In vitro genotoxicity assessment of MTES, GPTES and TEOS, three precursors intended for use in food contact coatings

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A B S T R A C T

Organoalkoxysilanes are precursors that are used increasingly in the synthesis of food contact coatings. To comply with the EU regulation, their potential toxicity must be assessed, and very little information is known. The genotoxicity of three common precursors was studied, namely, tetraethylorthosilicate (TEOS), methyltriethoxysilane (MTES) and 3-glycidyloxypropyltrimethoxysilane (GPTES). By the Ames test, MTES and TEOS were not mutagenic for bacteria. A significant positive response was observed with GPTES in the TA100 and TA1535 strains. The mutagenic effect was more pronounced in the presence of the exogenous metabolic activation system with an increase of the induction factor (ten-fold higher for the TA1535 strain). In the micronucleus assay performed with a human hepatoma cell line (HepG2 cells), GPTES gave negative results even in the presence of an exogenous activation system. To ascertain the possibility of using this precursor in food contact material, its migration must be monitored according to the coating formulation because migration might result in hazardous human exposure.

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1. Introduction

Various materials (e.g., polymers, metals, and ceramics) are used in food contact (e.g., food contact packaging and cookware articles). These materials have different properties, which can be favorable or detrimental, depending on the final application. For example, polymeric materials are appreciated for their lightness; however, they are generally quite permeable to O2 or water vapor and are thus less suitable for food contact packaging.

A common way to enhance the properties or to overcome the important drawbacks of a material is to deposit a thin functional coating on the substrate. Many papers in the literature report the preparation of such coatings on many different types of substrate (Gallardo et al., 2000; De and Kundu, 2001; Davis et al., 2003; Song et al., 2003). One of the easiest methods of synthesizing a coating is the developing industrial sol–gel route method based on hydrolysis and condensation reactions that transform a liquid solution into a solid material. This process is easy to implement and involves soft chemistry. Regarding coatings, hybrid organic/inorganic silica-based coatings are often good candidates; the inorganic part constitutes the network, displaying elevated hardness and mechanical resistance, whereas the organic part provides flexibility and new functionalities to the network. The initial reagents in sol–gel chemistry are water, solvent, acid, and metallic alkoxides. The organoalkoxysilanes are the easiest organometallic alkoxides to implement. First, they are less reactive than the other metallic alkoxides, enabling their handling under air with no need of for an inert atmosphere. A strong link is present between the organic and inorganic parts, they are quite inexpensive, and a large variety of precursors are commercially available. These precursors allow the preparation of thin, adherent, non-porous and dense coatings, with excellent mechanical properties such as elevated hardness, abrasion and scratch resistance (Soloukhin et al., 2002; Robertson et al., 2003; Chen et al., 2008), which are very interesting characteristics for food contact packaging or culinary articles. On polymeric substrates such as polyamide, polyethylene, polycarbonate, these coatings can be deposited to achieve low gas permeability (Iwashita et al., 1996; Tadanaga et al., 1996; Toselli et al., 2007; Lee et al., 2009), whereas on metallic substrates such as aluminum or steel, which are already quite impermeable, they are often used to enhance their mechanical properties (Berrux and Barcickowski, 2010), to avoid corrosion (Sayilkan et al., 2003; Tan et al., 2005; Tavandashti et al., 2009) and to provide easy-to-clean properties (Wu et al., 2005; Perillon and Dubanchet, 2008). In the preparation of sol–gel coatings, tetraethoxysilicate (TEOS), methyltriethoxysilane
(MTES) and 3-glycidoxypropyltriethoxysilane (GPTES) are commonly used as precursors (Lee and Jo, 2002; Wu et al., 2008). After the drying and curing of a coating, a densified network is obtained and no initial precursor should remain. This possibility, as well as the potential presence of broken bonds formed during annealing, cannot be completely ruled out. Regarding food contact, materials should be in compliance with article 3 of the EU regulation 1935/2004, which specifies that the constituents of materials should not be transferred to food in quantities that could endanger human health. Unlike sol–gel components such as water, solvents or acids, the organoalkoxysilanes are not listed in the “Union list of authorized monomers, other starting substances, macromolecules obtained from microbial fermentation, additives and polymer production aids” (Regulation 10/2011) because their toxicity had never been assessed. The TEOS and MTES precursors are mentioned in patent WO2010/07312 (Berrux et Barcikowski, 2010) on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite cookware comprising a vitreous protective coating and in the council of Europe resolutions on the production of a composite 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number of cells with greater than 2 nuclei \times 3)]/\text{total number of cells scored}. The cytotoxicity was determined using the following formula: cytotoxicity = 100–100 × [C0 (CBPI treated culture) – 1]/[CPI vehicle control culture – 1].

