European Journal of Oral Sciences

Eur J Oral Sci 2016; 124: 511–525
DOI: 10.1111/eos.12309

European Journal of Oral Sciences

Review

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

Bo W. Nilsen[1], Ulf Örtengren[1,2], Jaione Simon-Santamaria[3], Karen K. Sørensen[3], Vibeke B. Michelsen[4]

[1]Department of Clinical Dentistry, UiT - The Arctic University of Norway, Tromsø, Norway; [2]Department of Cariology, Institute of Odontology/Sahlgrenska Academy, Göteborg, Sweden; [3]Department of Medical Biology, UiT - The Arctic University of Norway, Tromsø; [4]Department of Clinical Dentistry, University of Bergen, Bergen, Norway

Nilsen BW, Örtengren U, Simon-Santamaria J, Sørensen KK, Michelsen VB. Methods and terminology used in cell-culture studies of low-dose effects of matrix constituents of polymer resin-based dental materials.
Eur J Oral Sci 2016; 124: 511–525. © 2016 Eur J Oral Sci

General comprehension of terms and confounding factors associated with in vitro experiments can maximize the potential of in vitro testing of substances. In this systematic review, we present an overview of the terms and methods used to determine low-dose effects of matrix constituents in polymer resin-based dental materials in cell-culture studies and discuss the findings in light of how they may influence the comprehension and interpretation of results. Articles published between 1996 and 2015 were identified by searches in the Scopus, Web of Science, MEDLINE, PubMed, and Embase databases using keywords associated with low-dose effects, polymer resin-based materials, in vitro parameters, and dental materials. Twenty-nine articles were included. Subtoxic (n = 11), sublethal (n = 10), and nontoxic (n = 6) were the terms most commonly used to describe the low-dose effects of methacrylates. However, definition of terms varied. Most (82%) studies employed only one method to define the exposure scenario, and no agreement was seen between studies on the use of solvents. Prophylactic use of antibiotics was widespread, and mycoplasma screening was not reported. In conclusion, cell-culture conditions and tests used to define exposure scenarios have changed little in the last decades, despite development in recommendations. Nomenclature alignment is needed for a better understanding of possible biohazards of methacrylates.

Bo W. Nilsen, PO Box 6050 Langnes, No-9037 Tromsø, Norway

E-mail: bo.w.nilsen@uit.no

Key words: dental materials; methacrylates; research design; toxicity tests; triethylene glycol dimethacrylate

Accepted for publication August 2016

The increased use of polymer resin-based dental materials (PRMs) in dentistry has warranted hazard-evaluation of their ingredients. Methacrylic monomers, the main matrix constituents of most PRMs, have in particular been identified as chemicals of interest, as their electrophilic, unsaturated α,β carbonyl structure (1) enables them to react with bionucleophiles such as DNA and proteins (2, 3).

To assess the effects of methacrylic monomers on cells, data on cytotoxicity are necessary (4). However, cytotoxicity assays – the main tools used to define suitable dose and duration time of exposure, hereby referred to as the exposure scenario – harbour many challenges owing to the complex nature of cytotoxicity. Data on cytotoxicity are in general obtained by testing substances for their ability to produce gross cytotoxic events such as cytostasis (inhibition of metabolic activity and cell proliferation), necrosis, and/or apoptosis (5). Assessment of these events includes analysis of cell morphology, cell proliferation, plasma membrane integrity, and cell metabolic activity. Bioassays (i.e. assays that determine a specific biological activity), are readily used owing to their simplicity and ability to yield rapid results (5). Yet, several bioassays, such as the much-used MTT assay – a colorimetric assay for assessing cell metabolic activity – only provide an indication of cytotoxicity, as cells determined to be metabolically inactive are not necessarily apoptotic or necrotic. In addition, the results can vary 100-fold between similar exposure scenarios measured using different metabolic assays (6). Cytotoxicity is therefore a product of the method used to determine it. Consequently, the lack of gross cytotoxic events does not signify that a substance is non-toxic; it only represents a negative measurement. Therefore, care should be taken when interpreting assay results, especially if complementary methods are not used (7).

Cytotoxic data, and thus the exposure scenarios, are further influenced by experimental design. Experimental conditions that previously have been considered irrelevant may cause epiphenomena and artifacts when increasingly sensitive methods are employed to measure

changes in the cell transcriptome, proteome, or metabolome. Examples of experimental design reported to influence cytotoxic results include cell passage number (8, 9), density of parent stock culture of cells and density of cells per well in assay (10), dose metrics (concentration–effect relationships) (11), the use of prophylactic antibiotics in cell-culture medium (12), and solvents (12–14). Undetected mycoplasma infections, and/or other infections in culture may also severely affect the results (15, 16). A transparent and complete presentation of material and methods is therefore vital for the ability of peers to interpret and compare published data. Incomplete description of methods, as well as the use of unstandardized nomenclature, are detrimental for a universal understanding of cellular events (7).

With this background, the aim of this review was two-fold. First, to present an overview of terms and experimental parameters used in the literature during the last two decades (1996–2015) to describe and determine low-dose effects of matrix constituents in polymer resin-based dental materials in cell studies. Second, to discuss how the use of expressions and methods may influence the interpretation of results.

Material and methods

A systematic search of the literature was performed in PubMed [National Center for Biotechnology Information (NCBI), US National Library of Medicine, Bethesda, MD, USA], MEDLINE (US National Library of Medicine), Web of Science (Thomson Reuters, New York, NY, USA), Scopus (Elsevier, Amsterdam, the Netherlands), and Embase (Elsevier) search engines. Inclusion and exclusion criteria are presented in Fig. 1A. The publication date range, 1996–2015, was chosen to follow the development in methods and terminology used to describe and determine low-dose effects of methacrylate monomers. Prestudy searches were conducted to identify an ideal search process. Search terms associated with low-dose effects, PRMs, in vitro cell studies, and dental materials were identified for the Scopus search engine before adapting these terms to the indexing of the MEDLINE, Embase, and PubMed search engines (Table 1).

