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In vitro toxicity assessment of extracts derived from sol–gel coatings on polycarbonate intended to be used in food contact applications

Isabelle Séverin [d, *], Krystelle Lionti [b], Laurence Dahbi [d], Catherine Loriot [c], Bérangère Toury [a], Marie-Christine Chagnon [d]

[a] Laboratoire des Multimatériaux et Interfaces UMR 5615, University of Lyon, Villeurbanne, 69100, France
[b] IBM Almaden Research Center, San Jose, CA 95120, USA
[c] Laboratoire National de Metrologie et d'essais, Trappes 78197, France
[d] Derttech « Packtox » LNC UMRU866, Nutox Team, Agrosupdijon, Université Bourgogne-Franche-Comté, Dijon, 21000, France

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ABSTRACT

Polycarbonate is a widely used polymer in food contact applications all around the world. However, due to the potential release of Bisphenol A (BPA) during repeated washing cycles, its use becomes compromised as BPA is known for being an endocrine disruptor for rodents. In order to tackle this issue, sol–gel coatings based on organoalkoxysiloxane were developed on PC, to act as a physical barrier. To this end, two sol–gel systems based on tetraethylorthosilicate (TEOS), methyltriethoxysilane (MTES) and 3-glycidyloxypropyltriethoxysilane (GPTES), three common sol–gel precursors, were prepared. The coatings derived from the latter two systems were then studied with regards to their potential toxicity *in vitro*. Migration tests were performed in food simulants, and the maximal migration was obtained in ethanol 10% (v/v) for one system and in isooctane for the other one. *In vitro* genotoxicity was assessed with the Ames test (OECD 471) and the micronucleus assay (OECD 487), and no genotoxic effect was observed. Moreover, the estrogenic activity of the extracts was studied with a transcriptional activation assay using transient transfection in human cells; none of the extracts was found estrogenic. These negative *in vitro* results are highly promising for the future use of these new barrier coating formulations onto food contact materials.

1. Introduction

Polycarbonate (PC) is a widely used polymer in a variety of applications such as electronics, optics, automobile or even construction, which clearly shows the diversity of the use of PC (over two million tons produced each year). As a food grade material, PC is also very present in the alimentary field (bottles, containers …), the latter benefiting from its transparency, lightness and exceptional impact resistance at a reasonable cost (Wu et al., 2008). Unfortunately, PC displays poor scratch and solvent resistance along with a fairly high sensitivity to hydrolysis, which significantly limit the polymer's lifetime. In addition, the potential release of bisphenol A (BPA), a component monomer of PC, is currently of toxicological concern. It is worth pointing out that while the use of BPA in food contact materials is permitted in the European Union (EU) under Regulation (EU) No 10/2011[2], it became restricted in January 2011 when the European Commission adopted regulation (EU) No 321/2011/EU (Commission implementing regulation (EU), 2011), that prohibits to use PC in infant feeding bottles manufacturing.

To mitigate PC hydrolysis, thus BPA release, one could envision modifying PC's bulk directly, to achieve higher chemical stability. Although no such studies are reported in the literature, it is well-known that loading specific additives during PC formulation (Fabbri et al., 2008), or preparing PC copolymers (Li and Shimizu, 2011; Zhang et al., 2010) can successfully tune PC properties. However, these compositional modifications typically impact other useful properties of the material as well. Another path to achieve the same is surface modification *via* the deposition of protective

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

* Corresponding author.
E-mail address: isabelle.severin@agrosupdijon.fr (I. Séverin).

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films: barrier coatings with improved mechanical properties (as compared to raw PC) could largely increase PC's lifetime and prevent BPA leakage, maintaining PC suitable in food contact applications.

In that regard, dense oxide-like barrier coatings are excellent candidates as high mechanical properties can be obtained using industry-compatible techniques such as Plasma-Enhanced Chemical Vapor Deposition (PECVD) (Bursikova et al., 2007), Diamond-Like Carbon (DLC) deposition (Damasceno et al., 2002), plasma-ion assisted deposition (Schulz et al., 2008), atmospheric plasma (Cui et al., 2014) or the sol–gel route (Lionti et al., 2013a, 2013b; Le Bail et al., 2015a; Le Bail et al., 2015b). Due to the rapid, low-cost and easy to implement nature of the latter technique, we focused our efforts on the preparation of hybrid silica coatings by sol–gel, on PC. ORMOSILs (organically modified silica, i.e. class II hybrid O/I silica precursors), which display a non-hydrolysable Si–C bond ensuring chemical linkage between inorganic and organic networks, were used as starting precursors (Sanchez et al., 2005). ORMOSILs particularly fit our specifications well due to the dual nature of hybrid O/I silica, the latter offering elevated hardness and resistance through the inorganic part, as well as softness/flexibility or even specific characteristics (hydrophobia, anti-microbial resistance …) through the organic one. It is worth noting that, among the commercially available ORMOSILs, only ethoxy-ending precursors (as opposed to methoxy-ending ones) were used in the sol preparation, in order to release ethanol (harmless) as a sol–gel secondary product rather than methanol (highly toxic).

