizable resin, filler, and the filler-resin interface (Cramer et al., 2011). Radicals in the resin-phase will induce polymerization when exposed to visible light (photopolymerization) by extracting an electron from the double bond and leaving the other electron unpaired, forming a new free radical site in the molecule, thus starting a reaction which keeps adding monomers to the growing polymer chain (Anusavice et al., 2013). The products are polymerizable monomers which will convert from a liquid to a highly crosslinked polymer, crosslinking via the methacrylate monomers' vinyl groups. The filler particles main purpose is to strengthen the composite and reduce overall resin volume, thus lowering polymerization shrinkage. Lastly, the filler-resin interface couples the polymerizable moieties to the particle surface.

1.2.2 BisGMA

Bisphenol A-glycidyl dimethacrylate (BisGMA) is one of the most commonly used methacrylate monomers (Yoshii, 1997). It was first synthesized and published by Bowen (1963), and is often referred to as Bowen's Resin in the literature.

Through an addition reaction between one mole bisphenol-A (BPA) and two moles glycidyl methacrylate, the high-viscosity BisGMA monomer is formed. By using a tertiary amine as a catalyst, the addition of the phenolic hydroxyl groups to the epoxide groups connects the two molecules together. The BisGMA monomeric resin matrix is polymerized, using the energy from a blue-light source in combination with an initiator system which will create free radicals to start the polymerization process. The monomers

are joined together by chemical bonds; again, extensive cross-linking between vinyl-groups forms a robust polymer network.

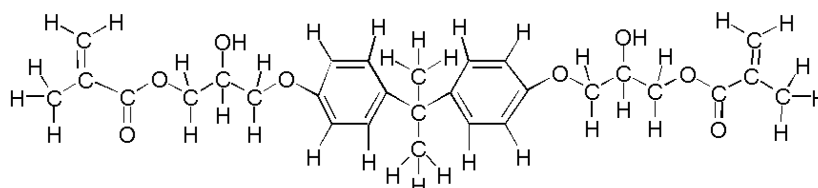


Figure 1-2 Molecular structure of bisphenol A-diglycidyl methacrylate (BisGMA).

Bisphenol-A (BPA) is an integral component of BisGMA, as the molecule consists of a BPA-core connected to two glycidyl methacrylate groups. BPA is reported to form adducts with DNA and bind to the estrogen receptor (Pulgar et al., 2000), and the oestrogenic activity of BPA is a cause for the restriction in its use in several areas, albeit the effect of BPA on estrogenic receptors are many-fold lower than the effect of actual oestrogens (Soderholm and Mariotti, 1999). Studies regarding the use of BPA-based monomers in dental restorative materials have found these materials unable to induce changes in oestrogen-sensitive organs and cells (Van Noort, 2013), and even though BPA can be effectively released from composites and sealants containing BisGMA, the amount reached is supposedly insignificant (Eramo et al., 2010). Nevertheless, Pulgar et al. (2000) have confirmed leaching of estrogenic monomers into the environment from BisGMA-based composites and sealants, in concentrations of biological significance. More recent studies concerning the release of BPA and its derivatives from dental restorative materials raise doubts as to their safety; even though the amount of BPA released is small, even small amounts can indicate paracrine activity, by way of affecting estrogen receptors, disrupt spermatogenesis and oogenesis and the disturb differentiation of tissue (Malkiewicz et al., 2015).

1.3 Exposure and toxicity

The exposure to components of dental restorative materials is likely to occur for both dental personnel and patients. Dental personnel can be exposed to components during both handling and placement, while patients typically during treatment and *in-vivo* leaching from the material over longer periods (Söderholm et al., 2000). For patients, there is also

the supposed release of particulates due to wear, degradation and leaching processes (Ansteinsson et al., 2013).

Dermal exposure and inhalation are the most likely exposure routes for dental personnel, where they are exposed to higher concentrations during shorter periods of time, and methacrylate monomers have been detected in the air of dental practices (Hagberg et al., 2005). In contrast to occupational exposure, patients are mainly exposed through the oral cavity during treatment and subsequent leaching, and as a result the gastro-intestinal (GI) – tract. Further, methacrylate monomers may migrate to the pulp and thus reach the bloodstream (fig. 1-3), and therefore virtually all organs in the body (Blasiak et al., 2012). As an example, studies suggest that BisGMA have the potential to destroy the gingival epithelium cells in the oral cavity, leading to periodontal disease (Reichl et al., 2008).

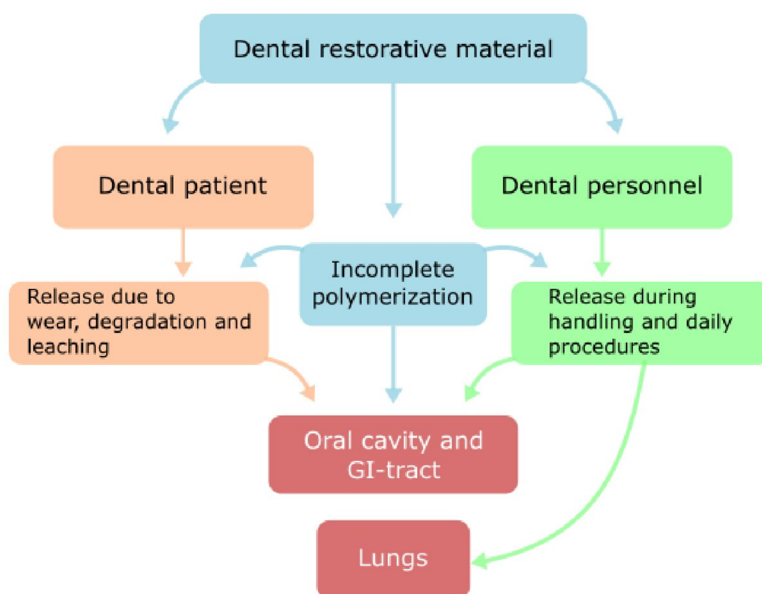


Figure 1-3 Diagram of main routes of exposure and exposure sites for dental patients and dental personnel. The oral cavity and GI-tract is a shared exposure site for both patients and personnel, whereas inhalation of released particulates predominantly occurs to dental personnel

The methacrylates are polymerized *in situ*, but unpolymerized components are released after the material has been set, as the polymerization process never is complete (Santerre et al., 2001). The degree of conversion, the fraction of carbon double-bonds that react during polymerization, typically does not exceed 70% (Atai et al., 2007), and some studies have shown that unpolymerized BisGMA could account for nearly 60% of the BisGMA content (Gajewski et al., 2012). Indeed, monomers from methacrylates, including BisGMA, have

been detected in the saliva after restorative therapy, with samples collected 10 mins after procedure (Michelsen et al., 2012). The accumulation of unpolymerized BisGMA monomers is owed to its low rate of conversion as well as the leaching-out from the polymeric networks (Zhu et al., 2015).

As a result, precursors of these synthetic polymers can react with biologically important molecules (Pulgar et al., 2000). For these reasons, scientific studies regarding the safety and molecular toxicology of substances released have been performed by numerous researchers: Bakopoulou et al. (2009) reviewed the cytotoxic and genotoxic effects of substances released by resin-based dental restorative materials, using several tools for risk assessment, and they conclude that the effects are biologically significant and can lead to irreversible disturbance of basic cellular functions.

1.4 Biological responses

1.4.1 Oxidative stress

Reactive oxygen species (ROS) are oxygen containing molecules which are chemically reactive and may cause oxidative damage to cellular macromolecules, which in turn can lead to disease. All organisms are regularly exposed to ROS, both as a natural by-product of oxygen metabolism and cell signalling, and as a response to environmental stress. Oxidation is one of the most significant contributors to DNA damage, and is considered an initiator of cancer (Ames and Shigenaga, 1992, De Bont and van Larebeke, 2004). The normal metabolic and biochemical reactions using oxygen in the living cell results in the continuous formation of ROS (De Bont and van Larebeke, 2004). And yet, the immune system also harnesses the reactivity of ROS to supplement its arsenal of molecular weaponry (Dickinson and Chang, 2011). Excessive amounts of ROS in the cell leads to oxidative stress and is associated with ageing and death (Barnham et al., 2004).

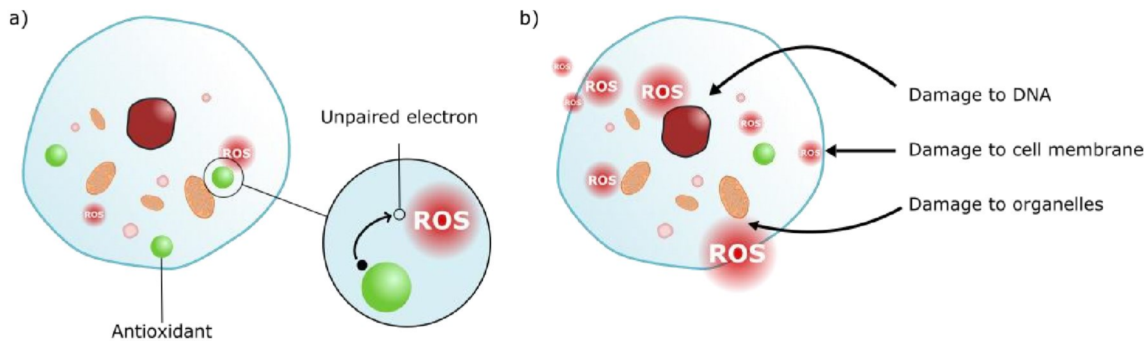


Figure 1-4 (a) Antioxidants in the cell acts as ROS scavengers, donating an electron to the ROS' unpaired electron. Consequently, the presence of an unpaired electron which can disrupt important cellular macromolecules is avoided. (b) But under conditions of oxidative stress, the antioxidant defence in the cell is unable to restrain the burden of the growing ROS. The result can be damage to proteins, lipids and DNA.

Oxidative stress (fig.1-4a) is defined as "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage" (Sies, 1997). Nucleic acids, proteins and lipids are examples of cellular macromolecules that can be damaged by exposure to excessive ROS (Kern and Kehrer, 2005). The oxidative degradation of lipids (lipid peroxidation) for instance, typically damages the cell membrane.

The potential damaging effects of ROS are under normal conditions regulated by antioxidant systems in the cell (fig. 1-4b). Halliwell (1989) state that an antioxidant is "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". Cellular antioxidants such as the enzymes superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase are used by the body to counteract increases in ROS levels (Ullevig et al., 2013).

Additionally, the vitamins E and C, carotenoids and, most importantly, glutathione (GSH) are some of the molecules that are a part of the antioxidant arsenal available to the cell, functioning as ROS scavengers in the cell. GSH is the principal hydrophilic cysteine containing antioxidant that protects cells against exogenous and endogenous toxins, including ROS (Aquilano et al., 2014). The nucleophilic sulfhydryl group (SH) of cysteine will react with electrophiles; in this way GSH protects cells by removing reactive metabolites (Timbrell, 2009). GSH also have roles in the metabolism of toxicants, where it takes part in conjugation reactions with metabolites that may be chemically reactive, metabolic products of the body's own drug metabolism or a foreign compound. These

conjugation reactions are usually an additional step in the drug metabolism process as the individual conjugates are more hydrophilic than the parent compounds, which facilitates excretion and reduces the likelihood of toxicity (Timbrell, 2009). A direct interaction between GSH and methacrylates have been found; other methacrylate monomers, such as HEMA and GDMA, have been shown to form adducts with GSH (Samuelsen et al., 2007, Schweikl et al., 2014), depleting the cellular content of GSH, thus lowering the cell's ability to respond to oxidative damage.