Articles that passed the title, abstract, and full-text screening were examined for substance tested, method used to determine cytotoxicity, solvent (type and concentration), use of prophylactic antibiotics, mycoplasma testing of cell cultures, cell origin and passage number, dose metrics, and terminology and definition used to describe the effects of the test agent.

Results

Twenty-nine articles were included in the final review. The identification, screening, and selection processes are presented in Fig. 1B. The number of articles found using the different search engines ranged from 65 (Scopus) to 13 (MEDLINE). Among the 187 articles found in the initial identification process, only 40 were duplicates, which supports the use of several search engines.

Table 2 presents a summary of the terminology used in the 29 articles were reviewed. 'Subtoxic' (n = 11), 'sublethal' (n = 10), and 'nontoxic' (n = 6) were the most commonly used expressions to describe low-dose effects of PRMs. These terms were defined in most of the articles; however, the definition varied among the authors. For example, subtoxic could be defined as no effect compared with the control (17, 18) or as the concentration that yielded 50% of the maximal toxic effect

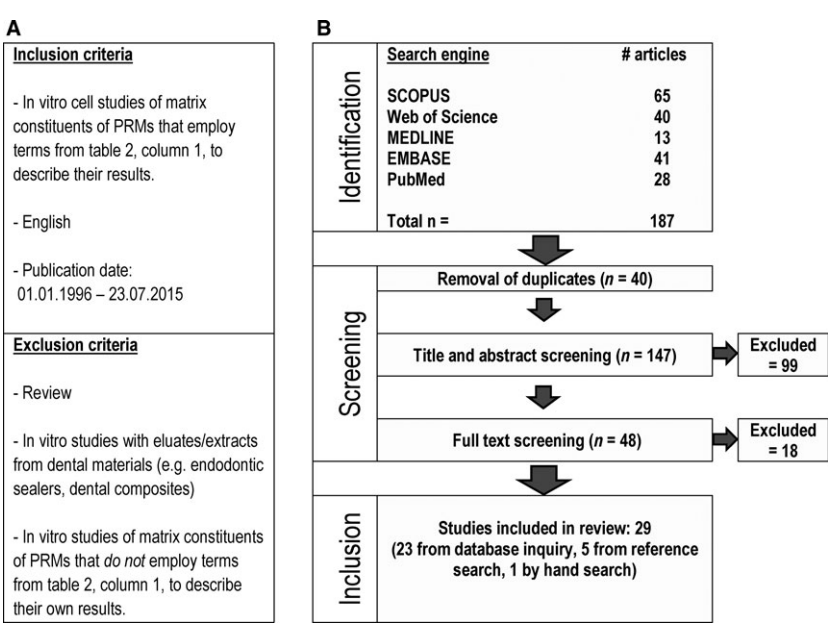

Fig. 1. The identification, screening, and inclusion processes. (A) Overview of the exclusion and inclusion criteria applied to articles to be included in the review. (B) Overview of the number of articles included and excluded during the identification, screening, and inclusion processes. PRMs, polymer resin-based dental materials.

Table 1

Search-terms used in the different search engines

Search engine	Terms associated with low-dose effects		Terms associated with polymer-based materials		Terms associated with in vitro parameters		Terms associated with dental materials
SCOPUS†	Subtoxic OR Nontoxic OR Sublethal OR Subcytotoxic OR (Small adj2 dose) OR (Low adj2 dose) OR Nonlethal	AND	Acrylate* OR monomer OR Methacryl* OR resin* OR Additive OR Silorane	AND	Cell culture OR In Vitro study OR Assay OR cell survival	AND	Dental or dental material or Dentin-Bonding Agents or HEMA or TEGDMA or Bis-GMA or UDMA or CQ or 4-META or MMA or DMAEMA
Web of Science	Subtoxic OR Nontoxic OR Sublethal OR Subcytotoxic OR (Small adj2 dose) OR (Low adj2 dose) OR Nonlethal	AND	Acrylate* OR monomer OR Methacryl* OR resin* OR Additive OR Silorane	AND	Cell culture OR In Vitro study OR Assay OR cell survival	AND	Dental or dental material or Dentin-Bonding Agents or HEMA or TEGDMA or Bis-GMA or UDMA or CQ or 4-META or MMA or DMAEMA
PubMed	Subtoxic OR Nontoxic OR Sublethal OR Subcytotoxic OR (Small-dose OR 'small dose') OR (Low-dose OR 'low dose') OR Nonlethal	AND	'Acrylates'[Mesh] OR 'Resins, Synthetic'[Mesh] OR additives or resinous monomers or monomers	AND	'In Vitro Techniques'[Mesh] OR 'Cell Line'[Mesh] OR 'Cell Survival'[Mesh] OR ('Toxicity Tests'[Mesh] AND 'In vitro')	AND	Dental or dental material or Dentin-Bonding Agents or HEMA or TEGDMA or Bis-GMA or UDMA or CQ or 4-META or MMA or DMAEMA
MEDLINE	Subtoxic OR Nontoxic OR Sublethal OR Subcytotoxic OR (Small adj2 dose) OR (Low adj2 dose) OR Nonlethal	AND	Exp Acrylates/ OR exp Resins, Synthetic/ OR photoinitiator OR exp Silorane Resins/ or additives	AND	In vitro techniques/ or exp cell culture techniques/ OR exp Toxicity Tests/ or exp Cell Survival/	AND	Dental Materials/ or Dental Cements/ or Dental Bonding/ or dental or exp Dentistry/ or Dentin-Bonding Agents or Dentin-Bonding Agents or HEMA or TEGDMA or Bis-GMA or UDMA or CQ or 4-META or MMA or DMAEMA
EMBASE	Subtoxic OR Nontoxic OR Sublethal OR Subcytotoxic OR (Small adj2 dose) OR (Low adj2 dose) OR Nonlethal	AND	Exp resin/ or exp silorane/ OR exp methacrylic acid derivative/ or photoinitiator/ or exp monomer/	AND	In vitro study OR exp cell line/ OR exp cell survival/ OR exp toxicity testing/	AND	Dental or exp 'biomedical and dental materials'/ or exp dental material/ or exp composite material/ or Dentin-Bonding Agents or HEMA or TEGDMA or Bis-GMA or UDMA or CQ or 4-META or MMA or DMAEMA

*Truncation symbol.

