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Research paper

Toxicity assessment of organomodified clays used in food contact materials on human target cell lines

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ABSTRACT

Nowadays, the incorporation of organomodified clays based on montmorillonite into polymers intended for packaging industry is a reality. The final result is a polymer nanocomposite with enhanced barrier properties. Different organomodified clays are already commercially available and others new ones are being developed; however little is known about their safety.

In the present work, the cytotoxic effects (a tetrazolium salt reduction and protein content) of three organomodified clays, Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified clays developed by the Packaging, Transport, & Logistics Research Institute, were evaluated in Caco-2 and HepG2 cells after 24 and 48 h of exposure. Our results showed that only Clay 2 induced toxic effects in both cell lines. The mean effective concentration was calculated for each case, showing Caco-2 to be more sensitive than HepG2. Moreover, in order to elucidate the toxicity mechanisms of Clay 2, different mechanistic biomarkers were investigated. Interleukin leakage and generation of intracellular reactive oxygen species were not observed, whereas glutathione content decreased in HepG2. DNA damage (comet assay) was induced in both cell lines at the highest concentration tested. Overall, results show that the type of clay, the concentrations range and the type of cell line play an important role in the toxicity observed.

1. Introduction

Over the last decades, the use of polymers as food packaging materials has increased enormously due to their advantages over other traditional materials (Jordan et al., 2005; Silvestre et al., 2011). The latest innovations include the use of "improved" food contact materials; this is the addition of different reinforcements, for example nanoparticles, to the polymeric matrix in order to enhance the functional properties of packaging materials, and thus improve the shelf life of food and beverage products. The resulting nanocomposites are a multi-phase material in which the majority of the dispersed phase components have one or more dimensions of the order of 100 nm or less (SCENIHR., 2007).

Smectite clays, such as montmorillonite (MMT), belong to the structural family called 2:1 phyllosilicates, which present a structure composed by two tetrahedral layers formed by Si and O atoms, fused with an octahedral layer with aluminum and magnesium atoms bonded to oxygen and hydroxyl groups (Jordá-Beneyto et al., 2008). They are one of the main choices for designing polymer nanocomposites due to their low cost and rich intercalation chemistry allowing them to be chemically modified (organoclays) and to improve the compatibility with the polymer matrix (Bitinis et al., 2011). Once the final clay nanocomposites are ready, the resulting material presents a specific disposition of the clays, such as platelets, giving a tortuous path to the gas permeant, forcing it to travel a longer path to diffuse through the film (Nielsen, 1967). Several authors reviewed the advantages, and in few cases the limitations, of the layered nanocomposites. Great improvements in thermal, mechanical and barrier (permeability) properties are presented, as well as strength, stiffness, dimensional stability, and heat resistance (De Azeredo, 2009; Duncan, 2011; Hatzigrigoriou and Pasparydes, 2011; Hetzer and De Kee, 2008). Moreover, they also offer good barrier properties under different packing, handling, shipping, and storage conditions (Avella et al., 2005; Brody, 2006; Ray et al., 2006; Duncan, 2011; Volpe, 2005), therefore they could improve the quality and safety of packaged food (Lagaron, 2006).

Although there is a lot of evidence for the good technological performance of nanocomposites, safety issues are also of importance. Available data on clay's toxicity is still scarce, but different authors have already described toxic effects induced by montmorillonite and organoclays (Lordan et al., 2011; Maisanaba et al., 2013a, in press; Sharma et al., 2010). Moreover, the modifier used to synthesize the organoclay has a role in the toxicity observed (Maisanaba et al., 2013a).

In the case of organoclays, the oral pathway is the most important entrance route for the consumers, as they are exposed to the possible

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migrants from packaging nanocomposites to the food products, and they should know the possible effects of the ingestion of these nanosubstances to the gastrointestinal tract (Silvestre et al., 2011). When the nanoparticles reach the blood circulation, the liver is among the main organs for distribution (SCENIHR., 2009), so for clays imbued in the nanocomposite material this possibility cannot be discarded.

Taking into account that the production of nanocomposites is going to increase in the near future, the human exposure will also increase. Therefore, potential toxic effects should be investigated in order to guarantee the safety of the food products.

The aim of this study is to evaluate the toxicity of three modified montmorillonite clays used in the production of nanocomposites with applications as food contact materials in two target human cell lines of the digestive system, Caco-2 and HepG2 from intestinal and hepatocellular origin, respectively. For this issue, basal cytotoxicity biomarkers and mechanistic biomarkers of inflammation, oxidative stress and genotoxicity have been investigated with Cloisite®20A, a commercial clay, and Clay 1 and Clay 2, two novel modified organoclays developed by the Technological Institute of Packaging, Transport and Logistics (ITENE).

