Applied Clay Science 150 (2017) 98–105

Contents lists available at ScienceDirect

Applied Clay Science

journal homepage: www.elsevier.com/locate/clay

Research paper

Toxicological assessment of two silane-modified clay minerals with potential use as food contact materials in human hepatoma cells and *Salmonella typhimurium* strains

Sara Maisanaba[a,*], María Llana-Ruíz-Cabello[a], Silvia Pichardo[a], Ana Isabel Prieto[a], Ana M. Cameán[a], María Jordá-Beneyto[b], Ángeles Jos[a]

[a] Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Profesor García González n°2, 41012 Seville, Spain
[b] Area of Packaging Materials and Systems, ITENE, C/Albert Einstein 1, 46980 Paterna, Valencia, Spain

ARTICLE INFO

Keywords:
Silane-modified clay minerals
HepG2 cells
Cytotoxicity
Cell death
Oxidative stress
Muta/genotoxicity

ABSTRACT

Reinforced technological properties can be achieved by adding small amounts of organo-modified clay minerals into traditional polymeric matrices. The result is a final product with improved characteristics destined to several applications, among them, food packaging. However, it is mandatory to evaluate the toxicity of novel food contact materials, as well as all new substances destined to this purpose. The present work shows for the first time in a human target cell line from hepatic origin (HepG2), and in *Salmonella typhimurium*, a prokaryotic system, the *in vitro* toxicological evaluation of Clay3 and Clay4, two silane-modified montmorillonites intended to be incorporated into food containers. Cytotoxic effects were evaluated (0 to 250 μg/mL) after 24 and 48 h of exposure and only Clay4 showed toxic effects. Once the mean effective concentration was calculated, different mechanistic biomarkers were investigated for Clay4: cell death, reactive oxygen species (ROS) production, glutathione content, mutagenicity, and standard and oxidative genotoxicity. Only a significant ROS production after both times of exposure, as well as a significant oxidative DNA damage after the longer time assayed were obtained with Clay4. Between both silane-modified clay minerals studied Clay3 is the better candidate to develop new reinforced packaging materials in base to its good toxicological profile.

1. Introduction

Since the end of the last century the expanding use of clay minerals in a variety of applications in industrial (*i.e.* oil drilling, ceramics and paper industry), engineering and scientific fields (*i.e.* catalysts, decoloration agents and adsorbents) resulted in a continuous development in different areas (Kotal and Bhowmick, 2015), one of them, the food industry. Currently, the incorporation of organomodified clay minerals into polymers intended for the packaging industry is a great alternative in order to enhance the product's perdurability.

Clay minerals or layered silicates have a typically stacked arrangement of silicate layers (nanoplatelets) with nanometric thickness; hence they are named as nanoclays. The nanometric size, their features and the extraordinary high surface area of the dispersed clay minerals are related to the improvements in the physical properties of polymers, such as tensile strength and modulus, gas permeability, lower coefficient of thermal expansion giving stability, and the absence of changes in the optical homogeneity of the material (Arora and Padua, 2010; Kotal and Bhowmick, 2015). In fact, the platelets create a tortuous path that prevents the passage of water, oxygen, aroma and tainting compounds, leading to a decrease of the rate of diffusion. Often, incorporation of only a low mass fraction such as a few percent in the nanocomposites can increase barrier properties many-fold compared to the polymer alone (Smolander and Chaudhry, 2010; Bradley et al., 2011).

Montmorillonite (Mt), one of the most used clay minerals for this purpose, is a layered silicate belonging to the structural family of the 2:1 phyllosilicates. The presence of inorganic cations on the planar surface of Mt. layers makes them hydrophilic and ineffective in hydrophobic polymers. Cation exchange reactions are one of the possibilities to resolve this inconvenience because these inorganic cations could be replaced with organic cationic surfactants (Jordá-Beneyto et al., 2014). These organic cationic surfactants act as modifiers and they intercalate into the clay mineral gallery, resulting in an increase of the interlayer spacing, giving a hydrophobic surface and improving interactions with organic polymers (De Azeredo, 2013). However, this

* Corresponding author.
E-mail address: saramh@us.es (S. Maisanaba).

http://dx.doi.org/10.1016/j.clay.2017.09.018
Received 1 September 2017; Received in revised form 13 September 2017; Accepted 17 September 2017

kind of modifiers is degraded at high temperatures which the plastics are commonly processed, being a remarkable disadvantage. Therefore, the grafting silylation reaction is a real alternative, which could solve the above-mentioned handicap, being an irreversible reaction and giving also to the polymers the improvements in the technological aspects. The presence of broken bonds on the platelet edges are common for layered silicates, and it leads to the formation of hydroxyl groups, which can be used for chemical modification by silylation reaction. Through the use of an organosilane as modifier, it is then possible to covalently bond the organic functional groups onto the layer surface (Pisticelli et al., 2010; Bujdák et al., 2012). There are many studies that have described the consequences of the technical changes produced in nanocomposites obtained with silane-modified clay minerals and the improvements acquired (Di Gianni et al., 2008; Pisticelli et al., 2010; Silva et al., 2011).