1.4.2 DNA damage

Exogenous chemical agents have the capability to damage the genetic information within a cell, producing deleterious mutations. When these mutations affect genes behind important protein products, such as those responsible for cell growth regulation, DNA-repair or apoptosis, the result can ultimately be cancer (Hanahan and Weinberg, 2000). The genotoxic substances induce DNA-damage through various interactions with the DNA sequence and structure, such as covalently binding to DNA (adducts), producing lesions, breaks, cross-links and different chromosomal aberrations, with oxidative modifications of DNA being one example. DNA damage will, when recognized by the many cellular control proteins that guard the DNA, start a signalling cascade that will eventually result in altered cell cycle control, cell death (apoptosis) or DNA repair (Ishikawa et al., 2006). The signalling cascade consists of many different players, starting with a sensor protein which detects the DNA damage, followed by activation through a sensor and a transducer, and ending with an effect mediated by an effector (fig. 1-5). Transducers such as CHk1 and CHk2, two serine/threonine kinases, are activated in response to various genotoxic insults (McGowan 2002), where CHk2 appears to be mainly activated in response to double-strand breaks and CHk1(Bartek and Lukas, 2003). P-Rb is the protein product of the retinoblastoma tumor suppressor gene, and its main role is to act as a signal transducer connecting the cell cycle clock with the transcriptional machinery (Weinberg, 1995). Analysis of signalling activity will provide information on DNA damage.

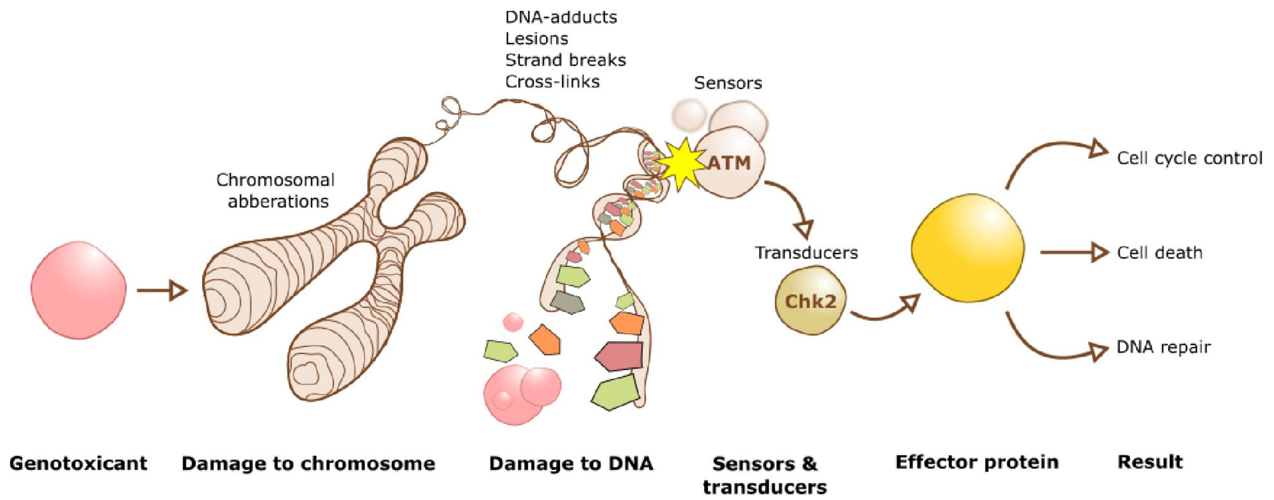


Figure 1-5 Signalling cascade initiated by DNA damage: A genotoxicant, like ROS, will induce damage to the DNA sequence and structure and can, if the damage is not recognized and mediated by the several sensor proteins guarding the DNA, ultimately lead to the development of cancer.

Cell survival and proliferation are reliant on the cell's ability to maintain genomic integrity, and inaccuracy in DNA replication and maintenance can result in deleterious mutations leading to cell death or, in multicellular organisms, cancer (Shackelford et al., 1999). Moreover, during cell division, the single stranded DNA is without base-pairing, nucleosomes, or histones and is more sensitive to damage than double-stranded DNA (Ames et al., 1993).

Normally in the eukaryotic cell cycle (fig.1-6), the majority of cells are quiescent. When signalled to initiate dividing it enters G_1-phase which precedes the synthesis of DNA (S-phase). The G_2-phase, where the cell is prepared for mitosis, is followed by the M-phase, where DNA-content is divided between the daughter cells.

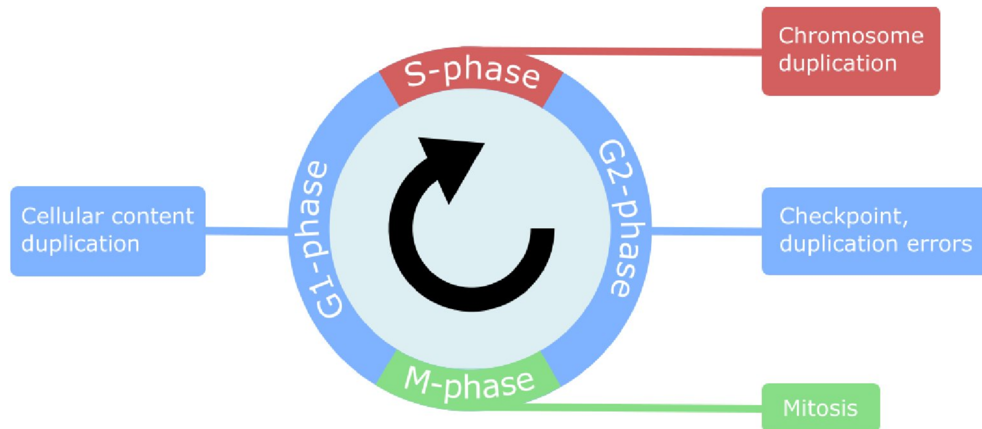


Figure 1-6 The eukaryotic cell cycle. During the gap-phases, several control mechanisms called checkpoints, ensures that everything is ready to duplicate DNA (S-phase) without errors and to start cell division (M-phase).

Under normal conditions the cell cycle proceeds without interruptions, but when the numerous surveillance control mechanisms which monitors the cell cycle discovers damage, it enables the cell to arrest proliferation in the G_1, S or G_2-phase and then resume proliferation after the damage has been repaired (Shackelford et al., 1999). Alternatively, the cell may undergo apoptosis if the damage is too extensive to be repaired (Niida and Nakanishi, 2006). DNA damage can be triggered by exposure to exogenous chemicals, attained through chemical events such as hydrolysis, exposure to ROS or other reactive metabolites (De Bont and van Larebeke, 2004).

The ability of cells to pause transiently during the cell cycle in response to agents that cause damage, principally to DNA, gives researchers the opportunity to observe toxic responses through the cell cycle, where for instance an increase in the ratio of G_1 and S to G_2 can indicate cell injury.

1.4.3 Inflammation

Inflammation is a part of the innate (non-specific) immune response that occurs in response to any type of damage to the human body (Ferrero-Miliani et al., 2007). As such, inflammation and its mediators, the cytokines, acts as a first response to cellular injury. Cytokines are small signalling molecules released by cells in response to external stimuli and they affect the behaviour of other cells by binding to their specific receptors on cell

membranes. This activates a signalling cascade causing specific gene expressions. Throughout an inflammatory response, different pro-inflammatory cytokines are produced to assist and warn other cells, or to down-regulate the response. They are primarily produced and secreted by macrophages, T-cells and B-cells, but also by cell types like endothelial and epithelial cells (Takizawa, 1998). Interleukins are a group of cytokines essential for the immune system; several of them are secreted by immune cells and are important mediators of inflammation and overall immune response (Alberts et al., 2015).

Interleukin-1 beta (Il-1ß) is mainly produced by blood monocytes and it is one of the most important soluble mediators of inflammation (Ferrero-Miliani et al., 2007). Interleukin-6 (IL-6) is secondary cytokine; given that it its secretion is initiated by the cytokine Il-1ß (Ferrero-Miliani et al., 2007). IL-6 activity is vital for the transition from acute inflammation to acquired immunity or chronic inflammatory disease, and has both pro- and anti-inflammatory properties by reducing the levels of other pro-inflammatory cytokines (Schindler et al., 1990). Interleukin-8 (IL-8) is secreted by many cell types, including monocytes. Il-8 is produced in response to pro-inflammatory stimuli such as exposure to IL-1, TNF, LPS, and viruses.

1.4.4 Cell death

There are two distinct ways in which a cell can die; by apoptosis or necrosis (fig. 1-7), and this is the most widely used classification of cell death (Hotchkiss et al., 2009). Apoptosis is an organized process of cell death that is critical in embryonic development and is involved in cell turn-over in healthy adult tissues, as well as being triggered by noxious agents and participating in the body’s defence against cancer (Kerr et al., 1972). The primary morphologic feature of apoptosis is shrinkage of the cell and its nucleus, chromatin condensation, nuclear fragmentation, and the formation of plasma-membrane blebs (Hotchkiss et al., 2009). Apoptosis is an active removal of damaged cells which occurs without inducing inflammation (Casarett and Doull, 2013).These processes rapidly remove contents of the cell that can spill out onto surrounding cells and inflict damage.

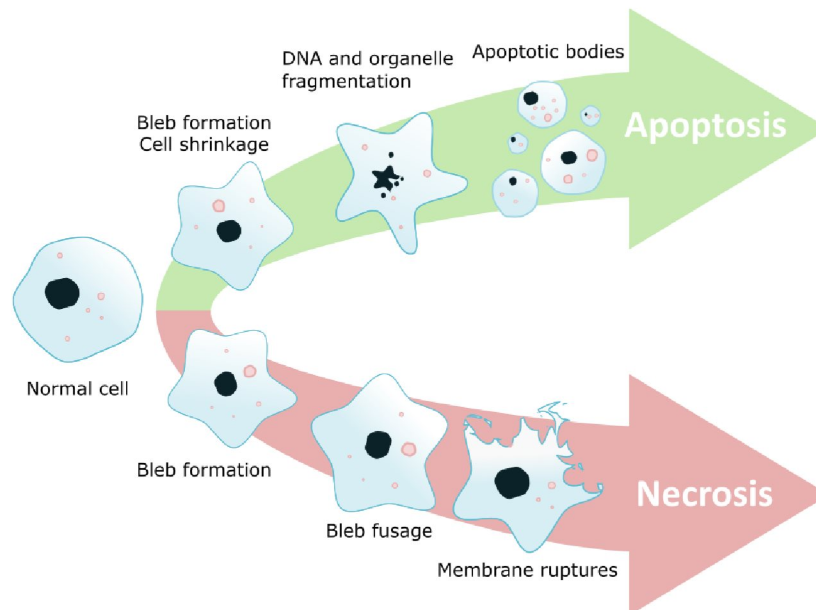


Figure 1-7 Cell death – apoptotic and necrotic pathway. While apoptosis is a regular method of discarding unwanted cells, necrosis is a disorganized process leading to inflammation and disruption to the cells local environment.

Dysregulation of apoptosis occurs in multiple diseases including neurodegenerative diseases, heart and lung and autoimmune diseases, as well as cancer (Anathy et al., 2012). Necrosis, on the other hand, is a disorganized process involving swelling and rupturing of membranes and dissolution of organized structures in the absence of cellular ATP (Denecker et al., 2001). Mainly, the rupturing of the cell membrane in necrosis will initiate inflammation, a fact which separates it from apoptosis (Fulda, 2010). And so, the early loss of integrity of the plasma membrane permits an influx of extracellular ions and fluid, with resulting swelling of the cell and its organelles (Hotchkiss et al., 2009).

1.5 Aims of study

In this study we hypothesized that the mechanism of BisGMA toxicity differs from other methacrylates previously studied.