†Scopus search-terms were used as a template for the other searches, and were altered on a one-to-one basis to suit the indexing of the other search engines.

4-META, 4-methacryloyloxyethy trimellitate anhydride; adj, number of words that can appear between keywords; Bis-GMA, bisphenyl A glycidyl methacrylate; CQ, camphorquinone; DMAEMA, 2-(Dimethylamino)ethyl methacrylate; exp, explode concept; HEMA, 2-hydroxyethyl methacrylate; MMA, methyl methacrylate; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate.

Table 2

Terminology used to describe low-dose effects of matrix constituents of polymer resin-based dental materials in the articles included in the review

Terms	No. of articles using the term	No. of definitions	Variance in definition (in relation to EC*,†)
Subtoxic	12	4	EC0 to EC50
Sublethal	10	4	EC0 to EC50
Nontoxic	6	2	EC0 to EC15
Non-lethal	1	–	EC0
Non-cytotoxic	1	–	EC0
Subcytotoxic	1	–	EC20

*EC, effective concentration. EC50 represents the concentration of a substance that yields a 50% effect in comparison with the maximal effect in a given assay – usually a negative control. EC0 is the no-effect concentration.
†The EC values represent our interpretation of the information/data presented in the articles reviewed.

(19, 20). All articles used nominal dose metric (added dose) to describe the concentration of the matrix constituents.

Table 3 presents a summary of experimental parameters. Triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) were the monomeric methacrylates most commonly studied and the effect of these two substances was tested in 59% and 45% of the studies, respectively. Seventy nine per cent of the articles used only one method to define the low-dose exposure scenario. The MTT assay, which measures the ability of cells to reduce the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble formazan, was the most commonly used bioassay, and was employed in 69% of the studies. Dimethylsulphoxide (DMSO), ethanol, acetone, and tetrahydrofuran were used as solvents. However, the concentration of solvent used for similar methacrylic monomers varied between studies (not shown).

A summary of cell types and cell-culture conditions is given in Table 4. Almost 80% of the cell lines used for cytotoxicity testing were of human origin. The immortalized human monocyte cell line, THP-1, was used in 34% of the studies. The majority (60%) of the studies were performed with immortalized cells. Passage number was reported for 93% of the non-immortalized cells, whereas only one study reported passage number for immortalized cells (21). Most cells (i.e. 71% of the immortalized cells, and all non-immortalized cells) were cultured in medium with antibiotics.

A comprehensive summary of methods and terminology is presented in Table 5.

Table 3

Experimental parameters used in the 29 articles included in the review

Variable	Value*
Matrix constituents tested†	
TEGDMA	59% (17)
HEMA	45% (13)
UDMA	14% (4)
Bis-GMA	21% (6)
Exposure time used	
<12 h	24% (7)
12 to <24 h	7% (2)
24 h	66% (19)
48 h	10% (3)
72 h	10% (3)
>72 h	31% (9)
Number of methods used to determine cytotoxicity	
1	79% (23)
≥2	7% (2)
No information	14% (4)
Bioassay	
MTT	69% (20)
ATP	7% (2)
Other‡	17% (5)
No information	14% (4)

*Percent of the 29 reviewed articles that used the variable (number).
†These were the four most common matrix constituents tested.
‡PI, MTS, XTT, Annexin V-FITC and LDH.
Annexin V-FITC, Annexin V-FITC Apoptosis Detection Kit (several parameters); ATP, adenosine triphosphate based assay (viability assay); Bis-GMA, Bisphenol A and glycidyl methacrylate; HEMA, 2-hydroxyethyl methacrylate; LDH, lactate dehydrogenase (cytolysis assay); MTS, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium- 5-carboxanilide (viability assay); MTT, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (viability assay); PI, propidium iodide (cytolysis assay); TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate; XTT, 2,3-bis- (2-methoxy-4-nitro-5-sulphophenyl)- 2H-tetrazolium-5-carboxanilide (viability assay).

Discussion

The nomenclature used to describe low-grade toxic effects of PRMs varied between the studies reviewed. Moreover, the terms assessed were non-uniformly defined and definitions overlapped between terms. Definitions of the terms used to describe low-grade effects of potentially cytotoxic substances varied also between dictionaries (Table 6), suggesting that this type of nomenclature should be used with caution. Regardless of this, non-standardized expressions are heavily adopted in the general scientific literature, as illustrated by a PubMed search on the use of the terms subtoxic, sublethal, or non-toxic in the period 01.01.1996–31.12.2015, that gave 16,623 hits, with progressively more results towards the present day.