The HepG2 cells were seeded at 5 × 10^5 cells/well in a 6-well microplate and incubated overnight. The cells were treated with GPTES dissolved in DMSO (the final concentration in a culture medium of 0.25% from 0.3125 to 0.5 mg/mL for 4 h with the S9 mix and for 24 h without the S9 mix. Vinblastine (0.625 mg/mL) and cyclophosphamide (10 µg/mL) were used as the positive controls without or with the S9 mix, respectively. The cells at the end of the treatment were washed, and the fresh medium containing cytochalasin (4 µg/mL) was added for 44 h.

All the treatments were duplicated at each concentration. Approximately 1 h prior to harvest, the cultures were rinsed with PBS, refed with the MEM medium, and returned to the incubator for an additional hour. The cells were trypanized, fixed with a methanol:acetic acid solution (3:1 v/v), and spotted on a glass slide and stained with acridine orange (0.1%) diluted in Sorensen Buffer (1/15, v/v) just before the microscopic analysis.

The micronucleus frequencies were analyzed in at least 2000 binucleate cells per concentration (at least 1000 binucleate cells from each culture for the identification of the micronuclei, and the criteria of Kirsch-Volders et al. (2000) were applied as follows: the micronuclei should have a diameter of less than one-third of the main nucleus, should be clearly distinguishable from the main nucleus and should have the identical staining as the main nucleus. The data were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test, and the differences were considered significant for \( p < 0.05 \).

3. Results

3.1. Ames test

The results of the Ames test (with/without exogenous metabolic activation) were validated by the presence of the negative and positive controls included in the historical values of the laboratory. As expected, the respective positive control of each strain produced a great mutagenic response.

The MTES and TEOS precursors were not mutagenic for the bacteria compared to the respective negative controls even at the highest concentration (5000 µg/plate) (data not shown). The GPTES precursor did not show any mutagenic effect in the TA 98 and TA 1537 (±S9) and in the WP2 uvrA pKM101 (−S9) strains (Fig. 1).

In contrast, GPTES was mutagenic with a dose response relationship in TA 100 and TA 1535 (±S9), and it exceeded the critical value of 2, with all quotients ranged below 1.6, in WP2uvrA pKM101 (+S9 mix) at concentrations ≥312.5 µg/plate.

Without the S9 mix, the highest response was observed with TA100, whereas the most important effect was observed with TA 1535 in the presence of the S9 mix. At the highest concentration (5000 µg/plate), the mutant frequency was more than fivefold higher than the response with the positive control, AA at 2.5 µg/plate.

3.2. Micronucleus assay

The number of micronuclei per 1000 binucleated cells (MN/1000 BNC) was assessed as a measure of the chromosomal abnormalities in the micronucleus assay of HepG2 cells exposed to a GPTES concentration range. In the presence of enzymatic activities (the S9 mix), an increase of MN was observed when the cells were exposed to the positive control, cyclophosphamide (10 µg/mL) with 33 MN/1000 BNC compared to the negative control (10 MN/1000 BNC). A slight increase in cytotoxicity was detected at the highest concentration tested (25%); however, it remained under the cytosis threshold of 55% established in the OECD 487 guideline (Fig. 2A). There was no GPTES induction of any micronuclei increase in the HepG2 cells at any concentration (0.03125 up to 0.50 mg/mL).

