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

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ABSTRACT

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-glycidyloxypropyltriethoxysilane (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.

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 O_2 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 Barcikowski, 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, tetraethylorthosilicate (TEOS), methyltriethoxysilane

Abbreviations: BNC, binucleated cell; EPHX, epoxide hydrolase; FBS, fetal bovine serum; FCM, food contact materials; GPTES, 3-glycidyloxypropyltriethoxysilane; IF, induction factor; MEM, minimum essential medium; MN, micronucleus; MTES, triethoxymethylsilane; PBS, phosphate buffered saline; OECD, organization for economic co-operation and development; TEOS, tetraethylorthosilicate.

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(MTES) and 3-glycidyloxypropyltriethoxysilane (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 AP(2004)5 on silicones and AP(2002) on papers and boards.

The objective of this work was to ascertain the toxicity of the initial precursors. In this study, because methanol is known to be toxic, only organoalkoxysilanes with ethoxy endings were tested to avoid methanol release. This paper reports the data obtained from the Ames test performed according to the OECD no. 471 guideline of up to a maximal concentration of 5000 μg/plate with MTES, TEOS and GPTES. This assay, which detects the mutagenic effects on bacteria, is required by EFSA (2008) regardless of the substance migration level. A second genotoxicity test, the micronucleus assay, which is also in the battery of tests required by EFSA, was performed according to the OECD 487 guideline on a human hepatoma cell line (HepG2 cells) to detect abnormalities on the structure or in the number of chromosomes. The interest of this study is to assess the genotoxicity of three precursors used in food contact coatings because these compounds could be present as traces in the final packaging if the polymerization step is incomplete or/and if the precursors are released during their life cycle. Genotoxicity is the prerequisite test required by EFSA regardless of the migration of the substance intended for contact with food (EFSA, 2008).

2. Experimental procedures

2.1. Chemicals and medium

The tetraethylorthosilicate (TEOS, no. CAS: 78-10-4), triethoxymethylsilane (MTES, no. CAS: 2031-67-6), 3-glycidyloxypropyltriethoxysilane (GPTES, no. CAS: 2602-34-8) (see Table 1), dimethylsulfoxide (DMSO), cytochalasin B, all the positive controls (2-nitrofluorene (2-NF), sodium azide (SA), ICR191, 4-nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2-AA), cyclophosphamide (CP), vinblastine sulfate (VBS), Minimum Eagle's medium (MEM) and the 100× non-essential amino acids were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France); the L-glutamine (200 mM), heat-inactivated fetal bovine serum (FBS), phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and trypsin (0.05%)-EDTA (0.02%) solution were obtained from Invitrogen Laboratories (Cergy-Pontoise, France).

2.2. Metabolic activation system

The S9 fraction, prepared from male Sprague–Dawley rats dosed with phenobarbital and 5,6-benzoflavone to stimulate mixed-function oxidases in the liver was purchased from Trinova Biochem (Giessen, Germany).

The S9 mix present in the bacterial mutation assay consisted of 10% (v/v) S9 fraction, 33 mM of potassium chloride (KCl), 8 mM of magnesium chloride ($MgCl_2$), 4 mM of nicotinamide adenine dinucleotide phosphate (NADP) and 5 mM of glucose-6-phosphate (G-6-P) prepared in 100 mM of phosphate buffer (PBS, pH 7.4).

For the *in vitro* mammalian cell micronucleus assay, the S9 mix present in the culture medium (the final concentration during treatment) consisted of 5 mM of G-6-P, 0.3 mM of NADP, 1.5 mM of KCl and 2% (v/v) rat liver S9 fraction (Kirkland et al., 1989).

2.3. Bacterial reverse mutation test

The plate incorporation method with or without metabolic activation was conducted according to Maron and Ames (1983) and the OECD no. 471 guideline for the testing of chemicals OECD 471. The histidine-requiring *Salmonella typhimurium* strains TA98, TA100, TA135 and TA1537 were obtained from Dr. Bruce Ames (Berkeley, USA). The tryptophan-requiring *Escherichia coli* strain WP2*uvrA* (pKM101) was provided from Trinova Biochem (Giessen, Germany). The test strains were cultured in Oxoid nutrient broth no. 2 for 10 h at 37 °C under agitation.

Based on the results of the solubility and cytotoxicity evaluation, a range of GPTES, TEOS and MTES concentrations (dissolved in DMSO) was selected (312.5–5000 μg/plate) for the main study.

In the presence or absence of metabolic activation, each concentration of test substances was conducted in triplicate. The reference mutagens used as the positive controls were as follows: 2-NF (2 μg/plate) for TA98; SA (1 μg/plate) for TA100 and TA1535; ICR191 (1 μg/plate) for TA1537; 4-NQO (0.5 μg/plate) for WP2*uvrA*(pKM101) in the absence of the S9 mix; 2-AA (2.5 μg/plate) for TA98, TA100, TA1535 and TA1537; and 2-AA (25 μg/plate) for WP2*uvrA* (pKM101) in presence of the S9 mix. The mutagenic activities were expressed as induction factors, i.e., as multiples of the background levels.

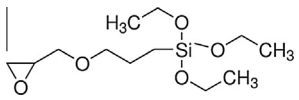
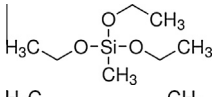
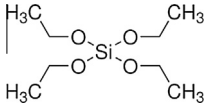
The test substance is considered positive in a bacterial reverse mutation assay when there is (a) an increase ($\geqslant$ twofold for TA98, TA100 and WP2*uvrA*(pKM101) or $\geqslant$ threefold for TA1535 and TA1537) of the spontaneous revertants compared with those of the negative control, or (b) a dose-dependent increase of the revertant colonies in at least one of the tester strains without cytotoxicity.

2.4. In vitro micronucleus test in HepG2 cells

The micronucleus test was conducted in accordance with the OECD no. 487 guideline for the testing of chemicals OECD 487. The HepG2 cell line was obtained from the ECACC (European Collection of Cell Culture, UK). Routine monitoring has shown the HepG2 cells to be mycoplasma free (the Mycoalert kit, Cambrex, Verviers, France). The cells were grown in monolayer culture in MEM supplemented with 2 mM of L-glutamine, 1% non-essential amino acids and 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. Continuous cultures were maintained by subculturing the flasks every 7 days at 2.2×10^6 cells/75 cm^2 flask by trypsinization during 10 passages.

To determine the highest GPTES concentration for the main study, its cytotoxicity was evaluated by the cytokinesis-block proliferative index (CBPI). CBPI = [(the number of cells with 1 nucleus × 1) + (the number of cells with 2 nuclei × 2) + (the

Table 1
Information on the organoalkoxysilanes studied.

Abbreviation	Full name	Formula	CAS
GPTES	3-Glycidyloxypropyltriethoxysilane		2602-34-8
MTES	Methyltriethoxysilane		2031-67-6
TEOS	Tetraethylorthosilicate		78-10-4