The drawback of using non-standardized nomenclature becomes particularly apparent when terms are not defined, used as synonyms, and/or cited from a previous study. In one of the articles included in the present review, it was stated in the material and methods section that 'The lethal concentration of TEGDMA in THP-1 monocytes was determined to be 1.5 mM for 72 h … Therefore, 1.25 mM, a sublethal concentration and 3 h, a short exposure time, were chosen for hydrolase activity determination.' (22). In this example, the method used to define the lethal or sublethal

Table 4

Cells and culture conditions in the 29 articles included in the review

Variable	Value*
Cell type used	
THP-1	34% (10)
HCP	10% (3)
HGF	21% (6)
DPSC	10% (3)
Other†	45% (13)
Cell origin	
Human	80% (28)
Non-human	20% (7)
Immortalized cells	60% (21)
Non-immortalized cells	40% (14)
Passage number reported	
Immortalized cells	
Yes	4% (1)
No	96% (20)
Non-immortalized cells	
Yes	93% (13)
No	7% (1)
Reported use of antibiotics	
Immortalized cells	
Yes	71% (15)
No	29% (6)
Non-immortalized cells	
Yes	100% (14)
No	0%

*Value differ by variable. Cell type used: Percent of the 29 reviewed articles that used the variable (number of articles). Cell origin: Percent of total number of cell studies. Passage number reported, and Reported use of antibiotics: Percent of total number of immortalized cells or non-immortalized cells.
†RK-13, BHK-21, TR146, EVC304, L929, U937.
BHK-21, Hamster Kidney fibroblast; DPSC, human dental pulp stem cells; EVC304, human umbilical vein endothelial cells; HCP, keratinizing hamster buccal cheek pouch epithelial cells; HGF, human gingival fibroblasts; L929, mouse C3H/An connective tissue; RK-13, rabbit kidney cells; THP-1, human monocytic cell line derived from an acute monocytic leukaemia; TR146, cell line of human buccal epithelial origin; U937, human leukaemic monocyte lymphoma cell line.

concentration was not described, and it was unclear whether the authors had extrapolated cytotoxic data from a study on THP-1 cells to U937 cells. The same group of authors may also use the same term (i.e. sublethal) differently between papers (23–25).

Phrases used to explain the assay results varied. However, while most authors used phrases that are descriptive of the effect measured, some used phrases like 'percent viable cells' (26), 'cell survival' (19), or 'mortality' (27) to describe results, despite the fact that this was not what the assays measured. The Nomenclature Committee on Cell Death (NCCD) has advised that assays should use expressions that clearly indicate what is measured to strengthen a common understanding of terms [e.g. the MTT assay assesses succinate dehydrogenase activity (not viability), a propidium iodide (PI) assay assesses PI-positive cells (not necrosis), and a caspase-3 assay measures caspase-3-positive cells (not apoptosis)] (7, 28). This is in line with abandoning terms such as non-toxic, subtoxic, and sublethal.

The next paragraphs will elaborate on how experimental design may influence the design of the exposure scenario and the results.

Assays

Cytotoxicity is a complex event that is not easily characterized. Cells deemed viable by one method may display characteristics that are not obvious (e.g. early apoptotic cells will still be metabolically active) (29). In addition, similar assays can differ in sensitivity, as well as in results, because of interferences and method weaknesses (6). Interestingly, cell-culture conditions, as well as the methods used to determine cytotoxicity of matrix constituents of PRMs, were relatively similar in articles published between 1999 and 2015, despite many methodological advancements in the same period and the publishing of the outline on Good Cell Culture Practice in 2002 (30).

In the reviewed articles, the MTT assay was the most frequently used bioassay. MTT assays have been frequently used in cell research since 1983 (31), and are cheap in use, but have some disadvantages compared with other assays. In contrast to, for example, ATP assays, MTT has to be incubated with viable cells before reading. During this period, cells are damaged by crystal formation. This, combined with the ability of MTT to react with certain chemicals, can contribute to artifacts when evaluating cytotoxicity (32, 33). While some studies report good correlation between MTT and ATP assays (34, 35), large variations also occur (6). Weyermann *et al*. reported a 100-fold difference between the MTT assay and an ATP bioluminescence assay used to estimate the half-maximal effective concentration of chloroquine (MTT assay, 10 mM; and ATP assay, 0.1 mM) and sodium azide (MTT assay, 300 mM; and ATP assay, 3.7 mM) (6). If only one of these results had been used to determine an appropriative exposure scenario, different states of the cells would have been assessed.

Toxic reactions need to be interpreted in light of an exposure scenario. The reason for this is that metabolites, transcripts, and cell functions will change in a time- and dose-dependent manner following chemical insults (4) (Fig. 2). Depending on the exposure scenario, the response measured may be partly because of non-specific adaptation mechanisms, and thus obscure the detection of relevant events (4). Therefore, NCCD encourages researchers to quantify cell-death events by more than one assay (7). However, the majority of the articles included in this review used only one method to define the exposure scenario. This finding was independent of the publishing date.

Cells and cell-culture conditions

Most studies used cells of human origin, including all articles published after 2007. This is in line with the National Research Council report, 'Toxicity testing in the 21st century: a vision and a strategy (21.tox)', which states that human biology ought to be the basis

Table 5

Overview of test substance, solvent, cell type, bioassays and terminology used in the reviewed articles

Author (ref. no.)	Concentration and substance referred to with low-dose term	Maximum solvent concentration used	Cells†	Method used to define concentration	Terminology used to describe low-dose effect	Criteria for use of dose or term	Other comments
Rakich *et al.* (1999) (17)	1,000 μM HEMA[A] 10 μM Bis-GMA[B] 10 μM UDMA[C] 100 μM 4-META[D]	0.5% ethanol	THP-1[a] (I)	MTT[1]	Subtoxic	'. . .highest concentration that had no effect on mitochondrial activity'	Solvent was not used for HEMA Toxicity data are from Rakich *et al.* 1998 (17). Subtoxic concentrations were not used in this article
Caughman *et al.* (1999) (53)	0.8 mM DMAEMA[E]	0.1% DMSO	HCP[b] (I)	No reference	Sublethal	'This DMAEMA concentration is fourfold less than the minimal growth-inhibitory concentration in this cell-culture model and produces no detectable cell growth or morphologic changes.'	No information regarding antibiotics Cell source was not stated
Lefebvre *et al.* (1999) (54)	25 μM Bis-GMA[B] 400 μM TEGDMA[F]	0.5% ethanol	THP-1[a] (I)	MTT[1]	Sublethal	'. . .the concentrations of TEGDMA and Bis-GMA were selected such that succinic dehydrogenase activity . . . remained above 50% of the controls after 5 wk.'	
Schuster *et al.* (1999) (55)	10 mM (HCP) and 5 mM (RK) HEMA[A]	Not specified	HCP[b](I) RK13[c](I) BHK-21[d] (I)	MTT[1]	Subtoxic and sublethal	'These concentrations of HEMA were the highest doses tested that: (i) did not result in an overtly toxic response; (ii) produced growth responses similar to control cultures using the MTT assay; and (iii) did not appear to cause the test cells to differ morphologically from control cells.'	MTT only performed for HCP and RK cells No information regarding antibiotics Cell source not stated
Schmalz *et al.* (2000) (56)	$NiCl_2$ $CoCl_2$ $PdCl_2$ 100 mM TEGDMA[F]	Not specified	TR146[e] (I)	MTT[1]	Non-toxic	'Cell survival rates that did not differ from controls'	No information regarding antibiotics