In the absence of metabolic enzymes (S9 mix), the positive control (vinblastine sulfate, 0.625 ng/mL) induced a clear positive effect with 55 MN/1000 BNC compared to the negative control (12 MN/1000 BNC). No cytotoxic effect was observed, except at the highest concentration tested (0.25 mg/mL), with a percentage of cytosis at 30%. In this new condition, GPTES did not induce any increase of micronuclei in the range of concentrations (0.03125–0.25 mg/mL) (Fig. 2B).
<p>The three precursors studied are common precursors used in sol–gel chemistry. The sol–gel route is based on two reactions. The first reaction is hydrolysis, which starts when the precursors are in contact with water and is followed by condensation. This second reaction can occur between hydrolyzed species, in which water is released or between hydrolyzed and unhydrolyzed species, in which ethanol is released (in the case of ethoxysilanes) (Scheme 1 and 2).

TEOS is a precursor displaying only hydrolyzable organic functions. Pure silica is formed after sol–gel reactions. Conversely, MTES and GPTES display nonhydrolyzable organic functions, and whereas MTES is a network modifier bringing hydrophobicity through the methyl groups (no chemical reaction involved), GPTES forms networks. In this case, the epoxy function could open and react. Different opening mechanisms could take part (Sowontharya et al., 2012), leading to the simultaneous presence of different states of the precursor. All the precursors should be hydrolyzed and condensed after the entire process in the final coating; however, the presence of some residual initial precursors cannot completely be ruled out. According to the annealing conditions, some changes, primarily in the organic part, can occur (degradation). Determining the toxicity of the initial precursors and the final coatings is highly important before any use for food contact. This paper focuses on the genotoxicity of the initial precursors with the Ames and micronucleus assays because they have never been used for food contact plastic materials and are not listed in the EU regulation no. 10/2011/EC.

The TEOS and MTES precursors failed to induce any frameshift mutations detected by TA98 and TA1537 or base substitution mutations detected by TA100, TA1535 and WP2uvrA KpnM101 in the absence or presence of an exogenous metabolic activation system (rat liver S9 mix).

The GPTES precursor induced base substitution mutations, which was shown with Ames test positivity for TA100, and particularly for the TA1535 strain, with or without the S9 mix. These strains contain the identical base pair substitution mutation (hisG46) and are suitable for detecting a limited number of single base pair changes, principally GC-AT at the mutant hisG46 CCC codon (Barnes et al., 1982). The GPTES precursor was positive asayed with the E. Coli WP2uvrA KpnM101 strain in the presence of the S9 mix only. This strain has an ochre mutation (UAA, stop codon) in the trpE gene, which enables detection of the base pair substitution and small deletions (Brusick et al., 1980).

These results are not surprising because the GPTES chemical structure shows a structural alert with an epoxy group which is well-known to be highly reactive with cell DNA leading to mutations (Koskinen and Pinta, 2000). The Ames test is appropriate for detection of this type of compound as Ashby and Tennant (1994) analyzed the correlation between the chemical structure, mutagenicity to Salmonella and carcinogenicity in rats and mice among 301 chemicals. In their study, all the listed chemicals with an epoxy function (such as allyl glycidyl ether, 1,2-epoxybutane, 4-vinyl-1-cyclohexene diepoxide, glycidol, and 1,2-propylene oxide) gave positive results in the Ames test and in carcinogenic studies on rats and/or mice. In the presence of exogenous activation enzymes, the responses in the reverse mutation assay were more pronounced (a 10-fold increase with the TA1535 strain), suggesting that exogenous enzyme activities increased the mutagenic potential of GPTES. These results could be explained by the fact that despite the presence of detoxification enzymes such as epoxide hydrolase (EPHX) in the rat S9 mix, an activation metabolism could occur.

The in vitro micronucleus assay was conducted to study the structural and numerical chromosomal aberrations in a human hepatoma cell line when exposed to GPTES. The HepG2 cells were chosen because these cells represent an interesting tool for genotoxicity screening. The HepG2 cell line was isolated from a primary hepatoblastoma from an 11-year old Argentine boy by Aden et al. (1979). These cells retain the morphological characteristics of liver parenchymal cells and specialized functions normally present in primary hepatocytes in culture, such as secretion of the major plasma proteins (Knowles et al., 1980). They express a variety of liver-specific metabolic enzymes (Westerink and Schoonen, 2007a,b)

![Scheme 1. The hydrolysis reaction of TEOS.](image-url)

Fig. 2. The MN and% of cytotoxicity data when the HepG2 cells were exposed to GPTES (ng/ml) with the S9 mix (3 h) (A) or without the S9 mix (24 h) (B). *P < 0.01, significantly different from the negative control. Positive controls: cyclophosphamide (2A) at 10 μg/ml and vinblastine sulfate (2B) at 0.625 ng/ml.