Here, we report on the toxicological testing of coatings derived from two different ORMOSILs based sols, with optimized mechanical properties and adhesion (Lionti et al., 2013a, 2013b). It is noteworthy that chemical and mechanical properties of sol–gel coatings are widely reported in the literature, but toxicology testing, which is of primary importance for food contact applications, is hardly ever carried out and reported. Since many parameters can be adjusted when making sol–gel formulations, potentially leading to coatings with different composition, structure, and properties, the compliance with the European commission regulation of each sol–gel coating derived from a specific set of sol–gel conditions, intended to be used in food contact applications, needs to be evaluated. Our first formulation is based on methyltriethoxysilane (MTES). Although numerous patents already report on the deposition of similar coatings for alimentary applications (Dubanchet et al., 2008; Jeon Bong et al., 2007; Chung Kwon et al., 2008), the nature of the substrate was different (metallic substrate) and no toxicological data were included. The second sol is based on glycidyloxypropyltriethoxysilane (GPTES) and tetraethylorthosilicate (TEOS): the possibility of using GPTES in coatings for food-contact applications was only mentioned twice before, in our previous patent (Toury et al., 2014) where no toxicology study had been intended to be conducted, and in our paper published in 2014 where we only looked at the toxicity of GPTES as a starting precursor (i.e. unreacted) (Lionti et al., 2014).

In this paper, coatings extracts derived from the two above mentioned sol–gel formulations were collected and tested, as they contain all the substances susceptible to migrate from the material to the food simulant, i.e. intentionally added substances (IAS) but also non-intentionally added substances (NIAS). Genotoxicity is the prerequisite steps required by EFSA irrespective of the migration level of the substance intended to be used in contact with food (EFSA, 2008). This paper reports the data obtained from the Ames test performed according to the OECD 471 guideline with both coatings extracts in order to detect genetic mutations in bacteria. A second genotoxicity test, the micronucleus assay, also part of the battery of tests required by EFSA, was performed according to the OECD 487 guideline on a human hepatoma cell line (HepG2 cells) in order to detect abnormalities on structure and/or in the number of chromosomes. In addition, estrogenic activity of the coatings was also tested with an *in vitro* estrogen receptor transcriptional activation (ERTA) assay which identifies chemicals that are able to activate the estrogen receptor (ERα) (i.e., ER agonists) on the human HepG2 cell line.

2. Material and methods

2.1. Chemicals and medium

Tetraethylorthosilicate (TEOS, n°CAS: 78-10-4), methyltriethoxysilane (MTES, n°CAS: 2031-67-6), 3-glycidyloxypropyltriethoxysilane (GPTES, n°CAS: 2602-34-8), Ludox AS-30 (colloidal silica, 30% (w/v) solid content, suspension in H_2O), glacial acetic acid, dimethylsulfoxyde (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 100× non-essential amino acids were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France) and used without any further purification. L-glutamin (200 mM), heat-inactivated fetal bovine serum (FBS), phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and trypsin (0.05% w/v)-EDTA (0.02% w/v) solution were obtained from Invitrogen laboratories (Cergy-Pontoise, France). Levasil 200E (colloidal silica, 20% (w/v) solid content, suspension in H_2O) was generously given by Akzo Nobel. Isopropanol was purchased from VWR. 2 mm-thick Bisphenol A-PC sheets, (molded from Mitsubishi's Lupilon S3000 UR pellets) were degreased with ethanol. A N_2/H_2-plasma treatment (atmospheric pressure, N_2/H_2 flow rate 330 mm/s), was performed on the PC substrate coated with GPTES and TEOS in order to increase the coating to substrate adhesion.

2.2. Coatings synthesis

Two different sol syntheses were done; all the steps were carried out at room temperature. MTES or (GPTES + TEOS) were mixed with acetic acid (referred to as solutions 1 and 1′, respectively). Ludox AS30 or levasil 200E and isopropanol were mixed together under stirring (solutions 2 and 2′ respectively). Solution 1 was then added to solution 2 (1 + 2 being referred to as A2 thereafter), and solution 1′ added to solution 2′ (1′+2′ being referred to as A8 thereafter), under stirring. Finally, the sols (liquid state) were left aside under stirring for 48 h to ensure full hydrolysis, and were then deposited by dip-coating (withdrawal speed of 1 mm/s) on 10 cm × 7.5 cm × 2 mm PC sheets. A2 was deposited on PC substrates degreased with ethanol; A8 was deposited on N_2/H_2 plasma treated PC substrates. Following deposition, the coated specimens were annealed at 135 °C in a ventilated oven to cure them and obtain dense hybrid silica films (solids). A final film thickness of ~4 μm was measured by profilometry for both systems.