Table 5 Continued

Author (ref. no.)	Concentration and substance referred to with low-dose term	Maximum solvent concentration used	Cells[†]	Method used to define concentration	Terminology used to describe low-dose effect	Criteria for use of dose or term	Other comments
Bouillaguet *et al.* (2000) (57)	0.25–1.5 mM HEMA[A]	Not specified	THP-1[a] (I)	MTT[1]	Sublethal	'Final concentrations of HEMA were selected such that succinic dehydrogenase (SDH) activity (see the procedure which follows) remained above 50% of controls after 4 wk.'	
Kostoryz *et al.* (2001) (21)	10 μM Bis-GMA[B] 500 μM Cyracure UVR 6105[G] 5,000 μM MMA[H]	0.1% DMSO	ECV 304[f] (I)	MTT[1]	Non-lethal, non-toxic, subtoxic	'...select doses that cause no apparent alteration in cellular metabolism...' (non-toxic) The articles refers to concentration up to EC50 as subtoxic	No information regarding antibiotics Human umbilical vein endothelial (EVC 304) could be a misidentified human urinary bladder carcinoma T24 cell line (58)
Engelmann *et al.* (2002) (59)	0.5 mM TEGDMA[F]	0.5% DMSO	HGF[g] (NI)	PI[2]	Subtoxic	None	
Noda *et al.* (2002[a]) (23)	0–10 mM HEMA[A] 0–1 mM TEGDMA[F]	Not specified	THP-1[a] (I)	MTT[1]	Sublethal	'at or below toxic levels at 24 h'–'not highly cytotoxic'	
Noda *et al.* (2002[b]) (60)	2 mM HEMA[A] 0.75 mM TEGDMA[F] Hg^{2+}, Ni^{2+}	0.5% ethanol	THP-1[a](I)	MTT[1]	Sublethal	None	Solvent was not used for HEMA
About *et al.* (2002) (61)	1 μM UDMA[C] 10 μM HEMA[A] 10 μM TEGDMA[F] 1 μM BisGMA[B]	Not specified	HPF[h](NI)	No reference	Nontoxic	None	Cell abbreviation not used by author
Noda *et al.* (2003) (43)	1.2 mM HEMA[A] 0.75 mM TEGDMA[F] Hg^{2+}, Ni^{2+}	1:1,000 Ethanol	THP-1[a] (I)	MTT[1]	Sublethal	None	Solvent was not used for HEMA
Kostoryz *et al.* (2004) (19)	100 μM araldite GY 281[I] 20 μM Bis-GMA[B] 500 μM DECHE-TOSU[J] 100 μM Cyracure UVR	0.4% DMSO	L929[i] (I)	MTT[1]	Subtoxic	'However, the doses to produce DNA damage were of a lower magnitude than the doses to produce 50% cell viability, confirming that DNA damage occurred after exposure to subtoxic concentrations in mammalian L929 cells...'	

Table 5 Continued

Author (ref. no.)	Concentration and substance referred to with low-dose term	Maximum solvent concentration used	Cells†	Method used to define concentration	Terminology used to describe low-dose effect	Criteria for use of dose or term	Other comments
Datar *et al.* (2005) (18)	0.5 mM CQ[K]	0.1 mM acetone	THP-1[a] (I) HCP[b](I)	MTS[3]	Subtoxic	'Non-inhibitory concentrations'	Cell source not described for HCPs
Noda *et al.* (2005) (24)	10 mM ≤ HEMA[A] 1 mM ≤ TEGDMA[F] 2 mM ≤ CQ[K] 15 μM ≤ BP[L]	0.5% ethanol	THP-1[a] (I)	MTT[1]	Sublethal	'From these tests, concentrations of the compounds were selected that inhibited SDH activity less than 50% of control cultures. Then these more focused concentra tions were evaluated to assure that they did not change THP-1 cell number by more than 50% over 24 h of exposure.'	Solvent was not used for HEMA
Kostoryz *et al.* (2007) (20)	138 μM OMP-5[M] 1,490 μM Cyracure UVR-6105[G] >200 μM TET-Sil[N] 57 μM PH-Sil[O]	0.4% DMSO 0.4% tetrahydrofuran	L929[I](I)	MTT[1]	Subtoxic	'The concentration that reduced the viability of 50% (TC50) of L929 cells was measured using the MTT assay and guided the selection of subtoxic doses for evaluation of DNA damage'	Tet-Sil was dissolved in tetrahydrofuran
Noda *et al.* (2007) (25)	0.4 mM CQ[J] 1 M DMPT[P]	Not specified	THP-1[a] (I)	MTT[1] CellTiterGlo[6]	Sublethal	'Camphoroquinone and dimethyl-p-toluidine were initially applied to monocytes in a broader range of concentrations (CQ, 0–2 mM; DMPT, 0–20 mM) to identify sublethal concentrations (<10% suppression of SDH activity)'	CellTiterGlo does not measure SDH activity
Reichl *et al.* (2008) (62)	1 mM TEGDMA[F] 0.1 mM UDMA[C] -HEMA[A] -BisGMA[B] 0.1 mM H_2O_2	1% DMSO	HGF-1[g] (I) HPF (NI)	XTT[4]	Non-toxic	EC0	None of the doses used for HEMA was labelled non-toxic Parts of the experiment were performed in co-exposure with H_2O_2
Gregson *et al.* (2008) (22)	1.25 mM TEGDMA[F]	Not specified	U937[j] (I) HGF[g] (NI) DPF[h] (NI)	MTT[1], as determined by Noda *et al.* (43) MTT[1], as determined by Stanislawski *et al.* 2002 (43)*	Sublethal**	None	Exposure under serum-free conditions **Sublethal concentration extrapolated from another cell line *Stanislawski *et al.* (63) did not use the term sublethal