4. Discussion

The three precursors studied are common precursors used in sol–gel chemistry. The sol–gel route is based on two reactions. The first reaction is hydrolysis, which starts when the precursors are in contact with water and is followed by condensation. This second reaction can occur between hydrolyzed species, in which water is released or between hydrolyzed and unhydrolyzed species, in which ethanol is released (in the case of ethoxysilanes) (Scheme 1 and 2).

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and could be useful to avoid false positive responses in in vitro genotoxicity assays (Kirkland et al., 2008; Knasmüller et al., 1998; Uhli et al., 1999, 2000; Yusuf et al., 2000). The HepG2 cells are p53-competent with a functional, transcribed and expressed p53 gene (Bressac et al., 1990). Cell lines that are not p53-competent or have a low proficiency in repairing DNA are considered to be hypersensitive and do not reflect normal mammalian cell behavior in which DNA damage might be repaired or lead to apoptosis. Human p53-competent cell lines are preferable in mammalian genotoxicity assays (EFSA, 2011; Kirkland et al., 2007).

The GPTES precursor did not induce any micronuclei formation in the HepG2 cells even in the presence of the S9 mix (after 4 h of exposure). First, this observation could be explained by the fact that the micronucleus assay detects different endpoints in terms of genotoxicity compared to the Ames test, suggesting that chromosomes are not affected by GPTES.

Second, these results could be explained by the presence of high levels of phase II enzymes involved in the detoxification pathway during xenobiotic metabolism in the human hepatoma cell line (Westerink and Schoonen, 2007a,b). The EPHX enzyme cuts the epoxy group and converts epoxides to trans-dihydrodiols, which can be further conjugated and excreted (detoxification process) from the body.

To confirm the mutagenicity observed in the Ames test, an in vivo test should be planned (EFSA, 2011). More recently, the use of the comet assay and the in vivo gene mutation assay with transgenic mice has increased, primarily because these tests detect genotoxic damage in (almost) every tissue. Kirkland and Speit (2008) reported that the comet assay and the transgenic mouse assay had a high sensitivity to identify carcinogens acting via the clastogenic (comet assay) and gene mutation (both assays) mechanisms. It would be of primary interest to ascertain the GPTES effect in a comet assay using the liver as the most important organ in term of enzyme metabolic activities.

Regarding the further use of GPTES, the conclusions of the EFSA Working Group on Genotoxicity Testing Strategies (2011) are relevant; the EFSA performed an analysis of the concordance between the in vitro and in vivo responses of chemicals by inspecting the data submitted to the former Scientific Committee on Food (SCF) or to the EFSA for approval of chemically defined food contact materials (FCM). The high incidence of positive in vitro results for FCM might reflect a high proportion of chemically reactive substances in this class of compounds because of their technological function (e.g., reactive monomers). In terms of a technology coating, precursors are intended to be densified and polymerized during the condensation step, and the toxic effect observed in the in vitro assays with the initial substances could disappear when the toxic structural alert is involved in the reactive part of the coating.

Regarding these data, before testing the toxicity of GPTES in vivo and with the objective of reducing the cost and the use of experimental animals, it would be very useful to ascertain the level of the GPTES migration as a residual precursor using analytical methods and in parallel to the genotoxic response. The genotoxicity of the overall migrating substances from a coating formulation containing GPTES could be assessed using the Ames assay.

The data on the coating formulation with the three precursors studied in this paper (MTES, GPTES and TEOS) will be published soon.

5. Conclusion

In this study, we demonstrated for the first time that MTES and TEOS were not mutagenic in the Ames test. In contrast, GPTES induced a positive response in the Ames test, demonstrating its mutagenicity on bacteria, with a more important effect on the presence of exogenous enzyme activities. The precursor GPTES did not induce any chromosomal aberration or number change in the HepG2 cell line when the micronucleus assay was performed. To ascertain the possibility of using this precursor in food contact material, its migration must be monitored according to the coating formulation. Any release from the coating in worst case conditions including temperature and UV conditions must be ascertained with the analytical method.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Resolution ResAP(2004)5 on silicones used for food contact applications.