2.3. Migration tests

The overall migration tests were carried out according to either the European standard NF EN 1186-3 for the aqueous food simulants or the European standard NF EN 1186-14 for isooctane or ethanol 95% (v/v) substitute simulants. The test conditions were selected in accordance with the European standard NF ISO 1186-1. Coated PC sheets of 0.5 dm^2 were placed in glass Petri dishes and immersed in 100 ml of the substitute food simulants under well-defined time and temperature exposure conditions: (i) 1 h at 100 °C for acetic acid 3% and ethanol 10% (v/v), (ii) 3 h at 60 °C for ethanol 95%, and (iii) 1 h at 60 °C for isooctane. To ensure a good

contact of the two faces of the plate with the simulants, a metallic stand was employed. In each food simulant, three replicates of the coated PC sheets as well as two blanks were tested. At the end of the test period, each PC sheet was removed from the substitute food simulant. Then, the food simulant was evaporated to dryness; the mass of the non-volatile residue was determined gravimetrically and expressed as mg/dm^2 of the surface area of the PC sheet.

The overall migration data is reported as the mean of three determinations on separate test PC sheets. The maximum authorized level is 10 mg/dm^2 with an analytical deviation of 2 mg/dm^2 for the aqueous and substitute simulants.

2.4. Sample preparation for toxicity tests

For each system (A2 and A8), the migration extracts showing the highest level of migration (most stringent experimental conditions) were used to perform the subsequent toxicity tests: for A2, the isooctane simulant in contact with A2 coated PC sheets was evaporated to dryness and the dried migration extract was then dissolved in 1 mL of ethanol 95% (v/v). The extract was concentrated 100 fold as ethanol (the vehicle for bioassays) is used at the final concentration of 1% in the culture medium. At higher concentration, ethanol is cytotoxic for the HepG2 cell line. For A8, the ethanol 10% (v/v) simulant in contact with A8 coated PC sheets was evaporated to dryness and the dried migration extract was then dissolved in 1 mL ethanol 10% (v/v), and concentrated 100 fold as well. Each extract in ethanol was then directly added to the culture medium. The final tested concentrations were 13.5 μg/mL and 41.5 μg/mL for A2 and A8 extracts, respectively. However, bacteria used in the Ames test being more resistant than human cells, the final concentration of ethanol in the agar was 3.8% instead of 1%. Thus, the maximal concentrations tested of A2 and A8 extracts in the Ames test were 51.3 μg/mL and 157.73 μg/mL, respectively.

2.5. Metabolic activation system

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

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

2.6. Bacterial reverse mutation test

The plate incorporation method with or without metabolic activation was conducted according to Maron and Ames (Maron and Ames, 1983) and the OECD guideline for the testing of chemicals n°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 n°2 for 10 h at 37 °C under agitation.

A range of A2 and A8 extracts concentrations was selected corresponding to 3.8%, 1.9% and 0.95% of ethanol extracts for the study. In the presence or absence of metabolic activation, each concentration of test substances was conducted in triplicate. The reference mutagens used as positive controls were as followings: 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 absence of S9 mix and 2-AA (2.5 μg/plate) for TA98, TA100, TA1535 and TA1537, 2-AA (25 μg/plate) for WP2*uvrA* (pKM101) in presence of S9 mix. The mutagenic activities were expressed as induction factors, i.e. as multiples of the background levels of the negative controls.

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

2.7. In vitro micronucleus test in HepG2 cells

The micronucleus test was conducted in accordance to the OECD guideline for the testing of chemicals n°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 (Mycoalert kit from Cambrex, Verviers, France). The cells were grown in monolayer culture in MEM supplemented with 2 mM L-glutamine, 1% non-essential amino acids and 10% FBS in a humidified atmosphere of 5% CO_2 and at 37 °C. Continuous cultures were maintained by sub-culturing flasks every 7 days at 2.2×10^6 cells/75 cm^2 flask by trypsinisation during 10 passages.

The HepG2 cells were seeded at 5×10^5 cells/well in a 6-wells microplate and incubated overnight. The cells were treated with A2 and A8 extracts dissolved respectively in ethanol 95% (v/v) and ethanol 10% (v/v) (final concentration in culture medium 1%) for 4 h with S9 mix and for 24 h without S9 mix. Vinblastine (0.625 ng/mL) and mitomycine C (25 ng/mL) were used as positive controls without S9 mix (24 h treatment) and cyclophosphamide (10 μg/mL) with S9 mix (4 h treatment). At the end of the treatment, the cells were washed and fresh medium containing cytochalasine B (4 μg/mL) was added for 44 h.