Table 5 Continued

Author (ref. no.)	Concentration and substance referred to with low-dose term	Maximum solvent concentration used	Cells[†]	Method used to define concentration	Terminology used to describe low-dose effect	Criteria for use of dose or term	Other comments
Mavrogonatou *et al.* (2010) (26)	0.5 mM TEGDMA[F]	0.5% (v/v) DMSO	HGF[g] (NI)	MTT[1]	Non-cytotoxic	'Selected the highest concentration of the monomer that, according to the MTT assay, did not influence cell viability'	
Galler *et al.* (2011) (64)	0.3 mM TEGDMA[F]	DMSO	DPSC[k] (NI)	Annexin V-FITC[5], as determined by Spagnuolo *et al.* (65)	Subtoxic	None	Solvent concentration was not specified
Bakopoulou *et al.* (2011) (66)	0.5 mM HEMA[A] 0.25 mM TEGDMA[F]	0.25% (v/v) ethanol	DPSC[k] (NI)	MTT[1]	Non-toxic	'For the odontogenic differentiation experiments DTSCs were exposed to concentrations of HEMA (0.05, 0.1 and 0.5 mM) and TEGDMA (0.05, 0.1 and 0.25 mM), which were found –based on the MTT analysis– to have minimal or no cytotoxicity to the cells (cell viability ≥85% for both monomers after 72-h exposure).'	
Kachi *et al.* (2011) (67)	0.5 mM DMAEM[E] 0.4 mM CQ[J]	Not specified	THP-1[a] (I)	DMAEM: CellTiterGlo (ATP[1]) CQ: MTT[1], as determined by Noda *et al.* 2005 (24)	Subtoxic	'Dose response experiments were performed initially to determine levels of DMAEM and CPtN that did not alter ATP levels in THP-1 by themselves in the unactivated (no blue light) state. Parallel levels of CQ were used based on previously published results using the same experimental conditions'*	*Referring to Noda *et al.* (24), that used the word sublethal to describe this concentration
Cataldi *et al.* (2012) (68)	3 mM HEMA[A]	Not specified	HGF[g] (NI) –	MTT[1] [as determined by Falconi *et al.* (69)]	Subtoxic	'we chose to test 3 mM HEMA according to previous studies in which it was demonstrated that 3 mM HEMA was responsible of a reduction of cell viability lower than 50%'***	***Referring to Falconi *et al.* (69). Falconi *et al.* did not use this term regarding this concentration

Table 5 Continued

Author (ref. no.)	Concentration and substance referred to with low-dose term	Maximum solvent concentration used	Cells†	Method used to define concentration	Terminology used to describe low-dose effect	Criteria for use of dose or term	Other comments
Di Nisio *et al.* (2013) (70)	3 mM HEMA	Not specified	HGF[g] (NI)	Not specified Referring to Cataldi *et al.* (68)	Subtoxic	'As already observed [Cataldi *et al.* (68)], 3 mmol l^{-1} HEMA is a subtoxic concentration, which is responsible for a reduction in cell viability lower than 50%.'	
Paschalidis *et al.* (2014) (71)	0.25–0.5 mM TEGDMA[F]	0.25% (v/v) DMSO	DPSC[k] (NI) –	MTT[1]	Non-toxic 'subtoxic'	'TEGDMA at 0.25 mM and 0.5 mM was not cytotoxic at any time-point (24–72 h).'	
Nisio *et al.* (2014) (72)	1 mM TEGDMA[F]	0.3% DMSO	HGF[g] (NI) –	No reference	Subtoxic	'Preliminary studies demonstrated that 3 mM can be considered TC50 – 48 h. 1 mM was therefore chosen as the concentration.'****	****Methods used in preliminary studies was not specified.
Nocca *et al.* (2014) (27)	4 mM HEMA[A] 0.7 mM TEGDMA[F] 0.2 mM UDMA[C] 0.4 mM BDDMA[Q]	0.1% DMSO	Human pulp cells (NI) –	MTT[1]	Subcytotoxic, sublethal	'values able to induce a mortality not higher than 20% in respect to control...'	Author did not describe the human pulp cells as fibroblasts, but referred to articles concerning HPFs [h]
Batarseh *et al.* (2014) (73)	0.25 mM TEGDMA	Not specified	HPF[h] (NI)	LDH[7]	Nontoxic	None	

*, **, ***, and **** are used to indicate relationship between specific information given in different columns.

†Immortalized (I) and non-immortalized (NI) cells.

[A]HEMA, 2-hydroxyethyl methacrylate; [B]Bis-GMA, bisphenyl A glycidyl methacrylate; [C]UDMA, urethane dimethacrylate; [D]4-META, 4-methacryloyloxyethy trimellitate anhydride; [E]DMAEMA/DMAEM, dimethylaminoethyl methacrylate; [F]TEGDMA, triethylene glycol dimethacrylate; [G]Cyracure UVR 1605, 3,4-epoxycyclohexylmethyl-3,4- epoxycyclohexylcarboxylate; [H]MMA, methyl methacrylate; [I]Araldite; [J]CQ, camphorquinone; [K]DMPT, dimethyl-p-toludine; [L]BP, benzoyl peroxide; [M] OMP-5, 1,3-bis[2-(2-oxiranylmethyl) phenoxy]pentane; [N] TET-Sil, 3,4-epoxyclohexylethyl-cyclopolymethylsiloxane ; [O] PH-Sil, epoxycyclohexylethyl-phenyl-methylsilane.

