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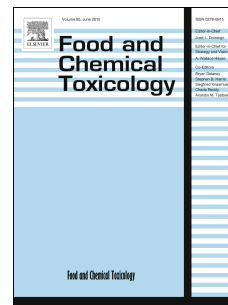
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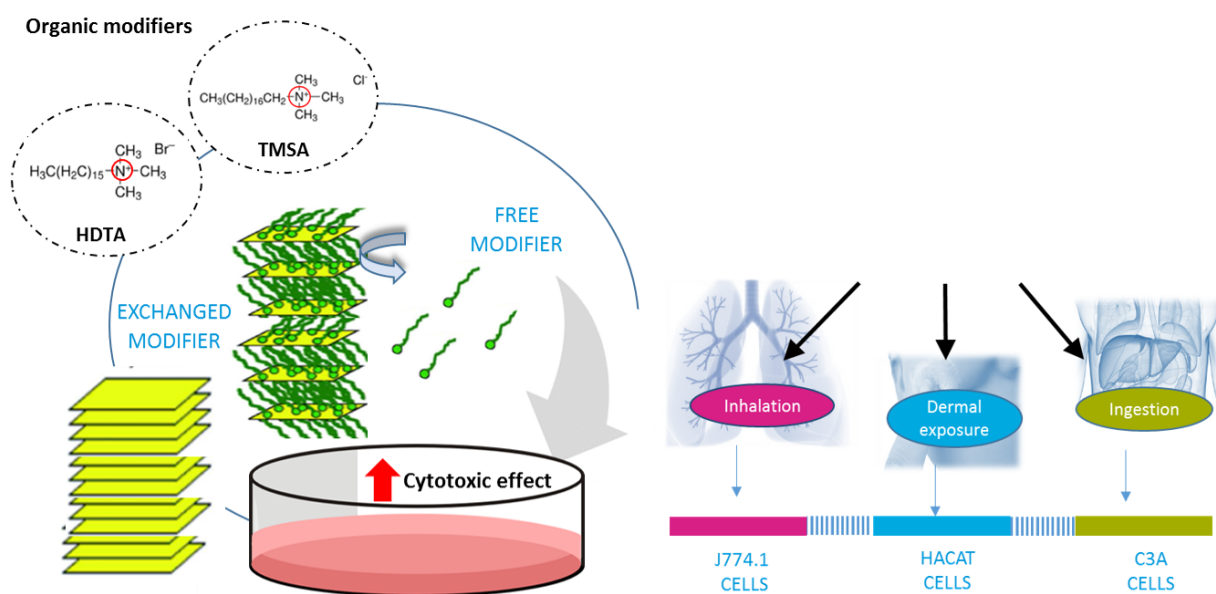
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**Title:** The influence of organic modification on the cytotoxicity of clay particles to keratinocytes, hepatocytes and macrophages; an investigation towards the safe use of polymer-clay nanocomposite packaging.