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

The cytotoxicity was evaluated by the cytokinesis-block proliferative index (CBPI). CBPI = [(number of cells with 1 nucleus × 1) + (number of cells with 2 nuclei × 2) + (number of cells with greater than 2 nuclei × 3)]/total number of cells scored. The cytotoxicity was determined by using the following formula: Cytotoxicity = 100 − 100 [(CBPI treated culture − 1)/(CBPI vehicle control culture − 1)].

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

Table 1
Results of experimental migration of A2 and A8 formulations, expressed in mg/dm^2.

A2 formulation	Food simulants	Individual values	Mean (mg/dm^2)
1 h, 100 °C	Acetic acid 3%	0.7; 1.0; 0.5	0.7
1 h, 100 °C	10% ethanol	0.9; 0.3; 0.8	0.7
3 h, 60 °C	95% ethanol	0.3; 1.6; 0.8	0.9
1 h, 60 °C	Isooctane	1.1; 2.3; 4.8	2.7
1 h, 100 °C	Olive oil	1.0; 0.2; 0.2	<1
A8 formulation	**Food simulants**	**Individual values**	**Mean (mg/dm^2)**
1 h, 100 °C	Acetic acid 3%	2.6; 6.8; 1.1	3.5
1 h, 100 °C	10% ethanol	6.1; 16.2; 2.7	8.3
3 h, 60 °C	95% ethanol	4.1; 1.1; <0.1	1.8
1 h, 60 °C	Isooctane	0.3; 0.1; <0.1	0.2
1 h, 100 °C	Olive oil	0.4; 0.8; 11.7	4

2.8. Transcriptional activation assay for estrogenic effect

HepG2 cells were transiently transfected, as previously described by Gasnier et al. (Gasnier et al., 2009), using the Exgen 500 procedure (Euromedex, Mundolsheim). Briefly, the wells of 24-well plates were seeded with 0.125 × 10^6 cells/well in MEM supplemented with 2 mM L-glutamine, 1% non-essential amino acid and 10% dextran-coated charcoal-treated FBS. Cells were transfected 24 h after plating, with 500 ng plasmid per well (100 ng pRST7-hERα, 100 ng ERE-TK-Luc, 100 ng pCMVβGal and 200 ng pSG5) first mixed with 2 μL Exgen 500 in 30 μL of 0.15 M NaCl. After 1 h, the transfection medium was removed and replaced with 1 mL of treatment medium, MEM without phenol red, 2 mM glutamine; 1% non-essential amino acid and without fetal calf serum in presence of ethanol extracts (13.5 μg/mL for A2 and 41.5 μg/mL for A8) for 24 h. 17-β-estradiol was used as positive control at 10 nM which is the saturating agonist concentration resulting in the maximal response in the bioassay.

At the end of the treatment, cells were lysed with Reporter Lysis Buffer (Promega) and frozen at −80 °C for at least 30 min. They were then scraped and placed into microtubes before three freezing (liquid nitrogen)/thawing (37 °C waterbath) cycles and centrifuged 5 min at 10,600 × g.

The resulting lysates were used to determine luciferase and β-galactosidase activities, together with protein levels, as described by Gasnier et al. (Gasnier et al., 2009). For luciferase activity measurement, 10 μL of lysate were mixed with 50 μL of luciferase assay reagent (Promega) into a white 96-well plate. The mixtures were immediately analyzed using a luminometer (TopCount NT, Packard). The β -Galactosidase activity was measured using chlorophenol-red-D-galactopyranoside (Roche Diagnostics GmbH, Mannheim, Germany). The chlorophenol-red product was measured with a spectrophotometer at 570 nm (MRX Dynex). Protein concentration determination was performed using 2 μL of the lysate according to Bradford (1976) method on a spectrophotometer at 595 nm. The luciferase activity was normalized against β-galactosidase activity and protein contents. The induction was compared to the negative control.

3. Results

3.1. Migration test

Under the defined analytical conditions and provided that the constituents of the PC sheets are allowed by the food contact European regulation, the overall migration in aqueous and substitute simulants of both A2 and A8 coated PC sheets were below the limits set up by the regulation on plastics materials intended to come into contact with food (10 mg/dm^2). The highest result of migration was obtained either for the A2 coated PC sheet in the isooctane simulant (2.7 mg/dm^2) or for the A8 coated-PC sheet in the 10% ethanol simulant (8.3 mg/dm^2) (See Table 1). Assuming an average film thickness of 4 μm and a density of 1.65 g/cm^3 for both coatings (previously measured by X-ray-reflectivity), these values

Table 2
Results of the Ames test with the strain TA98, TA 100, TA 1535, TA 1537 and *E. Coli* WP2 uvra pKM101 of three concentrations of A2 extract tested with and without exogenous activation system and expressed as revertants/plate and induction factors (i.e. multiple of negative control).