[a]THP-1, human monocytic cell line derived from a patient with acute monocytic leukaemia; [b]HCP, keratinizing hamster buccal cheek pouch epithelial cells; [c]RK-13, rabbit kidney cells; [d]BHK-21, hamster kidney fibroblast; [e]TR146, cell line of human buccal epithelial origin; [f]EVC304, human umbilical vein endothelial cells (EVC 304), but could be a misidentified human urinary bladder carcinoma T24 cell line (58); [g]HGF, human gingival fibroblasts; [h]HPF/DPF, human pulp fibroblasts; [i]L929, mouse C3H/An connective tissue; [j]U937, human leukaemic monocyte lymphoma cell line (U937); [k]DPSC, human dental pulp stem cells (DPSC).

[1]MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (viability assay); [2]PI, propidium iodide (cytolysis assay); [3]MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfonyl)-2H-tetrazo-lium (viability assay); [4]XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)- 2H-tetrazolium-5-carboxanilide (viability assay); [5]Annexin V-FITC, Annexin V-FITC Apoptosis Detection Kit (several parameters); [6]ATP, adenosine triphosphate based assay (viability assay); [7]LDH, lactate dehydrogenase (cytolysis assay).

Table 6

Dictionary definitions of terms

Term	Definition	Source
Subtoxic	1. 'A dose that causes consistent changes in haematological and biochemical parameters and might thus herald toxicity at the next higher dose level with prolonged duration'	1. Mould F. Richard. Introductory Medical Statistics, 3rd edition, 1998 (74)
	2. 'Less than toxic'	2. Wiktionary (75)
Sublethal	1. 'a dose of a potentially lethal substance that is not enough to cause death'	1. Mosby's Medical Dictionary, 9th edition (76)
	2. 'In medicine = not sufficient to cause death'	2. Stedman's Medical
	3. 'less than but usually only slightly less than lethal (a sublethal dose)'	Dictionary, 2002 (77) 3. Merriam-Webster Dictionary (78)
Non-toxic	1. 'not toxic'	1. Merriam-Webster Dictionary (79)
	2. 'not poisonous or not harmful to your health'	2. Oxford Advanced American Dictionary (80)
Non-lethal	'not causing death'	Oxford dictionaries (81)
Non-cytotoxic	'not toxic to cells'	Merriam-Webster Dictionary (82)
Subcytotoxic	'Of a dose or concentration. less than cytotoxic'	Wiktionary (83)

for in vitro toxicity assessment (36). The human THP-1 cell line and primary human gingival fibroblasts were the most commonly used cells. A trend towards increased use of non-immortalized or primary cell lines in the later years was also observed.

Cell type can greatly influence cytotoxic results. Mouse BALB/c fibroblasts have shown 2.7- to 14-fold higher sensitivity for HEMA, bisphenyl A glycidyl methacrylate (Bis-GMA), and urethane dimethacrylate (UDMA) compared with THP-1 cells (37, 38), whereas THP-1 cells have shown 5- to 10-times higher sensitivity for HEMA, TEGDMA, and Bis-GMA compared with primary peripheral blood monocytes (39). Extrapolation between cell types should therefore be carried out with caution. In the reviewed articles, such extrapolation was in general not evident, and only performed in one study referred to previously in the discussion (22). With regard to exposure scenarios, great variation can be seen when similar substances and cells are tested, even when the term used to describe these exposures suggests likeness – as illustrated in Fig. 3.

The passage number of cells in culture may also influence their response to xenobiotics (8). Passage number was reported for most non-immortalized cells, but in only one study using immortalized cells. However, high passage numbers have been reported to influence cytotoxic results also when using immortalized cell lines (8, 9), reflecting the instability of these cell systems.

All studies in non-immortalized cell lines, and 71% of those in immortalized cell lines, reported to have employed prophylactic antibiotics. In the latter category, all articles from 2002 on reported use of antibiotics, despite the publishing of good cell-culture practice guidelines in 2002 that discourage routine use of antibiotics in cell-culture media (40). Antibiotics have been suggested to interfere with cellular parameters of interest (12, 13, 40), as well as to increase the prevalence of antibiotic-resistant strains, and to suppress infections that would otherwise be detected (13, 40). In addition, antibiotics do not reliably protect against mycoplasma infections in culture, as 90% of mycoplasma strains are resistant to commonly used antibiotics (16, 40, 41). Interestingly, none of the included articles reported to have screened for mycoplasma. Mycoplasma has been, and still is, a widespread problem for the quality of in vitro cell research (15, 16, 41). The advance of guidelines for good cell-culture practice (30, 40), as well as development of more convenient mycoplasma screening methods, may have lessened the prevalence of mycoplasma. However, recent studies suggest that this infection is still prevalent in cell-culture systems and poses a risk to research quality (15).

Solvents

Many matrix constituents of PRMs are insoluble in cell-culture growth media and are therefore diluted in a polar solvent before toxicity testing. This may alter the toxicity of substances, as solvents interfere with cell-membrane permeability and increase the cellular uptake of chemicals (42). The majority of the studies reviewed reported use of ethanol, DMSO, or acetone to dissolve the test substances. However, the concentration of similar solvent varied between studies – also when an author used the same substance in different studies (23, 43). Findings by Nocca *et al.* suggest that solvents can affect the toxicity of methacrylates, as TEGDMA dissolved in DMSO or ethanol yielded significantly lower cell viability (as defined by an MTT assay) compared with TEGDMA added directly to the cell-culture growth medium (14). The same authors also questioned the need for solvents to dissolve TEGDMA in cell-culture medium (14). This question may be relevant for other methacrylate monomers as there seems to be no consensus in terms of the use of solvents for in vitro tests of toxicity regarding these substances.