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<sup>1</sup>Abbreviations

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Å: Armstrongs

AB: alamarBlue®

CAS no: Chemical abstracts service number

CEC: Cation exchange capacity

CFDA-AM: 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) probe

DLS: Dynamic Light Scattering

FTIR: Fourier Transform InfraRed

HDTA: Hexadecyl Trimethyl ammonium bromide

LOEC: Lowes observed effect concentration

NRU: Neutral red uptake

OECD: Organisation for Economic Co-operation and Development

QAC: Quaternary ammonium compound

REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals

TGA: Thermogravimetric analysis

TMSA: Octadecyl trimethyl Ammonium chloride

TNF- $\alpha$ : Tumor Necrosis Factor- $\alpha$

TEM: Transmission electron microscopy

XRD: X-ray diffraction

**Abstract**

Organically modified clays can be used as nanofillers in polymer-clay nanocomposites to create bio-based packaging with improved strength and barrier properties. The impact of organic modification on the physico-chemical properties and toxicity of clays has yet to be fully investigated but is essential to ensure their safe use. Two organoclays, named N116\_HDTA and N116\_TMSA, were prepared using a commercially available sodium bentonite clay and organic modifiers hexadecyl trimethyl ammonium bromide (HDTA) and octadecyl trimethyl ammonium chloride (TMSA). An *in vitro* hazard assessment was performed using HaCaT skin cells, C3A liver cells and J774.1 macrophage-like cells. Organic modification with HDTA and TMSA increased the hazard potential of the organoclays in all cell models, as evidenced by the higher levels of cytotoxicity measured. N116\_TMSA caused the greatest loss in viability with IC50 values of 3.2, 3.6 and 6.1  $\mu\text{g}/\text{cm}^2$  calculated using J774.1, HaCaT and C3A cell lines, respectively. Cytotoxic effects were dictated by the amount of free or displaced organic modifier present in the exposure suspensions. However, the parent bentonite clay also caused distinct cytotoxic effects in J774.1 macrophage-like cells with associated TNF- $\alpha$  release. Such information on the hazard profile of organoclays, can feed into risk assessments for these materials.

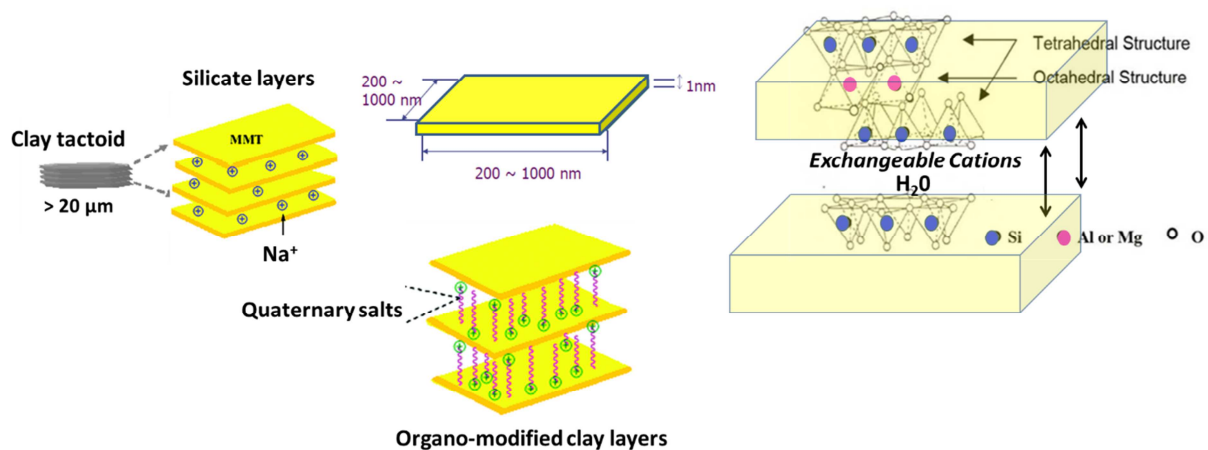
**Keywords;** bentonite clays; organic modification; nanofillers; hazard assessment; cytotoxicity; *in vitro*