Sample	TA 98		TA 100		TA 1535		TA 1537		*E. Coli* W2, uvra, pKM 101	
	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)
−S9 mix										
Negative control 100 μl 95% ethanol	20.0 ± 2.0	1	97.0 ± 9.5	1	16.3 ± 2.1	1	8.0 ± 2.6	1	152.7 ± 9.3	1
A2 1.3 μg/plate	20.0 ± 7.6	1.0	115.0 ± 14.7	1.2	15.3 ± 1.5	0.9	9.3 ± 3.1	1.2	149.0 ± 9.5	0.9
A2 2.57 μg/plate	29.0 ± 4.6	1.4	110.3 ± 2.9	1.1	17.7 ± 1.2	1.1	9.0 ± 1.7	1.1	147.7 ± 6.1	0.9
A2 5.13 μg/plate	29.7 ± 5.1	1.5	111.3 ± 15.0	1.1	15.0 ± 1.0	0.9	9.3 ± 5.1	1.2	146.3 ± 10.7	0.9
m(2-NF) = 2 μg/plate	671.0 ± 25.0	33.6	–	–	–	–	–	–	–	–
m(NaN3) = 1 μg/plate	–	–	935.0 ± 25.0	9.6	–	–	–	–	–	–
m(NaN3) = 1 μg/plate	–	–	–	–	774.5 ± 16.5	47.5	–	–	–	–
m(ICR191) = 1 μg/plate	–	–	–	–	–	–	618.5 ± 34.5	77.3	–	–
m(4-NQO) = 1 μg/plate	–	–	–	–	–	–	–	–	>2000	>13
+S9 mix										
Negative control 100 μl 95% ethanol	25.7 ± 2.1	1	118.0 ± 1.0	1	11.3 ± 0.6	1	4.0 ± 2.0	1	173.7 ± 14.6	1
A2 1.3 μg/plate	21.0 ± 2.6	0.8	115.0 ± 1.0	0.9	14.3 ± 0.6	1.3	5.7 ± 0.6	1.4	168.7 ± 6.0	0.9
A2 2.57 μg/plate	25.7 ± 3.5	1.0	137.7 ± 3.2	1.2	14.0 ± 1.0	1.2	4.0 ± 1.0	1.0	172.3 ± 8.7	1.0
A2 5.13 μg/plate	22.7 ± 2.1	0.9	116.0 ± 5.3	0.9	11.3 ± 3.2	1.0	3.0 ± 1.0	0.8	179.3 14.8	1.0
M(2AA) = 2.5 μg/plate	1120.5 ± 75.0	43.8	1193.0 ± 57.0	10.1	125.5 ± 5.5	12.1	77.5 ± 2.5	19.4	>1000	>5.7

Table 3
Results of the Ames test with the strain TA98, TA 100, TA 1535, TA 1537 and *E. Coli* WP2 uvra pKM101 of three concentrations of A8 extract tested with and without exogenous activation system and expressed as revertants/plate and induction factors (i.e. multiple of negative control).

Sample	TA 98		TA 100		TA 1535		TA 1537		*E. Coli* W2, uvra, pKM 101	
	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)	Revertants/plate (mean ± SD)	Induction factor (IF)
−S9 mix										
Negative control 100 µl 10% ethanol	27.0 ± 1.7	1	109.3 ± 3.0	1	19.7 ± 5.0	1	5.3 ± 0.6	1	135.7 ± 19.7	1
A8 3.95 µg/plate	30.0 ± 1.7	1.1	106.3 ± 5.1	0.9	16.0 ± 0.0	0.8	6.3 ± 1.5	1.2	149.0 ± 4.6	1.1
A8 7.89 µg/plate	25.0 ± 5.6	0.9	112.7 ± 16.6	1.1	14.7 ± 0.6	0.7	8.3 ± 3.0	1.6	165.3 ± 6.4	1.2
A8 15.77 µg/plate	26.7 ± 4.7	0.9	113.3 ± 4.7	1.0	15.3 ± 1.5	0.8	6.0 ± 1.0	1.1	144.7 ± 20.2	1.1
m(2-NF) = 2 µg/plate	671.0 ± 25.0	33.6	–	–	–	–	–	–	–	–
m(NaN3) = 1 µg/plate	–	–	935.0 ± 25.0	9.6	–	–	–	–	–	–
m(NaN3) = 1 µg/plate	–	–	–	–	774.5 ± 16.5	47.5	–	–	–	–
m(ICR191) = 1 µg/plate	–	–	–	–	–	–	618.5 ± 34.5	77.3	–	–
m(4-NQO) = 0.5 µg/plate	–	–	–	–	–	–	–	–	>2000	>13
+S9 mix										
Negative control 100 µl 10% ethanol	22.3 ± 5.7	1	116.0 ± 16.4	1	10.7 ± 0.6	1	3.7 ± 0.6	1	157.0 ± 15.6	1
A8 3.95 µg/plate	26.0 ± 2.0	1.2	124.7 ± 4.2	1.1	11.3 ± 1.5	1.1	4.7 ± 1.5	1.3	192.7 ± 6.8	1.2
A8 7.89 µg/plate	23.7 ± 1.5	1.1	122.3 ± 4.9	1.1	10.7 ± 0.6	1.0	5.7 ± 1.5	1.5	199.0 ± 14.9	1.3
A8 15.77 µg/plate	25.0 ± 1.0	1.1	125.7 ± 4.5	1.1	10.0 ± 1.7	0.9	6.3 ± 0.6	1.7	180.7 ± 6.0	1.1
M(2AA) = 2.5 µg/plate	1120.5 ± 75.0	43.8	1193.0 ± 57	10.1	125.5 ± 5.5	12.1	77.5 ± 2.5	19.4	>1000	>5.7

