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In vitro toxicological assessment of clays for their use in food packaging applications

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ABSTRACT

Montmorillonite based clays have a wide range of applications that are going to contribute to increase human exposure to these materials. One of the most promising uses of clays is the development of reinforced food contact materials that results in nanocomposites with improved barrier properties. Different organoclays have been developed introducing modifiers in the natural clay which is commercially available. However, the toxicological aspects of these materials have been scarcely studied so far. In the present study, the cytotoxic effects of a non-modified clay (Cloisite® Na+) and an organoclay (Cloisite® 30B) have been investigated in the hepatic cell line HepG2. Only Cloisite® 30B showed cytotoxicity. In order to elucidate the toxic mechanisms underlying these effects, apoptosis, inflammation, oxidative stress and genotoxicity biomarkers were assayed. Moreover, a morphology study with light and electron microscopy was performed. Results showed genotoxic effects and glutathione decrease. The most relevant ultraestructural alterations observed were mitochondrial degeneration, dilated endomembrane systems, heterophagosomes formation, fat droplets appearance and presence of nuclear lipid inclusions. Cloisite® 30B, therefore, induces toxic effects in HepG2 cells. Further research is needed to assess the risk of this clay on the human health.

1. Introduction

Clays have an array of commercial applications in different fields: the manufacture of inks, paints, greases and cosmetics, water treatment processes, the controlled release of therapeutic agents, food packaging, etc. (Lordan et al., 2011). In regard to food packaging applications, clays are used to improve the barrier properties of food contact materials. This results in a length of the storage time while keeping the product fresh. Polymers incorporating clay nanoparticles are among the first polymer nanocomposites to emerge on the market as improved materials for food packaging. This is due to the easy availability of the raw clay materials and because their cation exchange chemistry has been intensively studied. In addition, these clay nanoparticles have unique properties such as large surface areas, large aspect ratios and improved mechanical, thermal and optical properties (Utracki and Kamal, 2002; Pavlidou and Papaspyrides, 2008). The most frequently used clay in the preparation of polymer nanocomposites is montmorillonite, which is the major constituent of bentonite. Montmorillonite is a natural clay which occurs as plate-like particles called platelets. These platelets have an average thickness of only 1 nm, while its dimensions in length and width can be measured up to 1 mm (Lordan et al., 2011). One limitation of clays is the incompatibility between the hydrophilic clay and a hydrophobic polymer, which could cause agglomeration of clay in polymeric matrices (Elmore and Andersen, 2003; Zeng et al., 2005). Therefore, surface modification of clay minerals is an important step to achieve polymer nanocomposites. By cation exchange with organic cations, clays become hydrophobic and thereby compatible with polymers. Such modified clays are referred to as organoclays (Sharma et al., 2010). The most widely known theories to explain the improved barrier properties of polymer–clay nanocomposites are based on a theory developed by Nielsen (1967), which focuses on a tortuous path around the clay plates, forcing the gas permeant to travel a longer path to diffuse through the film.

Successful technical development of nanocomposites for food packaging has to overcome barriers in safety, technology, regulation, standardization, etc. (Silvestre et al., 2011). Regarding to safety, since the use of clays and organoclays is increasing, it is important to take into account their toxicity. Clays are natural materials but they are not free of possible side-effects. In this sense, toxicity studies of clays have been scarcely performed. The toxicological evaluation of these products can be faced using both

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in vivo and *in vitro* methods. The primary aim of *in vitro* testing is toxicity screening and the understanding of biological responses and underlying mechanisms (EFSA, 2011). Moreover, *in vitro* methods are encouraged in toxicological research for ethical reasons.

Toxic effects of clay minerals have been shown to occur mainly after inhalation (Carretero et al., 2006; Sharma et al., 2010). But also, one of the most likely routes of exposure to these clays for the general population is the oral pathway, since they are present in food contact materials. Tateo and Summa (2007) reported that the ingestion of clays is common at low doses in food preparations, in pharmaceuticals for oral administration, and as herbal remedies. Moreover, distribution studies performed with different nanoparticles showed that they can translocate to several organs such as liver, kidney, lungs. (Kim et al., 2008; Kwon et al., 2008; EFSA, 2009) so for clays this possibility cannot be discarded.

In view of the limited toxicological information of clays, in this study we aim to evaluate the toxicity of an unmodified (Cloisite® Na+) and an organomodified clay (Cloisite® 30B) in the human hapatocellular cell line HepG2. For this purpose, basal cytotoxicity biomarkers and mechanistic biomarkers of oxidative stress, inflammation and genotoxicity have been investigated. Moreover, a histopathological study has been also performed.

2. Materials and methods

2.1. Clay materials and characterization

Unmodified montmorillonite (Cloisite® Na+) and the organically modified one (Cloisite® 30B) were obtained from Southern Clay Products, INC. (modifier: methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium, concentration: 90 meq/100 g clay). Both clays were characterized by thermogravimetric analysis (TGA), X-ray diffraction (XRD) and particle size distribution (PSD).

TGA analysis of Cloisite Na+ and Cloisite 30B were performed on a Q5000IR thermobalance (TA Instruments) by heating the samples from room temperature up to 900 °C with heating rate of 10 °C/min, in nitrogen atmosphere. Approximately 7 mg of each finely ground sample was heated in a platinum crucible. Powder XRD analyses were performed using a D8ADVANCE A25 Bruker diffractometer. The clay powders were mounted on a sample holder with a large cavity and a smooth surface was obtained by pressing the powders with a glass plate. The X-ray diffraction patterns were measured from 1.5° to 25° (2θ) at a scan rate of 0.02 s. Particle size distributions of the samples were estimated with a Mastersizer 3000 (Malvern Instruments Ltd., UK). Dispersion in dry basis was the selected method to perform the different assays, with controllable airflow and feed rate. This method allows measuring particle sizes between the range 0.1–3500 μm.