In this regard, the Technological Institute of Packaging, Transport and Logistic (ITENE, Valencia) has developed two novel silane-modified clay minerals based on Mt. using as modifiers 3-aminopropyltriethoxysilane (APTES) and vinyltrimethoxysilane (VTMS), obtaining Clay3 and Clay4, respectively. Both of them are destined to be incorporated into polypropylene (PP), creating novel food packaging materials.

Data on the toxicological effects of food contact materials is required prior to its commercialization (EFSA, 2011; EFSA, 2016). This, joint to the increase in the manufacture of nanoclay-containing products with other applications, makes relevant the toxicological research of these compounds. Several studies are available in the literature in regard to the *in vitro* toxic potential of organomodified clay minerals with quaternary ammonium salts (Maisanaba et al., 2013; Houtman et al., 2014; Maisanaba et al., 2016), indicating in some cases that the chemical modification increase the toxicity. However, to the extent of our knowledge, the toxicological information of silane-modified clay minerals is very scarce in the literature with only some data of their effects on the human adenocarcinoma cell line Caco-2 (Maisanaba et al., 2018).

In this sense, new approaches and standardized test procedures to study the impact of organoclays on living cells are urgently needed for the evaluation of potential hazards relating to human exposure to these substances (Sekhon, 2010). Moreover, cytotoxic effects are frequently induced by high doses at acute exposure, and only inform about the viability status, being necessary to know the damage produced at lower concentrations that are overlooked (Singh et al., 2009). Considering all these facts, genotoxicity studies should be included in the toxicity assessment of new substances that humans could be routinely exposed to, such as clay minerals used in food packaging. Currently, European legislation in relation to the authorization of food contact materials and the European Food Safety Authority (EFSA) includes genotoxicity tests among the toxicological assays required (Commission Regulation (EU) 10/2011, 2011; EFSA, 2011; EFSA, 2016). Additionally, increases in the intracellular level of reactive oxygen species (ROS) represent a potential toxic mechanism, which could be related to the membrane dysfunction, lipid peroxidation which means a higher malondialdehyde level in the cell membrane, oxidative DNA damage and a drastic inactivation of proteins. Also, different markers are used to determine the cell death mechanism after exposure to a toxic substance. One of the most used is caspases activity (Maisanaba et al., 2015a).

In view of the limited toxicological information of these novel silane-modified clay minerals, the present study aims to evaluate the toxicity of Clay3 and Clay4 in the human hepatoma cell line HepG2 and *Salmonella typhimurium* strains. For this purpose, the basal cytotoxicity of both clay minerals was investigated as well as different mechanistic biomarkers for Clay4, due to its observed cytotoxic effects, including cell death, oxidative stress, mutagenicity and genotoxicity.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, fetal bovine serum (FBS) and cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain). Chemicals for the different assays were provided by Sigma-Aldrich (Spain) and VWR International Eurolab (Spain).

2.2. Clay minerals preparation and clay minerals test solutions

The silane-modified clay minerals, Clay3 and Clay 4, are two novel microsized clay minerals that have been developed and characterized by thermogravimetric analysis (TGA), Fourier transform infrared (FTIR) and wide-angle X-ray diffraction (WAXD) by the Institute of Packaging, Transport and Logistic (ITENE) from Valencia, according to Maisanaba et al. (2017). Clay3 contains as modifier, 3-aminopropyltriethoxysilane (APTES), and Clay4 vinyltrimethoxysilane (VTMS). Both clay minerals are obtained by grafting silylation reaction from one commercial Mt, Cloisite®Na^+ (CNa^+) (Southern Clay Products, Inc.).

The test concentrations for both clay minerals were selected taking into account previous dispersion experiments in cell culture medium in order to avoid interferences with the measurement system. The highest concentrations tested were 250 μg/mL for both clay minerals. Test solutions were prepared in serum-free medium for the HepG2 cells and in sterile MilliQ water for the bacteria. 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 cells (human hepatocellular carcinoma epithelial cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in monolayer in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% of FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin (Gibco, New Zealand). Cells were grown at 37 °C and 5% CO_2 in humidified atmosphere. Cells were used at passages between 7 and 12.

2.4. Cytotoxicity assays

From the initial stock solution (1000 μg/mL), serial dilutions in medium were prepared (0–250 μg/mL for Clay3 and Clay4). Culture medium without clay mineral was used as control group. After replacing the previous medium, the exposure solutions in culture medium without serum were added to the systems, and incubated at 37 °C for 24 and 48 h. The basal cytotoxicity endpoints assayed were supravital dye neutral red cellular uptake (NRU) and tetrazolium salt reduction (MTS).

The NRU biomarker 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). Furthermore, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good marker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). Both assays were carried out according to Maisanaba et al. (2013).

Exclusively, when cytotoxicity was observed, mechanistic biomarkers were determined. In this case, the mean effective concentration (EC50) (concentration of test chemical that modified each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls determined by linear interpolation) of the most sensitive cytotoxicity endpoint was chosen as the higher exposure concentration to investigate mechanistic biomarkers along with the fractions EC50/2 and EC50/4. In the present work, the EC50/4, EC50/2 and EC50 were only obtained for Clay4, being the values 53.25, 106.5 and 213 μg/mL,