correspond to a weight loss of ~4% (w/w) for A2 coating, and ~13% (w/w) for A8 coating.

3.2. Ames test

The results of the Ames test (with/without exogenous metabolic activation) were validated by the presence of negative and positive controls included in the historical values of the laboratory. As expected, the respective positive control of each strain produced a significant mutagenic response (Tables 2 and 3). The number of revertants was more than twofold higher for TA98 (33.6 without S9 mix, 43.8 with S9), TA100 (9.6 without S9 mix, 10.1 with S9 mix) and WP2*uvrA* (pKM101) (>13 without S9 mix, >5.7 with S9 mix) and threefold higher for TA1535 (47.5 without S9 mix, 12.1 with S9 mix) and TA1537 (77.3 without S9 mix, 19.4 with S9 mix).

Neither A2 nor A8 extracts were mutagenic for the bacteria compared to the respective negative controls even at the highest concentration tested (51.3 µg/mL for A2 and 157.73 µg/mL for A8): the induction factor never exceeds 2, irrespective of the conditions tested (Tables 2 and 3).

3.3. Micronucleus assay

The number of micronulei per 1000 binucleated cells (MN/1000 BNC) was assessed as a measure of chromosomal abnormalities in the micronucleus assay when HepG2 cells were exposed to 0.5 or 1% of the ethanol extract. In the presence of enzymatic activities (+S9 mix) and after a 4 h exposure of HepG2 cells, with the

Table 4
Percentage of cytotoxicity and number of micronuclei/1000 binucleated cells in HepG2 cells after a short or a long time of exposure to 1% of ethanol A2 and A8 extracts (13.5 µg/mL and 41.5 µg/mL, respectively).

Sample	Measured parameter	Short treatment with S9 mix	Long treatment without S9 mix
Negative control (culture medium)	% cytotoxicity	0%	0%
	MN/1000 BNC	**17**	**19**
A2 negative control (ethanol 95%)	% cytotoxicity	0%	0%
	MN/1000 BNC	**14**	**19**
A2 extract (41.5 µg/mL)	% cytotoxicity	4.42%	6.89%
	MN/1000 BNC	**20**	**18**
A8 negative control (ethanol 10%)	% cytotoxicity	0%	0%
	MN/1000 BNC	**18**	**17**
A8 extract (41.5 µg/mL)	% cytotoxicity	3.39%	3.33%
	MN/1000 BNC	**19**	**16**
Positive control Cyclophosphamide (10 µg/mL)	% cytotoxicity	15.48%	–
	MN/1000 BNC	**58**	–
Positive control mitomycine C (25 ng/mL)	% cytotoxicity	–	31.21%
	MN/1000 BNC	–	**194**
Positive control vinblastine sulfate (0.625 ng/mL)	% cytotoxicity	–	0.87%
	MN/1000 BNC	–	**63**

clastogenic reference cyclophosphamide (10 μg/ml), an increase of MN was observed with 58 MN/1000 BNC instead of 17 MN/1000 BNC for the negative control. In absence of metabolic enzymes (S9 mix), the positive aneugenic control (vinblastine sulfate, 0.625 ng/mL) induced a clear positive effect with 63 MN/1000 BNC compared to the negative control (19 MN/1000 BNC). The clastogenic control (mitomycine C 25 ng/mL) gave, as expected, an increase of the number of micronuclei (194 MN/1000 BNC). Irrespective of the conditions of treatment (short or long), no cytotoxic effect was observed on any extract. A2 and A8 extracts (13.5 μg/mL for A2 and 41.5 μg/mL for A8) did not induce any chromosomal aberrations in the HepG2 cell line (Table 4).

