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Cytotoxicity and mutagenicity studies on migration extracts from nanocomposites with potential use in food packaging

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ABSTRACT

Clays are used in the food packaging industry to obtain nanocomposites. The use of these new materials is a concern, because they could reach consumers by oral exposure through possible migration, and potential toxic effects could be derived. In the present study, several *in vitro* basal cytotoxicity and mutagenicity tests on migration extracts obtained from a nanocomposite material with poly (lactic) acid (PLA) and two modified clays, Clay1 and Clay2, are shown. Migration extracts in distilled water showed values of 0.1 ± 0.2 mg/dm^2 in all samples. Also, the content of characteristic metals of the clays structure (Al, Ca, Mg, Fe, Si) was studied and no statistical differences were observed. For the cytotoxicity assays, the human intestinal Caco-2 and human liver HepG2 cells were selected. Cells were exposed to concentrations between 2.5% and 100% extracts determining three different biomarkers of cellular viability. No significant differences were observed in the cytotoxicity assays. Finally, mutagenicity was evaluated by the Ames test and resulted in the absence of mutagenic response at all the concentrations assayed. Taking in account all above mentioned, these new materials show a good profile for their use in food packaging although further research is still needed.

1. Introduction

Nanotechnology is a science that involves research and technology development at the atomic, molecular and macromolecular levels, aimed at creating and using structures, devices, and systems with novel properties and functions based on their small size (Farhang, 2009). Nanotechnology has opened up new avenues of research and development in numerous fields, including medicine, cosmetic and agro-food area (Chaundry et al., 2008). The application of this technology in food and agriculture plays an important role in improving production, processing, storage, transportation, traceability, safety and security of food (Chaundry et al., 2008). Among the wide range of applications of nanomaterials in the food industry, the one with the main development in the near future is their employ as food contact materials (AESAN, 2010). Nanomaterials have demonstrated to possess novel characteristics that can be applied in developing new packaging with better properties than packaging produced with micromaterials (Pereira de Abreu et al., 2007). The resulting materials, which are frequently polymers with nanoparticles incorporated in their structure, present improvements of barrier properties, such as thermal, mechanical or permeability characteristics, providing innovative solutions to increase the performance of the polymers (Silvestre et al., 2011). Therefore, nanotechnology plays an important role in the preservation of perishable food.

Among the most commonly used materials in food packaging are nanocomposites based on organic polymers and inorganic clay minerals (Pereira de Abreu et al., 2007). These clays consist of silicate nanoplates whose structure presents platelet morphology. Once the clays have been incorporated into the polymer, the resulting platelets force gases and other external agents to follow a tortuous path through the material, decreasing and slowing the contact with the product obtaining more resistant plastics (Pereira de Abreu et al., 2007; AESAN, 2010).

The Technological Institute of Packaging, Transport and Logistic (ITENE) is developing modified clays based in montmorillonite (natural phyllosilicate), one of the main mineral clays used to be incorporated in food packaging. The use of functionalized clays, immersed and dispersed in the polymers for packaging results in nanocomposite materials, conformed by platelets with submicrometer dimensions, except for their thickness, which present

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nanometric dimensions (Hatzigrigoriou and Papaspyrides, 2011). These dispositions allow preventing the exchange of oxygen, carbon dioxide and humidity with the food, maintaining its organoleptic and nutritional characteristics for a longest period of time (Farhang, 2009).

The nanoscale formulation of materials change physicochemical properties compared with the bulk or microstructured material. Smaller size, higher surface to volume ratio and greater reactivity are properties that confer advantages to the use of nanomaterials. Moreover, the toxicity and toxic kinetic profile also change, since it cannot be inferred from data of their non-nanostructured homologous. Therefore, the potential risk for the health and the environment could be modified (EFSA, 2009), making necessary to investigate the toxicity profile of these modified clays, even more, taking into account the scarce toxicological information available so far.

Considering that the consumer exposure to these nanoclays is likely since they are present in the food packaging, it is interesting to evaluate not only the microstructured clay, but also the imbued nanomaterial in the polymer, due to the possible migration to the food product (Guillard et al., 2010; EFSA, 2011; Song et al., 2011).

The EU Commission Regulation No. 10/2011 and the Council Directive 82/711/EEC collect the aspects related to plastic materials and articles intended to come in contact with food and foodstuffs, as well as the simulants used in the migration assays, and it states that the risk assessment should cover the potential migration under worst foreseeable conditions of use and the toxicity. Moreover, the Commission Regulation EU No. 10/2011 also establish an overall migration limit of 10 mg/dm^2, this is plastic materials and articles shall not transfer their constituents to food simulants in quantities exceeding 10 mg of total constituents released per dm^2 of food contact surface.

As nanoclays are substances that could be potentially present in food, the oral pathway would be the most commonly entry for these substances in the organism. Thus, it would be interesting to establish the toxicological profile of the migration extracts with *in vitro* assays, and thereby contribute to the risk assessment of nanomaterials used in food industry, claimed by the European Food Safety Authority (EFSA, 2011). In this sense, the present study provides a toxicological evaluation of two migration extracts obtained from nanocomposite materials with PLA and two modified clays developed by ITENE, Clay1 and Clay2, through several cytotoxicity assays on two specific and target cell lines (Caco-2 and HepG2 cells, from intestinal and liver origin, respectively) and a mutagenicity assay, the Ames test.