Dose metric

Concentration–effect relationships are central to understand the potency of chemicals (11, 44). The amount of freely available substance in cell cultures determines the amount of substance that potentially can interact with cells (45). This may vary considerably from the added, nominal, dose, depending on the physicochemical

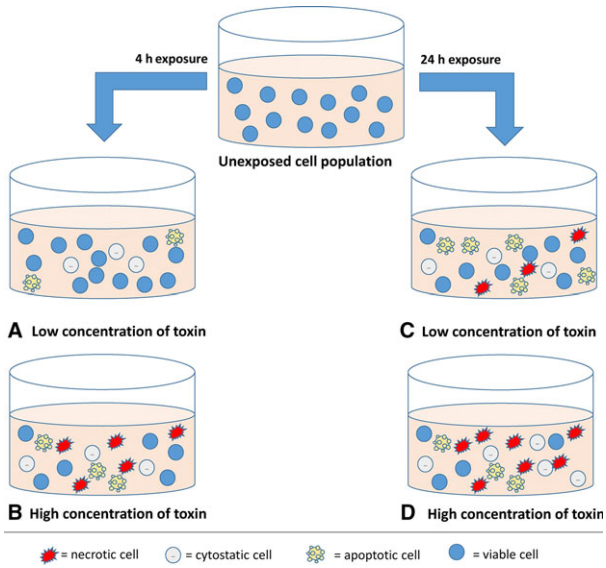

Fig. 2. Illustration of the relationship between measurable effect and exposure scenario. Before exposure, all cells are viable (blue circles). After exposure, different responses are measured depending on the scenario. The ability to detect gross cytotoxic events varies between methods, and one method alone is not sufficient to characterize the extent of cytotoxicity. Beside cytotoxicity, other effects can vary between exposure scenarios. Cells in A and B yield results reflecting changes in pathways that are affected by the initial chemical insult, whereas cells in C and D yield results that are related also to homeostatic/adapting mechanisms.

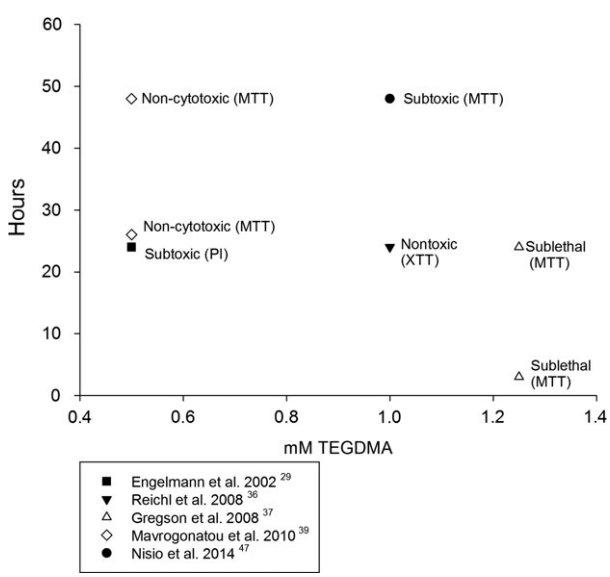

Fig. 3. Terms and methods used by authors to describe the effects of triethylene glycol dimethacrylate (TEGDMA) on human gingival fibroblasts in relation to concentration and exposure duration. Great variation is seen, even though the term used to describe exposures suggests likeness. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (viability assay); PI, propidium iodide (cytolysis or membrane leakage assay); XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)- 2H-tetrazolium-5-carboxanilide (viability assay).

properties of the substance tested, as well as the experimental set-up (e.g. exposure duration and medium composition) (45, 46). Nominal doses are currently the norm in cell research and are used in all the articles reviewed. However, alternative does metrics, such as freely available substance, are deemed an important role for successfully employing in vitro to in vivo extrapolation in the future, as they are not dependent on culture conditions in the way in which nominal doses are (4, 45).

Many cells are dependent on serum supplement in culture. At the same time, serum protein binding has been identified as the main cause of reduced bioavailability of substances in vitro (14, 47). The impact of this phenomenon on EC50 (i.e. the concentration of a substance that yields a 50% effect in comparison with the maximal effect in a given assay) values may vary from none to considerable, depending on the substance as well as on the serum concentration in the cell culture (44). The type of serum might also affect this value: fetal bovine serum has been reported to decrease toxicity less than human serum, probably as a result of different substance-binding properties (44). In the articles reviewed, the serum concentrations varied from 5% to 15%. This difference may have impact on the EC50 values obtained, especially for low-doses of substances ($\leq$600 μM), as albumin-binding sites are saturated at higher serum concentrations (48). Concerning matrix constituents of PRMs, variation in added and detectable concentrations of TEGDMA in cell cultures has been observed (14). Circumstantial evidence supports that protein binding of methacrylates occurs in eluates (47, 49). Studies in mouse reporting the ability of methacrylic compounds to form hapten–protein complexes, also support this notion (50–52). The dose of methacrylate monomers inducing biological effects may therefore be less than the added dose. Dose metrics further complicate the interpretation of in vitro research, and this underlines the importance of reporting results in line with the method used to obtain the results.

In conclusion, non-standardized nomenclature is still commonly used to describe the in vitro results of studies on the biological effects of PRMs. This may impair universal understanding of cytotoxic events. Cell-culture conditions and methods used to characterize exposure scenarios have remained largely unaltered during the last two decades, despite development of recommendation and guidelines. By adapting standards and guidelines when new research projects are initiated, a common, strong platform for in vitro hazard characterization may be achieved.

Acknowledgement – This work was supported in parts by a grant from the Norwegian Directorate of Health (14/1493).

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