3.4. Endocrine disruption activity

HepG2 were transiently co-transfected with ERE-TK-LUC and the hERα expression vector. 17β-estradiol (10 nM) was used as a positive reference and resulted in a statistically significant induction of the reporter gene activity. The activity of the extracts (13.5 μg/mL for A2 and 41.5 μg/mL for A8) as well as that of the positive control were expressed relatively to the negative control. Under our experimental conditions, no statistically significant increase in ERα transcriptional activity was observed when HepG2 cells were exposed to A2 or A8 extracts (induction of 2 and 1.4 respectively compared to the negative control), suggesting that neither A2 nor A8 extract presents an agonist ERα estrogenic activity (Fig. 1).

4. Discussion

The combination of negative results for all the toxicology tests performed indicates sufficient passivity to food simulants for both types of coating. Several factors can be invoked to explain these results.

First, migration results strongly depend on both coating to substrate adhesion, and coating network cohesion. Regarding the latter, it should be noted that ORMOSILs are great sol–gel precursors for food-contact applications: unlike class I hybrid materials which show weak interactions between the organic and inorganic phases, ORMOSIL precursors display a strong covalent bond between the two parts which (i) is maintained during the sol–gel reactions (soft chemistry) and (ii) favors high cohesion and network connectivity of the coating, minimizing network depolymerization. With respect to adhesion, it is clear that a bad (i.e. low) coating to substrate adhesion will cause the coating to peel off during the solvent immersion associated with migration tests, leading to extremely high migration levels. In the present case, the adhesion to PC of the coatings (G_c, in J/m^2) was previously measured by the Double Cantilever Beam technique (Lionti et al., 2013a). While A2 derived samples inherently show a relatively high and satisfying adhesion to PC ($G_c = 3.2\ J/m^2$), it is not the case of A8 derived samples ($G_c = 1.5\ J/m^2$). Indeed, values above 3 J/m (Commission regulation (EU), 2011) are indicative of strong covalent bonds at the interface, whereas values below 3 J/m (Commission regulation (EU), 2011) imply that weak interactions are dominant at the film/PC interface, suggesting mechanical adhesion mainly. In order to improve the adhesion of A8 derived coatings to PC, we have previously shown that nitrogen based plasmas are extremely effective: when depositing A8 sol on N_2/H_2 plasma treated PC, we observe a 15 fold increase in adhesion, with G_c values higher than 20 J/m (Commission regulation (EU), 2011). This preliminary plasma treatment step is thus likely to have an important role in the low migration level detected.

Bioassays such as the regulatory Ames test and micronucleus assay or a transcriptional activation assay were performed on the extracts (as mixture of migrating substances) for which the migration is the highest. In these most stringent experimental conditions, all results were found negative, constituting promising initial results to further continue the development of these coatings. In such conditions, the hazard assessment is performed on finished materials as the extracts contain both the intentionally added substances (IAS) used to produce the PC sheet and the coating, but also the non-intentionally added substances (NIAS) formed during the test process. Indeed, all of the substances able to migrate or the mixture of these substances didn't give a mutagenic effect on bacteria, or didn't induce chromosomal damage on a human cell line.

In the case of A2 derived samples, the negative results obtained were anticipated as (i) we previously showed that MTES induces negative responses in the Ames test and micronucleus assay (Lionti et al., 2014) and (ii) all other sol–gel reactants used in A2 synthesis are already EU or FDA approved substances. Regarding A8 derived samples, the negative results were not a foregone conclusion. Indeed, even if TEOS by itself was shown to be non-toxic, unreacted