The specific aims of this study were to:

1. Map cell viability and cell death in relation to GSH and ROS levels
2. Investigate DNA damage response

2 Materials and Methods

2.1 Cell culture and cell treatment

2.1.1 Cell line, THP-1

An *in vitro* cell line was used in this study, specifically the human leukaemia cell line, THP-1. It was originally cultured from the blood of a young boy with acute monocytic leukaemia and is a cell line with distinct monocytic markers, a fact which has been useful in studying the human immune response and mechanisms of macrophage differentiation (Tsuchiya et al., 1980).

After treatment with phorbol esters, phorbol 12-myristate 13-acetate (PMA), the cells will differentiate and behave like native monocyte-derived macrophages (Auwerx, 1991), thus giving researchers an opportunity to study macrophage involvement in inflammatory disease (Park et al., 2007), although this was not employed in the current study.

The myeloid leukaemia-derived cell lines do not express detectable levels of the tumor-suppressor gene p53 mRNA (Durland-Busbice and Reisman, 2002), a fact which makes experiments using p53 as an endpoint impractical.

2.1.2 Cell culturing

The cells were grown in suspension in 75 cm^2 cell culture flasks with a density of 600.000 cells per ml RPMI-1640 medium. The cells were split three times a week, Monday, Wednesday and Friday. Cells were counted and inserted in new flasks with fresh RPMI-1640 medium once a week, continuing a density of 600.000 cells per ml. When adding new medium, 500 ml of RPMI-1640 was supplemented with the additional constituents listed in table 2-1. All cell culture work was performed under sterile conditions.

Table 2-1 Additional constituents added to cell culture medium

5 ml (1M) HEPES buffer, for maintaining physiological pH-levels.
5 ml (100 mM) sodium pyruvate to provide an additional energy source.
50 ml FBS.
2.75 ml (10 mg/ml) of the antibiotic gentamycin.

The old medium was discarded and replaced with fresh medium every week. By centrifuging the cell culture at 50 g for 5 minutes, the cells would form a pellet and the residual supernatant consisting of the old medium can be discarded. The addition of new medium to the cell pellets was prepared so the renewed cell culture would display a cell density of 600.000 per ml.

Preceding the experiments, a sample of the cell culture was examined with a counting chamber, a hemocytometer, to determine cell density manually by counting the cells on a grid in a phase contrast microscope. 10 µl were inserted on each chamber slit and the average cell amount of 4 squares was calculated to measure the correct cell density. An automatic cell counter (MOXI Z, ORFLO Technologies) was also used at the end of the thesis period, where the cell density of the culture was determined prior to seeding by pipetting a 75 µl cell culture sample into the slot for analysis, at which point cell density was calculated by the cell counter. Finally, the cell culture was diluted in RPMI-1640 medium to obtain a cell density of 600.000 per ml.

2.1.3 Exposure to BisGMA

The cells (concentration 600 000 cells/ml) were seeded in cell culture dishes and incubated for 24 hours at 37°C, 5% CO_2 and 95% relative humidity. Stock solution of BisGMA diluted in the solvent dimethyl sulfoxide (DMSO) was prepared for exposure and further diluted in cell culture medium. A concentration gradient was arranged (table 2-2) and the concentrations were added to the designated wells and incubated for 24 hours at 37°C, 5% CO_2 and 95% relative humidity.

Table 2-2 BisGMA concentrations used in experiments

	Concentrations used in experiments					
BisGMA (µM)	50	25	12,5	6,25	3,125	0 (DMSO)

Visual inspection revealed that BisGMA was not soluble in the medium at concentrations above 50 µM. Preparing stock solution proved to be challenging, as the high viscosity BisGMA “solution” is difficult to measure and handle with precision. Therefore, BisGMA concentrations may vary somewhat among each stock solution prepared.

2.1.4 Antioxidants

The antioxidants L-ascorbic acid (vitamin-C), Trolox and N-acetyl cysteine (NAC) were added to the cell culture in a selection of the experiments, as they all are scavengers of ROS. This was done primarily to mimic the effects of the antioxidant arsenal accessible to the human body, and which is therefore not available in cell culture used in the *in vitro* experiments, and to reduce the anticipated effects of oxidative stress. Trolox is a water soluble vitamin E analogue, and NAC mimics the action of the vital antioxidant glutathione (GSH), through replenishing the GSH stores. Vitamin-C is a potent water-soluble antioxidant in humans, and must be ingested for survival (Padayatty et al., 2003).

The antioxidants were prepared according to table 2-3, with a new batch made prior to each experiment, and the antioxidants were mixed in either cell culture medium or solvent (DMSO) to obtain correct solution concentration.

Table 2-3 Preparation and solutions of antioxidants

Antioxidant	**Molecular weight**	**Solution**	**Resulting concentration**
Vitamin-C	176.12 g x mol^{-1}	10 mg vitamin-C + 5.7 ml cell medium	100 μM
NAC	163.19 g x mol^{-1}	100 mg NAC + 6.2 ml cell medium	1 mM
Trolox	250.29 g x mol^{-1}	10 mg Trolox + 400 μl DMSO	100 μM

2.2 Cytotoxicity assays

Overall cytotoxicity was measured with different assays (fig. 2-1) to establish the anticipated cytotoxicity of BisGMA, without initial concern to the exact mechanism of toxicity.

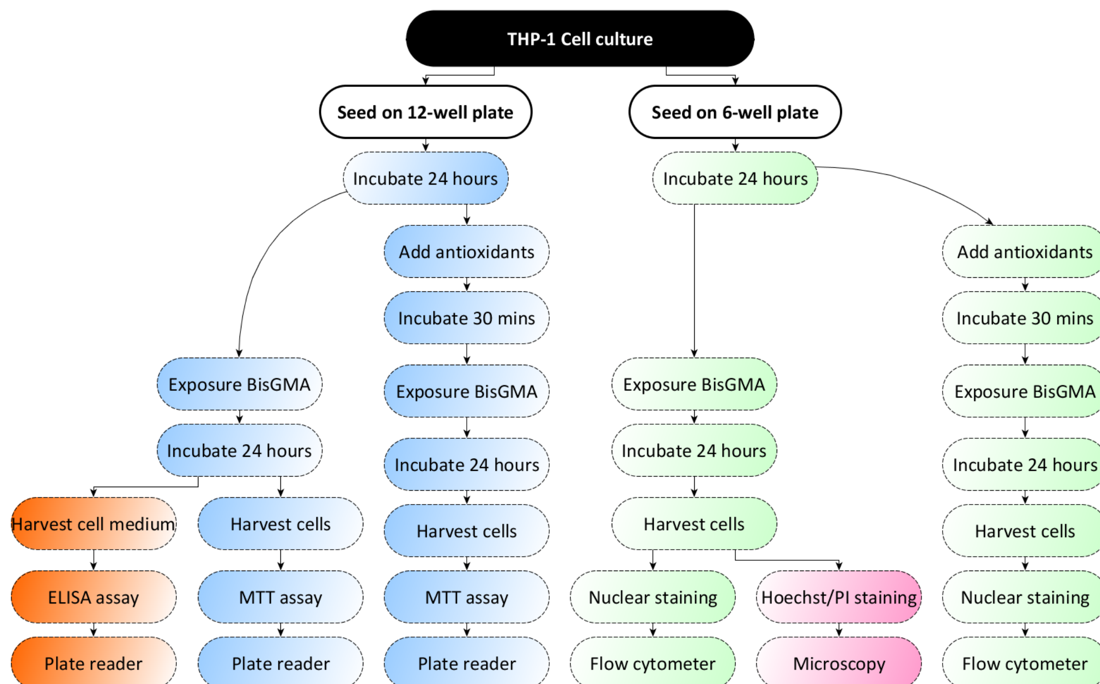


Figure 2-1 Outline of selected cytotoxicity assays. Experiments routinely started with seeding of cell culture, followed by exposure to BisGMA, and ending with harvest of exposed cell culture or medium and further analyses of obtained data

2.2.1 Cell toxicity assay, MTT

The MTT assay (fig. 2-2), a standard technique for measuring cell survival and proliferation, i.e. cell viability, was used to evaluate general cytotoxicity of BisGMA when exposed to the THP-1 cell culture. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of the yellow MTT substrate into visible blue formazan crystals by mitochondrial succinate-dehydrogenase (SDH) -enzymes (Mosmann, 1983). In view of the fact that the SDH enzyme is only active in living cells, this method allows researchers to estimate the amount of viable cells in an exposed cell culture based on the amount of formazan crystals present. And so, the amount of formazan corresponds approximately to the amount of viable cells, and the associated absorbance can be determined using a multiwell scanning spectrophotometer (fig. 2-3).

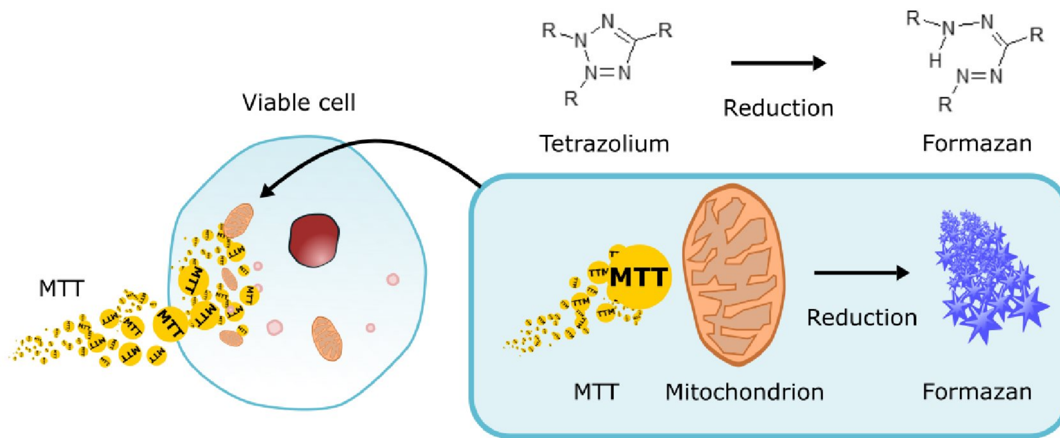


Figure 2-2 The yellow MTT substrate (a tetrazolium dye) is reduced by SDH, which is a membrane bound mitochondrial enzyme complex. Most importantly, it is only active in living cells; for this reason the absorbance of the resulting blue formazan crystals can be identified and used to quantify the amount of viable cells in a sample.

Procedure:

1. 75 µl MTT-assay were added to each well in a 12-well plate
2. 1 hour incubation at 37 ˚C
3. Each well transferred to a micro tube and centrifuged at 50 g for 5 minutes.
4. Supernatant removed
5. 500 µl of DMSO (Sigma-Aldrich, St. Louis, USA) added to each tube to dissolve the formazan crystals
6. 75 µl from each tube were inserted in two replicates to a 96-well plate and scanned in a multiwell scanning spectrophotometer (Synergy H1, BioTek Instruments Inc., Vermont, USA), which read the plate at 570 nm to determine absorbance

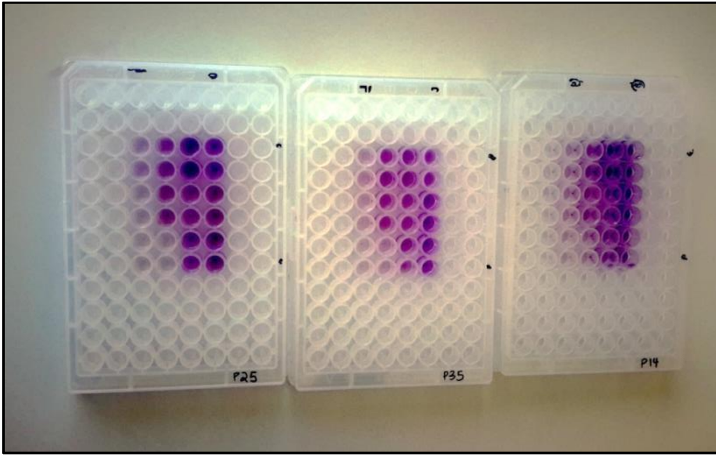


Figure 2-3 The blue colour from the formation of formazan crystals is clearly visible and is the foundation of the MTT-assay. Stronger colour correlates with higher cell viability, and the exposure gradient can practically be established from the photo.