2.2. Clays test solutions

The test concentrations for both clays were selected taking into account previous dispersion experiments in order to avoid interferences with the measurement system. The highest concentrations tested were 62.5 and 500 μg/mL for Cloisite® Na+ and Cloisite® 30B, respectively. Test solutions were prepared in serum-free medium. Three sonication steps of 10 s each one at an amplitude of 40% were performed using an ultrasonic tip (Dr. Hielscher, Germany) to disperse the test concentrations.

2.3. Cell culture

HepG2 (human hepatocellular carcinoma epithelial cell line) (HB-8065) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in monolayer in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% of fetal calf serum (FCS, Gibco, New Zealand), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, New Zealand). Cells were grown at 37 °C and 5% CO_2 in a humidified atmosphere.

2.4. Cytotoxicity assays

For cytotoxicity assays, exposure concentrations for Cloisite® Na+ were set at 0, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5 μg/mL and for Cloisite® 30B at 0, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 μg/mL. HepG2 cells were seeded at a density of 7.5×10^5 cell/mL in 96 wells plates and exposed to the clays for 24 and 48 h.

Total cellular protein content (PC) was quantified following the method of Bradford (1976). After exposure, cells were washed with phosphate buffer saline (PBS) and incubated with 0.1 N NaOH for 2 h at 37 °C. Later, a 22% Coomassie brilliant blue solution (Biorad, Spain) was added to the wells and within 30 min absorbance was read at 595 nm on a microplate reader (Biotek, USA).

Neutral Red (NR) uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Briefly, NR in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of viable cells. NR can be extracted from lysosomes for quantitative measurement at 540 nm.

The MTS tetrazolium reduction assay was performed according to a procedure based on Baltrop et al. (1991), being MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt bioreduced by cells into a colored formazan product soluble in culture medium. Cells were directly incubated with MTS tetrazolium salt for 2 h at 37 °C and absorbance was read at 492 nm.

Only when cytotoxicity was observed, mechanistic biomarkers were determined. In this case, the mean effective concentration (EC_{50}) of the most sensitive cytotoxicity endpoint was chosen as the higher exposure concentration to investigate mechanistic biomarkers along with the fractions $EC_{50}/2$ and $EC_{50}/4$.

2.5. Caspase-3/7 activity

Caspase-3 and -7 activities were used as apoptosis biomarkers in cells exposed for 24 and 48 h to the clays. Manufacturer instructions from the kit (Caspase-Glo® 3/7 Assay, Promega, USA) were followed.

2.6. ROS generation

The production of ROS was assessed in 96 wells microplates using the dichlorofluorescein (DCF) assay. Cells were incubated with 200 μL, 40 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in culture medium at 37 °C for 30 min, and then washed with PBS and resuspended in 200 μL of PBS. The formation of the fluorescence oxidized derivative of DCF-DA was monitored at emission wavelength of 535 nm and excitation wavelength of 485 nm. ROS production was expressed as fluorescence arbitrary units (Puerto et al., 2010).

2.7. GSH content

Cellular glutathione (GSH) content was evaluated by reaction with the fluorescent probe monochlorobimane (mBCl) (Jos et al., 2009). This molecule forms a thioether adduct with GSH in a reaction catalyzed by the enzyme glutathione-S-transferase (GST). After the cell exposure the medium was discarded and cells were incubated at 37 °C for 20 min in the presence of 40 μM mBCl. Later on cells were washed with PBS and the fluorescence was recorded in a spectrofluorometer (Biotek, USA) at the following excitation/emission wavelengths: 355/460. Results were expressed as fluorescence arbitrary units.

2.8. Interleukin-6 leakage

For this assay the culture medium of the cells after 24 and 48 h exposure to the clays was used. Manufacturer instructions from the kit (EH2IL6, Thermo Scientific, USA) were followed.

2.9. Comet assay

The comet assay was performed to detect DNA strand breaks. HepG2 cells were seeded into 12-well tissue culture treated plates (Corning Costar Corporation, New York, USA) and left overnight at 37 °C in 5% CO_2 to attach to the plates. Approximately 3.5×10^5 cells in each well were exposed with different concentrations of Cloisite® 30B (0, 22, 44 or 88 mg/mL) after 24 and 48 h to exposure.

In order to monitor the ongoing process of the assay, a negative control (cells treated with medium without fetal calf serum) and a positive control (cells treated with a solution of 100 μM H_2O_2) were included. After treatments cells were washed and detached in PBS. The comet assay was applied as previously described by Collins et al. (1997) with modifications (Corcuera et al., 2011). Briefly, cells were resuspended in PBS at a concentration of 2.5×106 cells/mL. This suspension were mixed with 1% low melting point agarose and placed on a microscope slide. Once the gels had become solid, the slides were dipped into lysis solution at 4 °C. All nucleotides were denatured in a high-pH buffer. Electrophoresis was carried out approximately at 25 V (300 mA) and the DNA was gently reneutralized in PBS and washed in H_2O. After neutralization, microscope slides are fixed in 96% ethanol and absolute ethanol. Finally, DNA was stained with SYBR Gold nuclei acid gel stain and was visualized with an Olympus BX61 fluorescence microscope (20× objective) coupled via a CCD camera to an image-analysis system (DP controller-DP manager). Images of randomly selected nuclei ($\geqslant 100$) per experimental point were analyzed with the image analysis software (Comet Assay IV, Perceptive Instruments, UK).