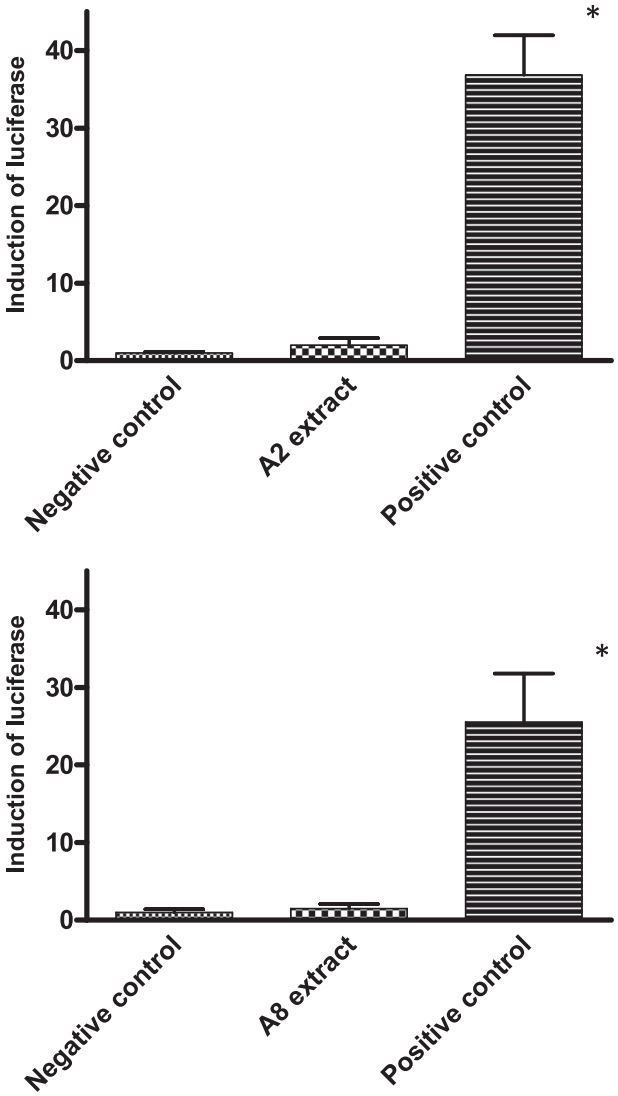

Fig. 1. Estrogenic activity in HepG2 cell line exposed to 1% of A2 or A8 extracts (13.5 μg/mL and 41.5 μg/mL respectively). Ethanol 95% (v/v) or ethanol 10% (v/v) were used as negative control for respectively A2 and A8 extracts, 17β-estradiol (10 nM) was used as positive control. The sign * indicates results statistically different from the negative control using a Fisher test. All experiments were performed in triplicate.

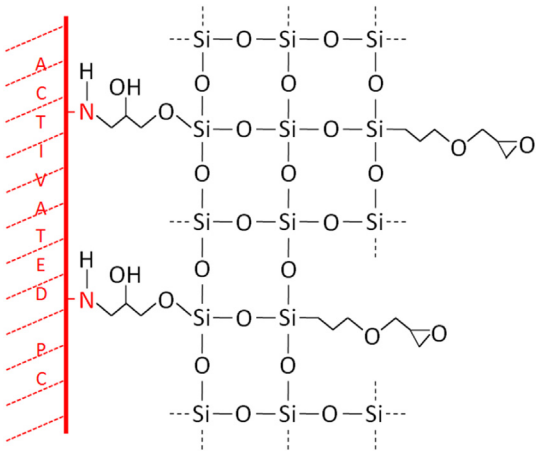

Fig. 2. Illustration of the bulk and interface of A8 derived samples deposited on plasma treated polycarbonate.

GPTES shows a mutagenic effect on bacteria in the Ames test (Lionti et al., 2014) due to its epoxy group, a well-known structural alert since it highly reacts with cell DNA (Ashby and Tennant, 1991). These negative results observed thus indicate that probably few or no unreacted GPTES molecules were present in the extracts, and two main assumptions can be made to explain it. The first one relates to epoxy reactivity: it is well-known that in presence of polar bonds such as C–NH_2, (formed on the PC surface during N_2/H_2 plasma treatment), epoxy rings can open (Innocenzi et al., 2005) and react with the latter, creating covalent bonds at the interface (Fig. 2). Therefore, in addition to improving the coating to substrate adhesion, the N_2/H_2 plasma treatment allows for epoxy group transformation which reduces the probability of observing mutagenic effect. Nonetheless, it is worth mentioning that even with a N_2/H_2 plasma treatment, the presence of unreacted epoxy groups in the coating cannot be ruled out: indeed, some of the GPTES molecules may not go to the interface to react with treated PC, but may instead be "trapped" in the bulk due to the reaction of GPTES hydrolysable functions (not involving epoxy groups) that lead to silica formation (Fig. 2). However, if the cohesion of the silica network is high enough and that no silica depolymerization occurs when the film is in contact with the aqueous food simulants involved in the migration tests, little or no epoxy groups will be present in the extracts, strongly limiting the risk to trigger positive responses in genotoxicity tests. The next step would then be to check if epoxy groups are present in the extracts using analytical methods, and to verify any release in an accelerated test at elevated temperature.

In conclusion, migration and bioassays such as those required by the European regulation on food contact materials were carried out on two different sol–gel coating extracts derived from commonly used ORMOSILs (MTES, and GPTES/TEOS respectively) with the aim of gathering initial information on whether these coatings could be qualified as food grade materials. The low levels of migration measured on these coatings in the different food simulants (below the maximum authorized level), which translate into low coating weight loss (4% (w/w) and 13% (w/w) for A2 and A8 respectively), suggest excellent coating cohesion and adhesion to polycarbonate. Genotoxicity tests complying with EFSA specifications were then performed: for each coating, the extract which gives the maximum migration was selected to carry out the Ames test, micronucleus assay and endocrine disruption assay. None of the extracts triggered positive responses in the bioassays, indicating that both coatings are neither mutagenic nor clastogenic/aneugenic (damage on chromosomes) in such experimental conditions. Furthermore, no estrogenic activity was detected *in vitro*. These promising results clearly show the potential of these sol–gel coatings as food grade materials. Additional tests, such as chemical analyses, or release study, are on the way to obtain the complete characterization of the extracts and to claim A2 and A8 derived coatings as food grade materials.

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