2.2.2 GSH measurements

It is possible to measure the concentration of GSH in cells by adding the reagent monobromobimane (mBrB), which will bind to GSH's sulfhydryl (SH) group by passing through the cell membrane. Using a flow cytometer one can determine the amount of fluorescence of the bound mBrB, with increased fluorescence relating to increased amount of GSH in each cell.

Procedure:

1. Cell culture is washed with PBS, centrifuged (50 g, 5 min) and supernatant removed
2. 400 µl PBS with 1% FBS and 40 µM mBrB is added to every sample
3. 15 min incubation in room temperature and darkness
4. The fluorescence is measured on a flow cytometer

2.2.3 ROS measurements

Using the reagent DCFH-DA (2,7-dichlorodihydrofluorescein diacetate), the amount of ROS in cells can be measured using a flow cytometer. The reagent is converted by cellular esterases to a more polar molecule (DCFH) which is further oxidized in contact with cellular ROS, producing a fluorescent molecule (DCF) which can be detected using a flow

cytometer. The amount of cellular ROS corresponds proportionally to the amount of DCF detected.

Procedure:

1. Incubate cells with 10 µl DCFH-DA (20 µM) the last 15 minutes exposure
2. Each cell is transferred to a micro tube
3. The fluorescence is measured on a flow cytometer

2.2.4 Classification of cell death by fluorescence microscopy

By staining the cells with fluorochromes, it is possible to visually identify cellular morphology using a fluorescence microscope. In this case, by adding the fluorochromes Hoechst 33342 and propidium iodide (PI), viable cells as well as necrotic and apoptotic cells can be identified and classified visually (fig. 2-4). Viable cells will be stained blue by Hoechst 33342, as only intact cell membranes will be penetrated. Likewise, the condensed chromosomes of apoptotic cells lead to a more intense blue fluorescence. In contrast, PI does not penetrate intact cell membranes, a characteristic of necrotic cells, and will stain these cells red.

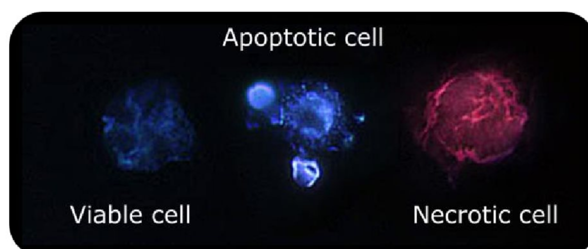


Figure 2-4 Stained cells were identified and classified according to morphology and colour in the microscope. Illustrative images taken from THP-1 cell culture exposed to 50 µM BisGMA.

Procedure:

1. Exposed cell culture is centrifuged (50 g, 4˚C, 5 min)
2. Supernatant removed
3. 100 µl FBS with Hoechst 33342 (10 µg/ml) and PI (10 µg/ml) is added per sample
4. 30 min incubation in room temperature, protected from light

5. One drop is pipetted onto a microscope slide and spread evenly out
6. When dry, the slide is studied in a fluorescence microscope (excitation filter 340 – 380 nm)
7. 300 cells counted from each sample

2.2.5 ELISA

Using the diagnostic tool enzyme-linked immunosorbent assay (ELISA), very small quantities of antigens, such as proteins, peptides, hormones, or antibodies, can be detected (Gan and Patel, 2013). This is achieved by the basic immunological concept of an antigen binding to its specific antibody. A secondary, enzyme-coupled antibody, detects the antibody connected to its antigen. This enzyme produces a visible fluorescence, employing a chromogenic substrate, which indicates the presence of the antigen (Gan and Patel, 2013). A colorimetric reading can then be performed using a multiwell scanning spectrophotometer, where the absorbance is correlated with the presence of the antigen in question.

The group of cytokines examined in this study was the interleukins Il-1ß, Il-6 and Il-8. Supernatants were set aside from cell harvest and stored in the freezer awaiting procedure. The release of pro-inflammatory cytokines were analysed in the supernatants after exposure using ELISA kits. The plate preparation and assay procedure is identical for the cytokines studied, but reagents vary and were prepared according to producer's protocol (table 8-4):

Plate preparation:

1. A 96-well microtiter plate was coated with 100 µl per well of the diluted capture antibody. Plate was sealed and incubated overnight at room temperature.
2. Each well was washed with the wash buffer using an autowasher, repeated 2 times.
3. Plates were blocked by applying 300 µl reagent diluent to each well. Incubated at room temperature for 1 hour.
4. Repeat wash procedure from step # 2.

Assay procedure:

1. 100 µl sample or standards are added to each well. Covered with adhesive strip and incubate at room temperature for 2 hours.
2. Wash procedure.
3. 100 µl of the detection antibody is applied to each well, covered with adhesive strip and incubated at room temperature for 2 hours.
4. Wash procedure.
5. 100 µl of streptadivin-HRP added to each well. Plate is covered and incubated for 20 minutes at room temperature.
6. Wash procedure.
7. 100 µl of substrate solution to each well. Incubated for 20 minutes at room temperature.
8. 50 µl stop solution to each well.
9. Using a multiwell scanning spectrophotometer, the optical density was determined at 450 nm wavelength.

2.2.6 Western blot

The western blot is a widely used analytical technique in cell biology aimed at detecting, separating and quantifying specific proteins (Mahmood and Yang, 2012). It is based on the movement of proteins through a polyacrylamide gel (SDS-PAGE) subjected to an electric field. The individual protein molecular weight and charge decides how far in the gel they will travel as the acrylamide mesh provides narrow pores in the gel, this allows smaller proteins to travel further than larger proteins, thus separating the proteins according to molecular weight, measured in kilodaltons. A protein marker with known molecular weights is added alongside the samples to function as a reference.

The separated proteins are then transferred and immobilized onto a nitrocellulose membrane. By applying an electric field for a second time, the negatively charged proteins move towards the positive field and the membrane.

The nitrocellulose membrane is incubated with a primary antibody specific to the protein which is studied, and a secondary antibody which binds to the primary antibody and will allow for visualization of the proteins in question.

Procedure:

1. SDS PAGE gels were prepared according to table 2-4.
2. 25 µl of each sample is first electrophoresed and then blotted onto a nitrocellulose membrane
3. Membrane is stained with Ponceau S
4. Membrane is blocked in 5 % BSA in TBS-T (TRIS-buffered saline with 0.1 % Tween) for 20 minutes
5. Incubation with primary antibody diluted (1:2500) in 1 % BSA in TBS-T at 8 ˚C overnight
6. Membrane washed three times (15 min) in TBS-T
7. Incubation with secondary antibody diluted (1:10 000) in 1 % BSA in TBS-T for 2 hours at room temperature
8. Scanning of membranes and results analysed in Image Studio software

Table 2-4: Constituents of separating- and stacking gel. Recipe generates 2 x gels

	Separating gel	**Stacking gel**
dH_2O	7.9 ml	6.1 ml
30% acrylamide	6.7 ml	1.3 ml
1.5 Tris (pH 8.8)	5 ml	
0.5 Tris (pH 6.8)		2.5 ml
SDS 10%	200 µl	100 µl
APS 10%	200 µl	50 µl
TEMED	8 µl	10 µl

2.2.7 Cell cycle analysis

Depending on the cells progression in the cell cycle, the DNA content in the cells will vary. The active phases of the eukaryotic cell cycle is; gap 1 (G_{1}), synthesis (S), gap 2 (G_{2}) and mitosis (M). During the gap-phases, several control mechanisms called checkpoints, ensures that everything is ready to duplicate DNA (S-phase) without errors and to start cell division (M-phase). Since the DNA content duplicates during the S-phase of the cell cycle, we can determine the phase in the cell cycle by measuring the amount of DNA present in each cell, as the fluorescence of cells in the G_2/M phase will be twice as high as that of cells in the G_1 phase.

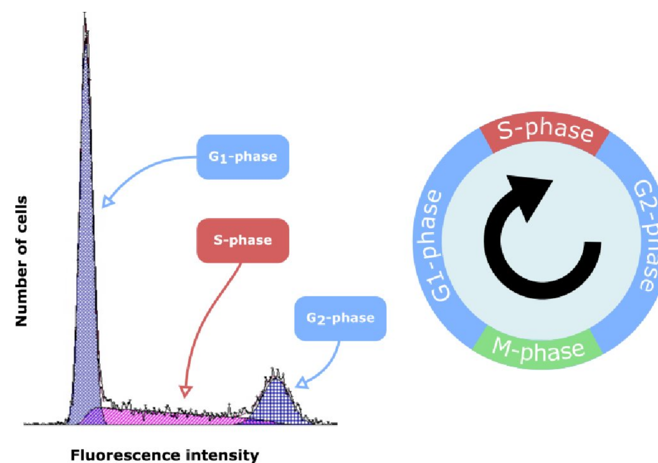


Figure 2-5 A DNA-histogram shows the number of cells which are located in the different cell cycle stages based on measurement of DNA-content in each cell by a flow cytometer

Fluorescence can be measured in a flow cytometer using 4',6-diamidino-2-phenylindole-dihyrochloride (NIM-DAPI), which binds to DNA in A-T rich regions. The fluorescence intensity correlates with the amount of DNA in each cell, in that way separating the results into cell cycle phases. The resulting DNA-histogram visualises the change in cell cycle phases (fig. 2-5)

Procedure:

1. Cells are centrifuged (50 g, 4 ˚C, 5 min) and supernatant is discarded
2. 100 µl citrate buffer is mixed with the cell pellet
3. Samples are kept in freezer for at least 24 hours
4. 300 µl staining solution (NIM-DAPI) was added to each tube and incubated in the dark for 15 minutes.

5. Fluorescence was measured using a flow, and based on the DNA content the different cell cycle phases were analysed and identified by Multicycle software

2.3 Statistical analysis

Statistical analysis was performed using the statistical software Graphpad Prism 4 and Microsoft Excel (with the Analysis ToolPak), where P-values were calculated using the unequal variance t-test (Welch's t-test), which does not assume equal variance. When testing whether the means of different groups are equal or not, common tests such as Student's t-test and ANOVA assumes equal variances among the tested groups. It is possible to test the assumption of equal variances with a standard F-test. However, it is not recommended to first test for equal variances (Zimmerman, 2004), as even when the variances are identical, the unequal variance t-test performs just as effectively as the Student's t-test (Ruxton, 2006). It is also believed that the unequal variance t-test performs better than the Student's t-test in terms of control of both Type I and Type II error rates (Ruxton, 2006), and many authors recommend it for almost all situations (Fagerland and Sandvik, 2009).

As a result, the unequal variance t-test was used throughout this thesis. P-values < 0.05 are considered significant.

2.4 Graphs and figures

The graphs representing the results were exported from Graphpad Prism 4. All supplementary figures in this thesis were created using the vector based graphics software Inkscape 0.91, and the flowcharts in yEd Graph Editor 3.14.4.

3 Results

3.1 SDH-activity

Cell viability was measured with the MTT-assay, which measures the SDH-activity in living cells. BisGMA reduced the SDH-activity in a dose-dependent manner (figure 3-1).