Moreover, the presence of characteristic metallic elements of the clays structure (Al, Ca, Fe, Mg, Si) was also determined in the migration extracts.

2. Materials and methods

2.1. Supplies and chemicals

Minimal Essential Medium (MEM) powder (1×) with Earlés Salts was obtained from PPA laboratories GmbH (Austria) and other cell culture reagents were supplied from Gibco (New Zealand). Chemicals for the different assays were provided by Sigma–Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Cell culture

Caco-2 cell line derived from human colon carcinoma (ATCC No. HTB-37) was maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire, Spain), in a medium consisting of Minimum Essential Eagle's medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 mg/mL gentamicin, 1.25 mg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. The cells were used at passages between 12 and 21. HepG2 derived from a liver hepatocellular carcinoma (ATCC No. HB-8065) was maintained at similar conditions in EMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested each two–three days with 0.25% trypsin. The cells passes used were 16–32.

2.3. Clays and polymer

Purified sodium montmorillonite (MMT) (Cloisite® Na^+), with cationic exchange capacity (CEC) of 116 meq/100 g was purchased in Southern Clay Products (COMITEX, S.A. Spain). Quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) ($C_{19}H_{42}BrN$, 364.46 g/mol, 98%) was supplied from CymitQuímica S.L. (Spain), acetylcholine chloride (ACO) ($C_7H_{16}ClNO_2$, 181.66 g/mol, ⩾99%) was provided by Sigma–Aldrich (Spain). Polylactide (PLA) pellets were purchased from Cargill Dow (NatureWorks ® PLA 7032 polymer) for the preparation of filled biopolymers (Fig. 1).

2.4. Organo-modified nanoclays

Organo-modified clays have been developed and characterized by ITENE based on previous works (Jordá-Beneyto et al., 2008; Jordá-Beneyto et al., 2009). Briefly, two different organo-modified clays were prepared by a cation-exchange method, which consists on a displacement of the sodium cations of Cloisite® Na^+ with the ammonium cations of the above mentioned salts, obtaining Clay1 and Clay2. Both clays were characterized by Fourier Transform InfraRed (FTIR), Wide-angle X-ray Diffraction and Termogravimetric Analysis (TG) following the methods described in Jorda-Beneyto et al. (2013).

2.5. Nanocomposites preparation

Different PLA nanocomposites samples have been obtained with the prepared modified clays, Clay1 (MMT + HDTA) and Clay2 (MMT + HDTA + ACO) with 4% clay content. For this purpose a twin screw extruder DSE 20/40 (COPERION) was used. The twin screw extruder was used to prepare the samples through melt-direct intercalation. PLA pellets (dried overnight at 90 °C and vacuum for 2 h (MANN HUMMEL PicolinoDehumidifier)) were blended with 4% in weight of Clay1 and Clay2, respectively. Bottles were obtained by injection stretch blow moulding (ISBM). An important part of the process is the mechanical stretching of the preform during the moulding process, which helps to increase the impact resistance of the container and also helps to produce a very thin walled container, affecting final mechanical, thermal and chemical properties of the nanocomposite material. The thermal, mechanical and barrier properties of the materials obtained were analyzed following the techniques described in Jorda-Beneyto et al. (2013).

2.6. Migration extracts

Migrations extracts of both nanocomposites were provided by ITENE. Migration tests were carried out according to UNE-EN 1186-9:2002 (AENOR, 2002). The samples, bottles of 150 mL of nanocomposite materials PLA + Clay1 and PLA + Clay2 (123,655 dm^2 and 590–598 µm thick) were filled with the simulant chosen according to the law (EU Commission Regulation No. 10/2011 and Council Directive 82/711/EEC), distilled water, at the specific conditions established by the regulations (Temperature (°C): 40; Time (days): 10). Then, all volume of simulants were poured into cell glasses and exposed to heat in order to the simulants were evaporated. Afterwards, the residues were weighed and compared with control cells glasses (without simulants) and the presented difference is the global migration from the simulants. Moreover, nanocomposites of PLA–Clay1, and, PLA–Clay2, were also filled with the specific culture medium of each cell line used as simulant. Each sample was evaluated by triplicate.

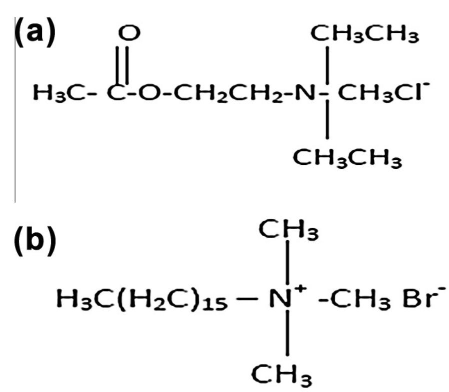

Fig. 1. Structure of the quaternary ammonium salts: acetylcholine chloride (a) and hexadecyltrimethylammonium bromide (b) used to modify the montmorillonite.