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). 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. Clays

Clay 1 and Clay 2 are two novel microsized clays that have been developed and characterized by thermogravimetric analysis (TGA) and Fourier transform infrared (FTIR), as described in Maisanaba et al. (submitted for publication) and Jordá-Beneyto et al. (2008, 2013). Clay 1 contains a modifier quaternary ammonium salt hexadecyltrimethyl-ammonium bromide (HDTA) and Clay 2 contains an HDTA and acetylcholine chloride (ACO). Both clays are obtained by cation exchange reaction from Cloisite®Na^+ (Southern Clay Products, Inc.). This raw clay has a typical dry particle size less than 25 μm (d50). Cloisite®20A was also obtained from Southern Clay Products, Inc. (modifier: dimethyl, dehydrogenated tallow, quaternary ammonium, concentration: 95 meq/100 g clay). This modified clay has a typical dry particle size less than 10 μm (d50) and has been characterized by thermogravimetric analysis (TGA) and Fourier transform infrared (FTIR).

TGA of Cloisite®20A and Cloisite®Na^+ (the original non-modified clay included as control) was performed on a Q5000IR thermobalance (TA Instruments) by heating the samples from room temperature up to 900 °C with a heating rate of 10 °C/min, in nitrogen atmosphere. Approximately 7 mg of each finely ground sample was heated in a platinum crucible.

FTIR spectra were obtained on an Equinox 55 spectrometer (Bruker), coupled to a microscope modulus with ATR objective (Hiperion, Bruker). This technique was used to characterize both clays Cloisite®Na^+ and Cloisite®20A. For each sample 128 scans were recorded with a resolution of 4 cm^{-1}.

2.3. Model systems

Caco-2 cell line derives from a human colon carcinoma (ATCC® HTB-37) and HepG2 is a human hepatocellular carcinoma epithelial cell line (ATCC® HB-8065). Both of them were obtained from the American Type Culture Collection. Caco-2 cell line was maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 50 μg/mL gentamicine, 2 mM L-glutamine, and 1 mM pyruvate. HepG2 cell line was maintained 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 at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer hemocytometer and viability was determined by the Trypan Blue exclusion test. The cells were used at passages between 10 and 21. Both cell lines were plated at a density of 7.5×10^5 cells/mL to perform all experiments.

2.4. Clays test solutions

Test concentrations of clays were determined individually in previous experiments in order to avoid interference with the method of measurement. With this purpose the absorbance of clay solutions (1000 μg/mL and serial 1/2 dilutions) were measured at 0, 24 and 48 h. The concentrations selected were the highest ones that did not show statistical differences versus the control. Thus, the maximum concentrations were 8 μg/mL for Clay 1, 125 μg/mL for Clay 2, and 62.5 μg/mL for Cloisite®20A. Test solutions were prepared in serum-free medium. An ultrasonic tip (Dr. Hielscher, Germany) at an amplitude of 40% for a total time of 30 s was employed to disperse the test concentrations.

2.5. Cytotoxicity assays

From the initial solutions, serial dilutions in medium without serum were prepared. Culture medium without clay was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37 °C for 24 and 48 h. The basal cytotoxicity endpoints assayed were tetrazolium salt reduction (MTS) and protein content (PC).

MTS reduction is carried out by dehydrogenases, enzymes present in mitochondria, making this endpoint a good marker for the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium salt) added to the medium is reduced by the enzymes to a colored formazan product soluble in culture medium and is measured by a spectrophotometer at 490 nm after 2 h of incubation in the dark.

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage with independence of the toxic mechanism involved (Pichardo et al., 2007). PC was quantified in situ, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the total cell number present in the wells. The culture medium was replaced by 200 μL NaOH and after 2 h of incubation at 37 °C, 180 μL was replaced by the same volume of a 22% Coomassie Brilliant Blue G-250 solution. After 30 min incubation at room temperature, absorbance was read at 595 nm in a microplate spectrophotometer (Tecan Infinite M200, Austria).

2.6. Oxidative stress assays

Considering that only Clay 2 showed remarkable cytotoxic effects, this clay was chosen to perform mechanistic studies. For this purpose low cytotoxic concentrations were selected: 0, 8.5, 17 and 34 μg/mL for Caco-2 cells and 22, 44 and 88 μg/mL for HepG2 cultures. These concentrations correspond to the mean effective concentration (EC_{50}) value obtained for the most sensitive cytotoxicity endpoint at 24 h along with the fractions $EC_{50}/2$ and $EC_{50}/4$. After replacing the previous medium, the exposure solutions were added to the cells, and incubated at 37 °C for 24 and 48 h. Culture medium without clay was used as control