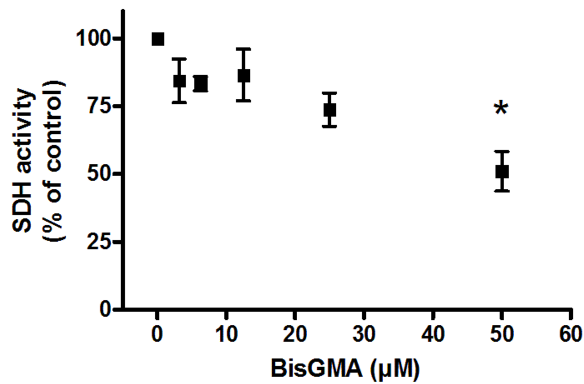


*Figure 3-1. Toxicity in THP-1 cells exposed to various concentrations of BisGMA. Cells were exposed to BisGMA 24 hours prior to analysis. The absorbance in the exposed samples is presented as percent of the controls. The results are shown as mean ± S.D (n ≥ 4). * denotes significant result compared to control.*

3.2 SDH-activity, antioxidants

To investigate if ROS is involved in BisGMA induced cytotoxicity, THP-1 cell culture was exposed to increasing concentrations of BisGMA (0, 25 and 50 μM) and the ROS scavengers vitamin-C (100 μM), Trolox (100 μM) and NAC (1 mM). There was significant change with vitamin-C at 25 μM, and NAC at 25 and 50 μM, when compared with control without antioxidants (fig. 3-2).

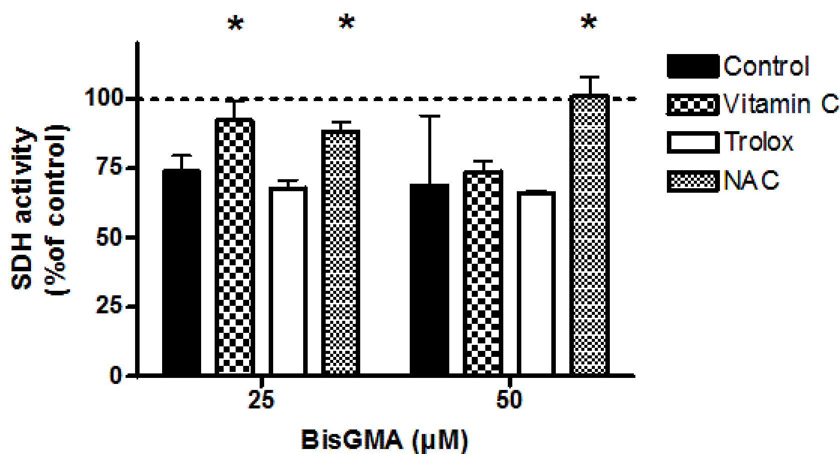


*Figure 3-2 SDH-activity in THP-1 cells exposed to BisGMA + antioxidants. Antioxidants were added to the cell culture 30 minutes prior to exposure to BisGMA. The absorbance in the exposed samples is presented as percent of the controls. Control level is set to 100 % and indicated in the chart and the results are shown as mean ± S.D ($N \geq 4$). There was significant change with vitamin-C at 25 μM, and NAC at 25 and 50 μM (indicated with *), when compared with the control without antioxidants (p >0.05).*

3.3 Cell death

Using fluorescence microscopy, cells stained with Hoechst 33342 and propidium iodide (PI) were catalogued according to colour and morphology (fig. 3-3) to evaluate the proportion of viable, apoptotic and necrotic cells. The number of apoptotic and necrotic cells increased considerably especially after concentrations of 25 µM BisGMA (fig. 3-4).

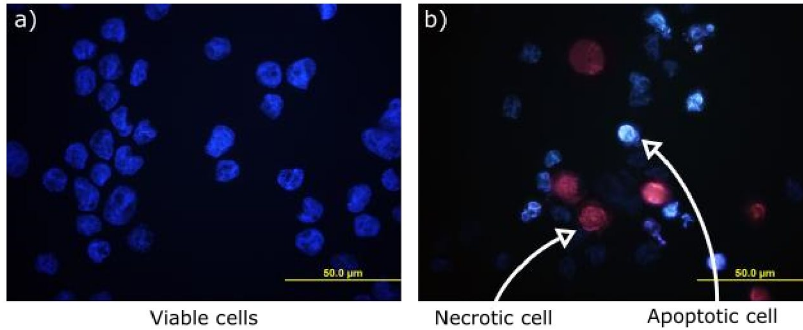


Figure 3-3 Representative microscopy images from exposed THP-1 cell culture: the control (a) shows blue stained viable cells, with no apoptotic and necrotic cells visible. The 50 µM BisGMA sample (b) shows a clearly larger percentage of apoptotic and necrotic cells.

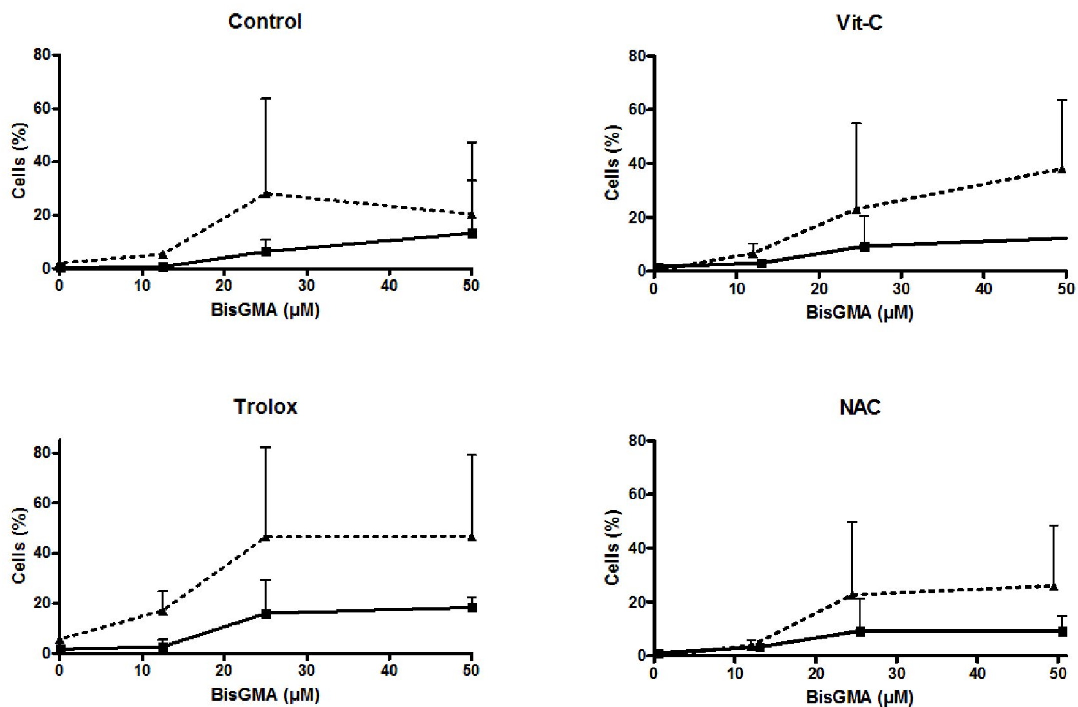


Figure 3-4 Cell death measurements by fluorescence microscopy: The cell culture was treated with the antioxidants vitamin-C, Trolox and N-acetyl cysteine (NAC) and incubated for 30 minutes. Percentage of apoptotic- and necrotic cells after 300 cells were counted in each sample. Dotted lines represent necrotic cells. The results are shown as mean ± S.D ($n \geq 3$).

3.4 GSH measurements

The GSH level in exposed THP-1 cell culture (fig. 3-5) was examined using a flow cytometer, by analysing monobromobimane treated cells. Exposure to BisGMA gave no significant reduction in GSH-levels. The positive control (5 mM HEMA) showed reduced GSH-level, as expected.

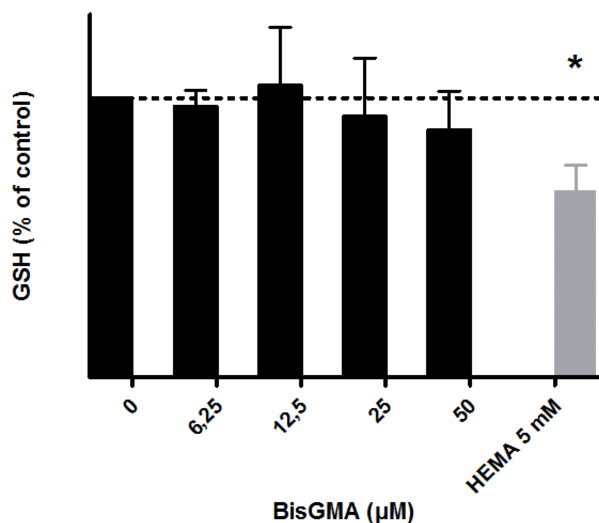


*Figure 3-5 GSH levels in THP-1 cells after 24 hour exposure to BisGMA. The results are shown as mean ± S.D (n ≥ 3). Control level is set to 100 % and indicated in the chart, HEMA (5 mM) was added as a positive control. *denotes significant change compared to the control.*

3.5 ROS measurements

Flow cytometric analysis of BisGMA exposed THP-1 cell culture did not produce significant results (fig. 3-6). There seems to be an indication of increase at 25 µM, it was however not significant. HEMA was employed as positive control and produced significant change in ROS levels.

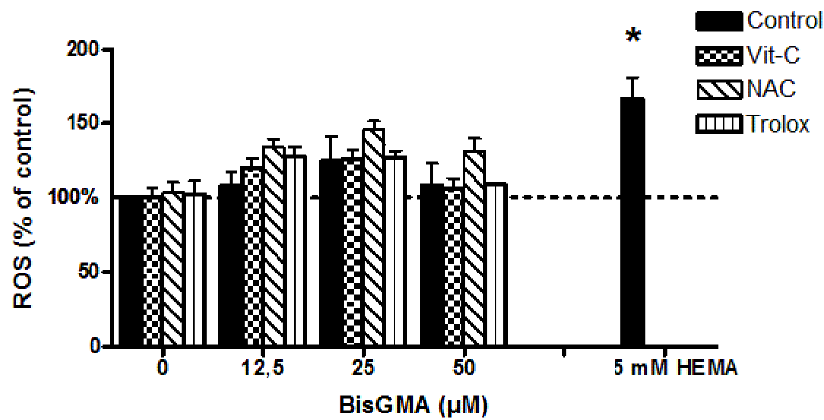


Figure 3-6 There was no significant changes in ROS generation with the antioxidants compared to the control. The results are shown as mean ± S.D (n ≥ 3). Control level is set to 100 % and indicated in the chart. HEMA (5 mM) was added as a positive control, and showed significant change (*) compared to the control.

3.6 Cell cycle distribution

The DNA-content of BisGMA exposed cell culture was analysed with flow cytometer to determine cell cycle progression. The cell cycle distribution after BisGMA exposure did not display significant change in the respective cell phases (fig. 3-7 and fig.3-8), in accordance with the results of previous studies.

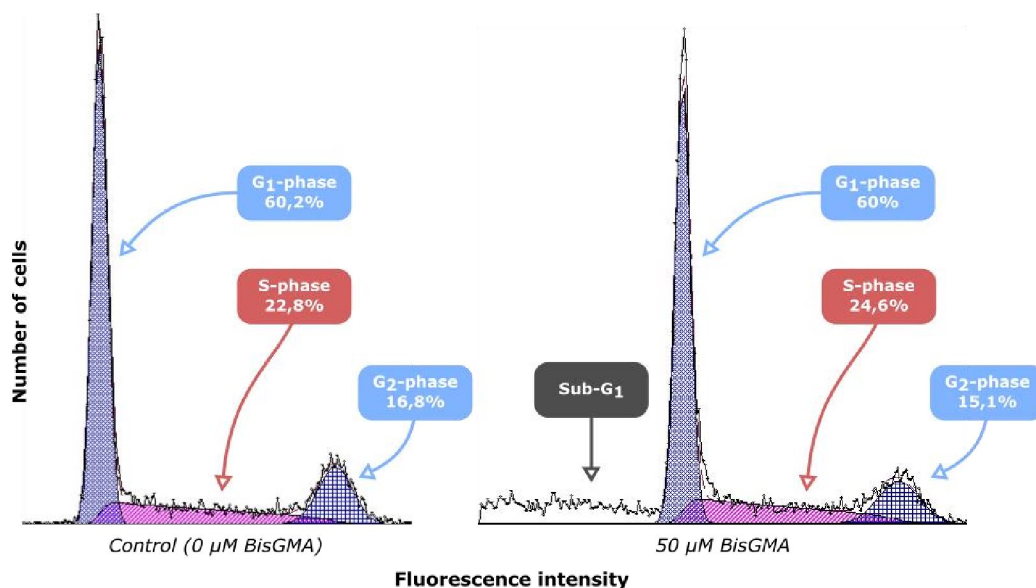


Figure 3-7 DNA-histograms, cell cycle distribution: control sample and 50 µM BisGMA. Sub-G1-segment is specified in the figure.

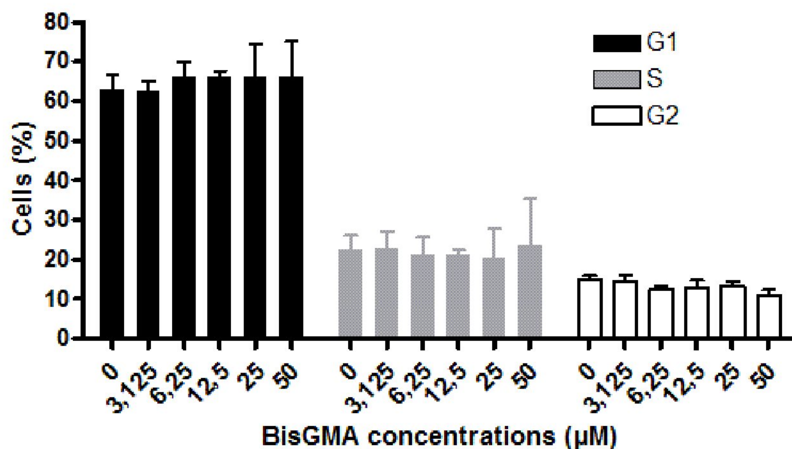


Figure 3-8 Percentage of THP-1 cells in G_1-, S- and G_2-phase after 24 hour exposure to BisGMA. There was no significant change in the cell cycle distribution compared to the control. The results are shown as mean ± S.D (n ≥ 4).

3.6.1 Cell cycle distribution, antioxidants

To investigate if ROS is involved in BisGMA induced cytotoxicity, THP-1 cell culture was exposed to increasing concentrations of BisGMA and the ROS scavengers vitamin-C (100 µM), Trolox (100 µM) and NAC (1 mM). There was no significant change in the cell cycle distribution with the antioxidants compared to the control (fig. 3-9).

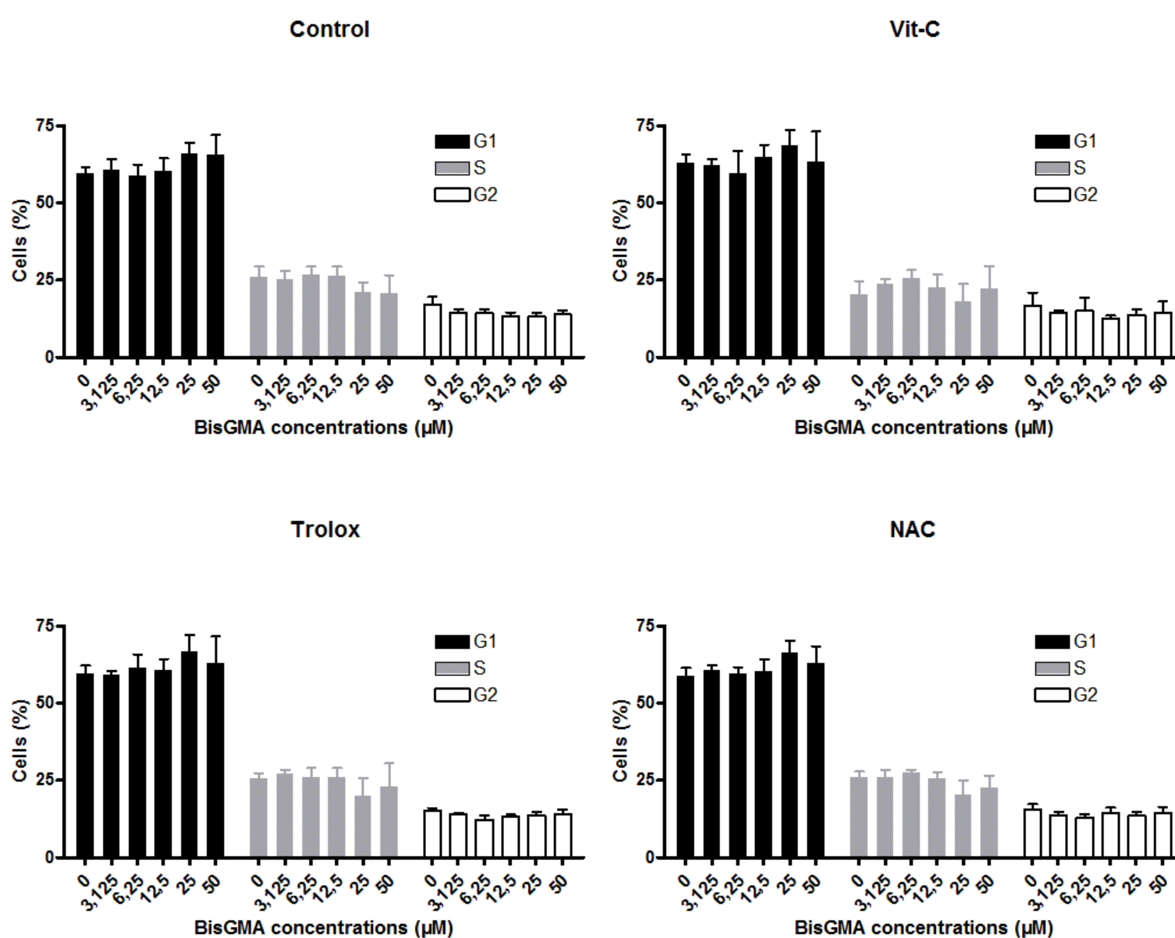


Figure 3-9 Percentage of THP-1 cells in G_1-, S- and G_2-phase after exposure to BisGMA. There was no significant change in the cell cycle distribution with the antioxidants compared to the control. The results are shown as mean ± S.D (n ≥ 4).

3.6.2 Cell cycle distribution, sub-G_1

The sub-G_1-segment appearing in a DNA histogram (illustrated in fig. 3-7) contains cells with a fractional DNA-content, often a characteristic of apoptotic cells (Darzynkiewicz and Zhao 2001), and serves as a crude indicator of BisGMA induced apoptosis. Sub-G_1 cells were analysed from DNA-histograms and the amount of cells as a percentage of total cells were calculated and the results shown presented in fig.3-10. There was no significant change with the antioxidants compared to the control.

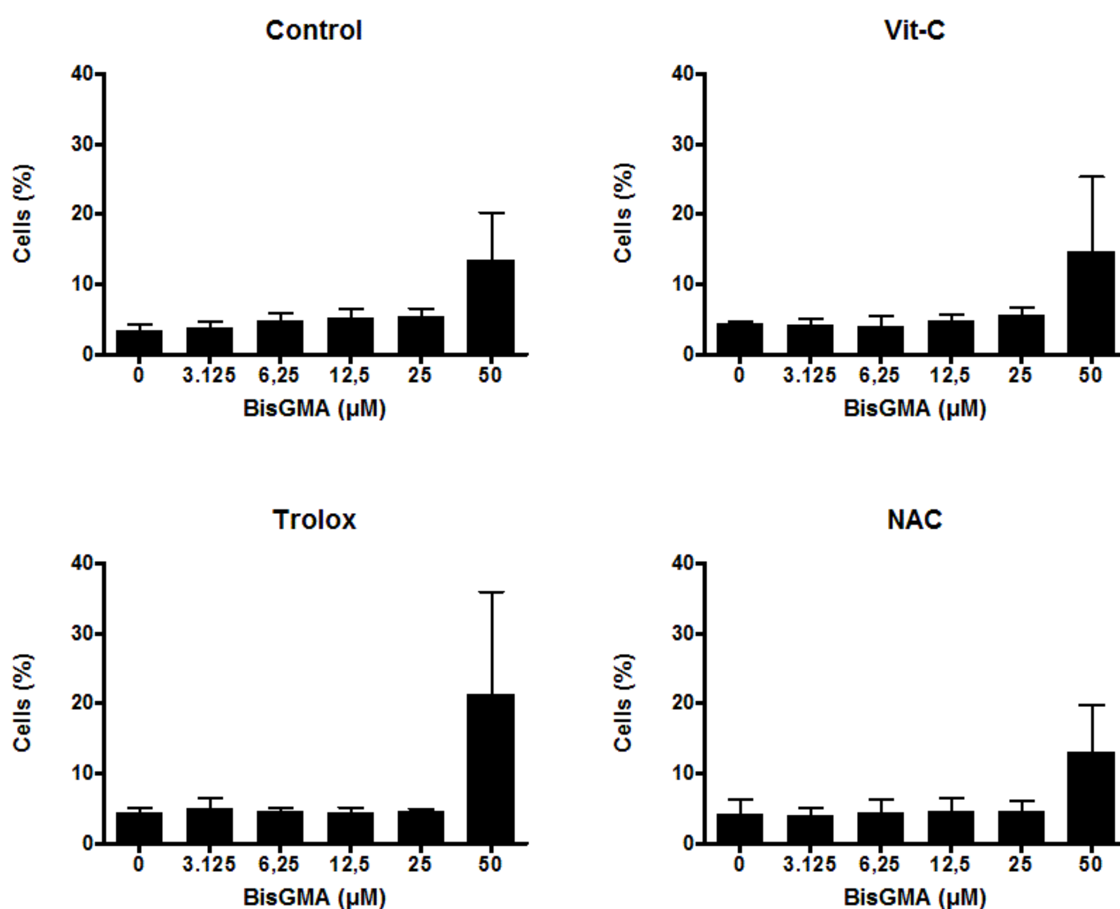
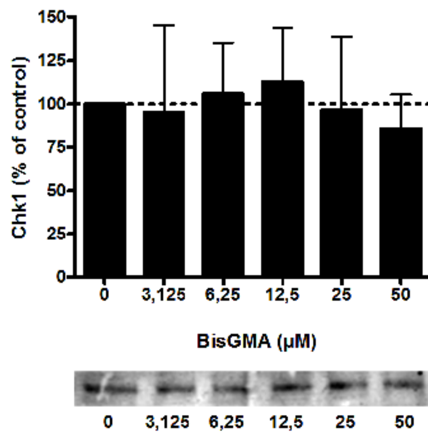


Figure 3-10 Percentage sub-G_1 cells in antioxidant treated cell culture. With concentrations higher than 25μM, the fraction of sub-G_1-cells increases considerably. There was no significant change with the antioxidants compared to the control. The results are shown as mean ± S.D ($n \geq 4$).

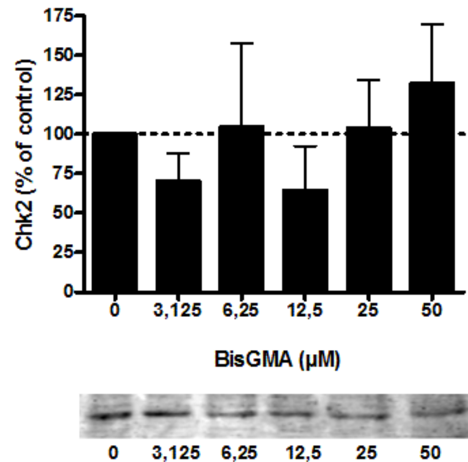
3.7 DNA damage response proteins

By using the western blot, an analytical technique implemented to detect specific proteins, the level of DNA damage response signalling proteins were measured in BisGMA exposed cell culture (fig. 3-11). The antibodies used are listed in table 8-3.

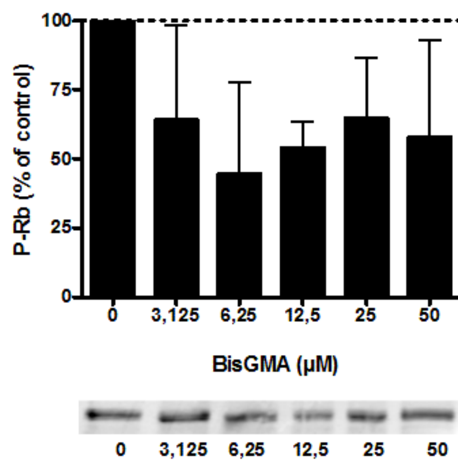
a) P-Chk1



b) P-Chk2



c) P-Rb



d) Rb

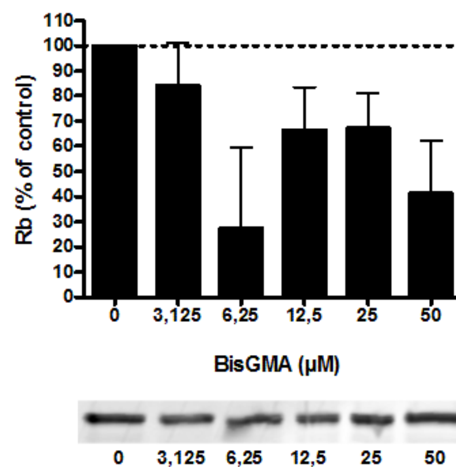


Figure 3-11 a) There was no significant change in the amount of P-Chk1 (a), P-Chk2 (b), P-Rb (c) or Rb (d) detected compared to the control. The results are shown as mean ± S.D ($n \geq 3$).

3.8 Cytokine release

The results for the different cytokines were, in the case of BisGMA exposure, very low compared to the positive control as well as the standard. Nicotine + HEMA was used as a positive control, as a study where human gingival fibroblasts were exposed to nicotine, it was demonstrated that the production of Il-6 and Il-8 were stimulated by nicotine and the cytokine production were upregulated accordingly (Wendell and Stein, 2001). But the results were distorted by the fact that the obtained data was parallel to that of the control, for BisGMA and nicotine + HEMA both. For these reasons, data was not shown.

4 Discussion

In this study, an *in vitro* cell line model was used as a model to examine the cytotoxicity induced by BisGMA, a methacrylate monomer used in a wide range of dental restorative materials. Endpoints like cell viability, GSH- and ROS-levels, release of inflammatory mediators, alterations in cell cycle, and lastly activation of proteins connected to DNA damage and cell cycle control, were measured. These are all parameters reported to be altered by several other methacrylates.

4.1 Methodological considerations

The human monocytic cell line THP-1 was used as a model system, in the current study. Immortalized cell lines, such as THP-1, are often used as model systems (Kaur and Dufour, 2012). They offer many advantages over *in vivo* models and animal studies; low cost, easy maintenance and culturing, plus controllable and easily reproducible culture conditions, the latter thanks to the consistent and pure population of cells. It also offers an infinite supply of material and avoids the ethical concerns associated with the use of animal and human tissue (Kaur and Dufour, 2012), as well as the use of animal studies. On the other hand, the homogeneity of an immortal cell line, owed to its formation during a mutation in single ancestral cell, cannot simulate the complexity and interactions of a biological organism. Additionally, an immortal cell line's theoretically infinite life span will alter its physical characteristics; aggregated mutations and a selection pressure for more proliferative cells further separates it from its *in vivo* counterpart (Masters, 2000). As a result, *in vitro* cell lines do not necessarily represent *in vivo* conditions appropriately, and potential results will only be an indicator of mechanisms of toxicity, which could further warrant studies using primary cells and as a final point animal studies.

BisGMA is often the major component of the resin, and have been shown to leach out from cured dental restorative materials. The BisGMA concentration used in this study are comparable with those measured in the saliva of patients after treatments, where Michelsen et al. (2012) detected a concentration of up to 18.8 μM 10 min after procedure. A concentration range between 0.01-0.5 mM BisGMA seems to be in accordance with what most studies employ and the concentrations suggested in relation to patient exposure (Engelmann et al., 2004, Van Landuyt et al., 2011).

To achieve a complete assessment of cytotoxicity, the MTT assay was first employed to determine general cell viability, where living cells are measured through their mitochondrial/metabolic activity. The assay is good for initial screening, but a potential effect can be explained both due to reduced viability by cell death, but also impaired proliferation and reduced mitochondrial activity. And so, accompanied by the visual observation of apoptotic and necrotic cells by fluorescence microscopy, one can further determine the accuracy of the cell viability assessment. Flow cytometry used to determine cell cycle distribution supplemented the MTT and the microscopy, as these methods will only provide information on cell viability and cell death, not the alteration of cell growth patterns. Lastly, proteins connected to DNA damage and cell cycle control were investigated with the western blot. This combination provides a thorough assessment of BisGMA induced toxicity, with an emphasis on gene toxicity.

4.2 Discussion of results

BisGMA reduced cell viability, as measured by MTT, in a dose-dependent manner, as other methacrylates have been shown to do (Samuelsen et al., 2008, Becher et al., 2006). In line with previous studies using epithelial cell lines (Ansteinsson et al., 2013, Yoshii, 1997), BisGMA induced cell death was prevalent at low concentrations compared to other methacrylates.

The measurement of cell death by fluorescence microscopy revealed increased apoptotic and necrotic cell death at BisGMA concentrations above 25 µM, although not significant. The antioxidants did not show significant change compared to the control, in contrast to the MTT data, where vitamin-C and NAC seemed to have an effect. These results demonstrate the toxic effect of BisGMA, and at a lower concentration compared to other methacrylates. Flow cytometric data regarding the cell cycle distribution did not produce significant changes compared to the control. The absence of obvious cell cycle alterations further indicates that DNA damage is not the mechanism at work. Changes in the patterns of cell division are expected with DNA damage, as the cell cycle is halted to repair damaged DNA. The amount of sub G_1-cells increased clearly around 50 µM BisGMA, a sign of cell death. This agrees with the microscopy data, where increased cell death was observed at similar concentrations, although not significant.

Interactions with DNA have been identified in other *in-vitro* methacrylates exposure studies. The formation of methacrylate-DNA adducts (Fude et al., 1999) via methacrylate epoxide formation (Durner et al., 2011, Seiss et al., 2007) have been a suggested mechanisms of toxicity. Cells normally respond to such changes by activating DNA damage response (DDR) pathways. Interestingly, Ansteinsson et al. (2013) reported increased phosphorylation of Chk2 in bronchial epithelial cells after HEMA exposure. Phosphorylation of Chk2 is normally related to DSBs. Further, in a recent study by Zhu et al (2015), G_1 cell cycle arrest and induction of apoptosis were observed in relation to BisGMA exposure. The authors suggest that the growth changes were a result of oxidative DNA-damage induced by BisGMA. Likewise, BisGMA concentrations of 1 mM have been shown to evoke a delay in S-phase in the cell cycle (Drozdz et al., 2011). Additionally, Blasiak et al. (2012) showed that a mixture of HEMA/BisGMA induced DSBs in HGFs, using the neutral comet assay and the H2AX assay; however this could be due to the DSBs as part of the apoptotic process and not DNA damage induced by BisGMA, as apoptosis seems to be prevalent at the studied concentrations.

We had difficulties dissolving more than 50 µM BisGMA in the cell culture medium. BisGMA is a very high viscous molecule, both due to the presence of strong hydrogen bonding interactions, and the non-covalent interactions given by the BPA-core (Gajewski et al., 2012, Lemon et al., 2007). Together with the hydrophobic character of BisGMA (Lemon et al., 2007), these physical properties will have an effect on an exposed cell culture as opposed to that of a higher viscosity, less hydrophobic molecule, especially in high molar concentrations. Due to a density higher than cell culture media, it is likely that the insoluble BisGMA will fall to the bottom and be in direct contact with the cells, resulting in a much higher local concentration, thus explaining the large variances observed. An additional feature of the BisGMA molecule is its large size, a parameter that affects its ability to penetrate membranes (Durner et al., 2011). The larger the molecule the more time is needed to penetrate the physical barriers like cell membranes (Tehrany and Desobry, 2004). This could possibly explain the lack of DNA damage at lower concentrations, as the BisGMA molecule is unable to enter the cell, or the cell nucleus. Then again, the higher toxicity of BisGMA compared to other methacrylates may be explained by its large liposolubility, making it more efficient to penetrate membranes (Tehrany and Desobry, 2004). This challenges the fact that DNA damage did not seem to be present in the current study, as the BisGMA molecule needs to penetrate membranes to

induce DNA damage. The high liposolubility of BisGMA can also lead to the assumption that lipid membrane damage can be part of the mechanism of toxicity.

The unequal expression of phase I biotransformation enzymes could be another possible explanation for the different observed toxicity. In most cases metabolism by biotransformation enzymes result in lower toxicity and increased removal of the toxicant. However, if not removed by phase II metabolism, the product of a phase I reaction may be more toxic than the initial compound (Casarett and Doull, 2013). Formation of epoxide-containing compounds is one example of phase I metabolism (Samuelsen et al., 2011). Epoxide formation is considered as a very carcinogenic/mutagenic agent that can induce DNA adducts and double-strand breaks (Durner et al., 2011). The expression of phase I metabolic enzymes is known to differ widely in different cells. Hence, a toxic response due to bioactivation would be cell type dependent, as the epoxide formation could explain the difference between the studies.

ROS have been suggested to be a key event in the toxicity in response to dental monomers (Eckhardt et al., 2009). Some studies show increased levels of ROS induced by exposure to BisGMA, where a concentration of 50 µM elevated the intracellular ROS levels (Chang et al., 2009). Protective property of vitamin-C in respect to methacrylate toxicity have been established by Blasiak et al. (2012), where the genotoxic effect of a mixture of HEMA/BisGMA were reduced when exposed to the antioxidant. NAC have as well been shown to decrease BisGMA induced toxicity, with a concentration of 5 mM NAC increasing the amount of viable cells after 24 h exposure (Zhu et al., 2015). However, the study showed no significant changes in cell viability in the concentration range used in the current study, as they only saw change in NAC-related viability in concentrations of 250-500 µM BisGMA. NAC counteracts oxidative stress through direct scavenging of ROS, and by donating cysteine to GSH and thereby acting as a precursor to GSH synthesis. Hence, NAC has the potential to increase phase II metabolic activity in cells. In fact, NAC is employed as an antidote in regards to drugs that will deplete GSH; overdose of the analgesic drug paracetamol can lead to serious liver injury, as one of its metabolites will deplete the liver's GSH, where NAC is given to help the patient restore natural GSH levels (Heard, 2008).

In the current study, the MTT data suggested a weak counteracting effect of some antioxidants of BisGMA induced toxicity. Vitamin-C did have a significant effect at 25 µM BisGMA, and NAC at 25 and 50 µM. The supposed effect of the antioxidants did not show up in the other experiments; no such changes were observed in cell cycle distribution or cell death measured by fluorescence microscopy, where the antioxidant data did not produce significant results. In other methacrylates, it is reported that NAC (a GSH analogue) will bind to the methacrylate group and thereby inactivate it (Spagnuolo et al., 2006), a possible explanation of the effect of NAC observed in this study. However, GSH levels does not seem to decrease, something which is expected when GSH adducts are formed. The contradictory results on antioxidants could be explained by a direct effect on SDH-activity independent of cell death and cell growth.

The difference in molar concentrations used in BisGMA assessment is wide-ranging; with most experiments being in the 100 µM to several mM ranges (Bakopoulou et al., 2009, Drozdz et al., 2011, Zhu et al., 2015). In fact, the experiments which yields the most in terms of positive results, seems to be in concentrations well over those indicated by measurements of actual exposure, both for dental personnel and patients. For instance, a broad spectrum of DNA damage has been indicated, including direct interactions (adducts), strand breaks and double-strand breaks (Drozdz et al., 2011), but only in ranges of 150 µM to 1 mM. At concentrations levels which yields extensive cell death, the DNA degradation will interfere with signals connected to DNA damage and thus skew the results (Redon et al., 2002). Also, the solubility issues concerning BisGMA will possibly interfere with the results at these concentrations. When exposed to primary human pulp cells, BisGMA induced cytotoxicity and marked morphological changes was prevalent at the concentration 75 µM (40% reduced viability), as measured with the MTT assay (Chang et al., 2009). Interestingly, they found that BisGMA concentrations over 250 µM produced almost total death of pulp cells, questioning whether the high concentrations used in some studies are clinically relevant.

The deviations among the results in the experiments have been large. The number of replicated experiments will matter, as the vulnerability to random fluctuations decreases with the number of experiments performed. As such, every experiment will suffer from inconsistent margins of error; when it comes to pipetting, preparation of exposure gradients, seeding of cells (obtained cell density). This may explain the variances observed between the experiments. Extreme observations have a large impact on the unequal

variance t-test (Fagerland and Sandvik, 2009), the statistical analysis used in the current study. This may have skewed some of the results. For instance, in the fluorescence microscopy experiments, some of the series suffered from a larger portion of both apoptotic and necrotic cells, possibly coming from variance in cell density between the experiments. Indeed, the number of experiments should be increased to validate the results. However, it should be emphasized that statistical significance is no concluding confirmation, but rather an indication of an effect, which should be investigated further.

The results from the cytokine release experiments (ELISA) were negative. The absorbance data did not suggest any release, when compared to the standard, for all three cytokines investigated (Il-1β, Il-6 and Il-8). HEMA + nicotine were used as a positive control throughout the experiments, but could not demonstrate conclusive results, as the value range was identical to that of the control for all three cytokines. Previous studies have demonstrated release of Il-6 and Il-8 from nicotine, using human gingival fibroblasts (Wendell and Stein, 2001). This could indicate that HEMA + nicotine are not suitable as positive controls in THP-1 cells. It is possible that no cytokines were released after BisGMA exposure, and that the assumed inflammatory response possibly is facilitated through other cytokines. The experiment itself could be ineffective in detecting the cytokines present, explaining the lack of definite results for all substances investigated. Il-1β has been shown to be induced in THP-1 cells in other studies (Ainscough et al., 2015).

5 Conclusion

BisGMA is the most common monomer used in dental restorative materials (Yoshii, 1997), and a possible adverse toxicity could have an impact on public health, considering the large amount of people with BisGMA based dental biomaterials. In this study, BisGMA was found to induce *in-vitro* cytotoxic responses in the THP-1 cell line, with reduced cell viability at relatively low concentrations (<50 μM), and an increase in cell death (50 μM). At the BisGMA concentrations employed in this thesis, ROS formation and DNA damage could not be verified as contributing factors in the observed cytotoxicity.

6 Future perspectives

Most studies regarding the toxicity of BisGMA have use rather high molar concentrations (>100 μM). There is a lack of studies using clinically relevant concentrations of BisGMA.

The absence of conclusive evidence of DNA damage, and the inability of BisGMA to significantly modulate ROS and GSH response, suggests an alternative to the mechanism of toxicity suggested in other studies. An interaction with the cell membrane could provide an explanation, for instance lipid peroxidation, the oxidative degradation of lipids. As well as the suggested mechanism, the formation of epoxides is another interesting hypothesis that should be investigated.

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8 Appendix

8.1 Reagents and chemicals

Table 8-1 List of chemicals

Chemical compound	Product number	Manufacturer
2-Mercaptoethanol (60-24-2)	M6250	Sigma-Aldrich, St. Louis, USA
30 % Acrylamide/Bis Solution	161-0158	Bio-Rad Laboratories, Inc., CA, USA
Ammonium persulfate (APS) (7727-54-0)	A3678	Sigma-Aldrich, St. Louis, USA
bisophenol A-diglycidylmethacrylate (BisGMA)	1565-94-2	Sigma-Aldrich, St. Louis, USA
Bovine serum albumin (BSA) (9048-46-8)	A7906	Sigma-Aldrich, St. Louis, USA
Buthionine sulfoximine (BSO) (83730-53-4)	B2515	Sigma-Aldrich, St. Louis, USA
Citrate buffer		Pre-made by NIOM
Dimethyl sulphoxide (DMSO) (67-68-5)	116743	Merck KGaA, Darmstadt, Germany
Fetal bovine serum (FBS)	F4135	Sigma-Aldrich, St. Louis, USA
Gentamicin	105030	MP Biomedical LLC, France
Hepes buffer 1M	Be17-737E	Lonza, Verviers, Belgium
Hoechst 33342 (23491-52-3)	B2261	Sigma-Aldrich, St. Louis, USA
L-ascorbic acid	50-81-7	Sigma Aldrich, USA
Methanol (67-56-1)	106007	Merck KGaA, Darmstadt, Germany
MTT solution (Thiazolyl Blue Tetrazolium Bromide) (298-93-1)	M2128	Sigma-Aldrich, St. Louis, USA
N-acetyl cysteine	616-91-1	Sigma-Aldrich, St. Louis, USA
Na-pyruvate	BE13-115E	Lonza, Verviers, Belgium
Nuclear isolations and staining solution (NIM-DAPI)	731085	NPE Systems, Florida, USA
Phosphate buffered saline (PBS)	BE17-516F	Lonza, Verviers, Belgium
Ponceau S (6226-79-5)	P-3504	Sigma-Aldrich, St. Louis, USA
Propidium iodide solution (PI) (25535-16-4)	70335	Sigma-Aldrich, St. Louis, USA
RPMI 1640 medium, 500 ml	BE12-702F	Lonza, Verviers, Belgium
Sodium dodecyl sulfate (SDS) (151-21-3)	L4390	Sigma-Aldrich, St. Louis, USA
Tetramethylethylenediamine (TEMED) (110-18-9)	T9281	Sigma-Aldrich, St. Louis, USA
THP-1 cell-line	88081201	Sigma-Aldrich, St. Louis, USA
Tricine (5704-04-1)	T5816	Sigma-Aldrich, St. Louis, USA
Trolox	53188-07-1	Sigma-Aldrich, St. Louis, USA
Tween 20 (9005-64-5)	822184	Merck KGaA, Darmstadt, Germany

8.2 Equipment and software

Table 8-2 List of equipment and software

Product	Producer
Accuri C6 Flow Cytometer	BD Bioscience, Bedford, MA, USA
Cell culture hood, SCANLAF, Mars safety Class 2	Labogene, Denmark
Centrifuge, Rotina 35R	Hettich, Germany
CO_2 incubator (SANYO MCO-18AIC(UV))	Surplus Solutions, LLC, USA
Electric pipette, Pipetboy	INTEGRA Biosciences AG, Switzerland
Electrophoresis systems and blotting module	TetraCell, Bio-Rad Inc., CA, USA
Flow cytometer, Cell Lab Quanta SC	Beckman Coulter, Florida, USA
GraphPad Prism 4 software	GraphPad Software, CA, USA
Hemocytometer, Bürker	Assistant, Germany
Inkscape 0.91	Free Software Foundation, Inc., Boston MA, USA
Microsoft Excel 2010	Microsoft corporation, USA
Microtiter plates 96 sample well-plates	Sarstedt, Germany
MOXI Z, Mini Automated Cell Counter	ORLFO Technologies, USA
Multicycle	Phoenics Flow Systems, CA, USA
Odyssey CLx Western blot scanner	LiCor Biosciences, Hamburg, Germany
Olympus BX51 fluorescence microscope	Olympus, Germany
Olympus C7070 camera	Olympus, Germany
Olympus CKX41 inverted phase contrast microscope	Olympus, Germany
Pipettes	Thermo SCIENTIFIC, USA
Spectrophotometric microtiter plate reader, Synergy H1	BioTek Instruments Inc., USA
Sterile 6 and 12 sample well-plates	Costar Cornar Incorporated, USA
Sterile cell culture bottles 75 cm^2	Falcon BD Biosciences, USA
Sterile fine pipette tips (5, 20, 200, 1000 µl)	Molecular BioProducts
Sterile pipettes (5, 10 25 ml)	Sarstedt, Germany
Water bath, 37 °C	LAUDA, Germany
yEd Graph Editor 3.14.4	yWorks GmbH, Germany

8.3 Antibodies

Table 8-3 List of antibodies used for western blotting

Antibody	Product number	Manufacturer
Chk1	#2341	Cell Signalling Technologies, USA
Chk2	#2661	Cell Signalling Technologies, USA
P-Rb	#8180	Cell Signalling Technologies, USA
Rb	#9309	Cell Signalling Technologies, USA
β-Actin	#4967	Cell Signalling Technologies, USA

Table 8-4 ELISA reagents and antibodies

	Il-1ß	**Il-6**	**Il-8**
Streptadivin-HRP	Diluted 1:40 in reagent diluent.	Diluted 1:40 in reagent diluent.	Diluted 1:40 in reagent diluent.
Capture Antibody	Reconstituted with 0.5 ml of PBS. Diluted to working concentration of 4.0 µg/ml in PBS.	Reconstituted with 0.5 ml of PBS. Diluted to a working concentration of 2.0 µg/ml in PBS	Reconstituted with 0.5 ml of PBS. Diluted to working concentration of 4.0 µg/ml in PBS.
Detection antibody	Reconstituted with 1.0 ml of reagent diluent. Diluted to working concentration of 200ng/ml in reagent diluent.	Reconstituted with 1.0 ml of reagent diluent. Diluted to a working concentration of 50 ng/mL in reagent diluent.	Reconstituted with 1.0 ml of reagent diluent. Diluted to a working concentration of 20 ng/mL in reagent diluent.
Standard	Reconstituted with 0.5 ml of distilled water. Seven point standard curve using 2-fold serial dilutions in reagent diluent with a high standard of 250 pg/ml is prepared. 1000 µl per assayed plate.	Reconstituted with 0.5 ml of distilled water. Seven point standard curve using 2-fold serial dilutions in reagent diluent with a high standard of 600 pg/ml is prepared. 1000 µl per assayed plate.	Reconstituted with 0.5 ml of distilled water. Seven point standard curve using 2-fold serial dilutions in reagent diluent with a high standard of 2000 pg/ml is prepared. 1000 µl per assayed plate.
Wash buffer	0.05% Tween 20 in PBS	0.05% Tween 20 in PBS	0.05% Tween 20 in PBS
Reagent diluent	1% BSA in PBS	1% BSA in PBS	0.1% BSA, 0.05% Tween® 20 in PBS
Blocking buffer	1% BSA in PBS	1% BSA in PBS	1% BSA in PBS

Other components:

96-well microplate

Plate sealers

PBS

Substrate solution

Stop solution: 2 N H_2SO_4