## 1. Introduction

Clays are found ubiquitously in the environment, particularly in soils and sediments. Natural forms of clays have been used in the past as topical antibacterial agents and even as oral treatments for gastrointestinal disorders (Williams and Haydel 2010). More recently, advances in modern-day material engineering has led to the use of both natural and synthetic clays in a wide variety of industries and sectors (Patel et al. 2006). The ease of chemical modification of the clays, by using exchangeable cations and organic species, is leading to a new cohort of modified clays becoming commercially available. Modifications can be used to alter the physico-chemical properties of clays such as particle size, surface area, surface charge, porosity, as well as absorptive and cation exchange capacity for wider applications. Applications of modified clays now range from additives in commercial products to enhance rheological properties (e.g. inks, paints and cosmetics) to their use in enzyme immobilisation platforms and waste-water treatment technologies which capitalise on their high absorptive capacity (Kurzbaum et al. 2017). As a result, clays now fall into the category of high volume production materials that are used in thousands of tons in modern industry (25,000-51,000 tons globally) with future production volumes expected to increase (Future Markets 2015). At present, the greatest single use for clays is as reinforcing nanofillers in the polymer nanocomposite industry (22 % of targeted market) (Future Markets 2011). Clays incorporated into different polymeric matrices can be used to confer strength, enhance barrier properties and flame-retardant properties to the finished polymer-clay nanocomposite materials (Nguyen and Baird 2006, Powell and Beall 2006). The clays used are typically modified to improve compatibility with polymer matrices by replacing the native interlayer cations (e.g.  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ) of the clays with organic ions (e.g.  $\text{Al}^{3+}$ ,  $\text{PH}^{4+}$ ,  $\text{NH}^{4+}$ ). A range of different alkylammonium bromides or chlorides, sulfonium, or phosphonium surfactants can be used in the organic modification process, and typically organoclays contain between 4-40% by weight of organic modifier (Southern Clay Products, USA). There is a very

large market for organoclay-reinforced materials in automotive and aerospace parts as well as in food packaging applications (Jorda Beneyto et al. 2014). The organoclays assessed in this study are being specifically investigated for their potential use in the production of biodegradable poly-lactic acid (PLA)-clay based nanocomposite packaging materials for the cosmetic industry.

Clays have a large variability in composition, exchangeable ions, accessory mineral constituents and transition metals (e.g. Fe, Mg, Ca) are present in small and varying amounts according to origin and purity. They can be defined loosely as hydrous silicates, classified based on geometry (e.g. sheet like, tubular or fibrous) and structure (layers), and distinguished based on the arrangement of structural units (octahedral and tetrahedral sheets) (Uddin 2008). The sodium bentonite clays investigated in this study are hydrous aluminosilicate layered clays formed from the devitrification of volcanic ash. They have sodium as the predominant exchange cation and they are most commonly used in polymer nanocomposites due to their excellent plasticity, good mechanical and chemical resistance and high ion exchange capacity. The main component of the sodium bentonite clay is the mineral montmorillonite. Its basic structural unit is composed of several high-aspect ratio sheets (~1nm thick and between 200-300 nm long) (two silica sheets and one alumina sheet) arranged in layers (2:1 type) held together by weak Van der Waals and electrostatic forces (figure 1). These platelet- type aluminium silicate clays have a very large specific surface area (600-800 m<sup>2</sup>/g) and high aspect ratio (100-150:1); characteristics that can confer toxicity (Fubini et al. 2011; Verma et al. 2012). Furthermore, bentonite clays can contain a fraction of quartz, a substance known to be carcinogenic and cause fibrosis upon inhalation (Perry 1959; Donaldson and Borm 1998).



**Figure 1** Diagrammatic representation of the structure of the sodium bentonite parent clay (N116) used in this study and the interlayer organic modification process through exchange of ions.

As well as their small size, large surface area and high aspect ratio, clays have high dustiness; a property that increases the likelihood for exposure, particularly via inhalation. The production and processing of large quantities of dust-like clays with a respirable fraction ( $<4 \mu\text{m}$ ) during nanocomposite manufacturing will result in potential lung exposure. In addition, skin contact in an occupational (e.g. at the polymer production site) setting is likely. Information on occupational exposure to clays during extraction, processing and application to polymer composites is limited. However, the presence of airborne clays through handling tasks performed during compounding of clays to formulate nanocomposites has been identified (Tsai et al. 2012). Concentrations of clay particles  $<560 \text{ nm}$  measured, ranged from  $12,390 \text{ particles/cm}^3$  -  $130,980 \text{ particles/cm}^3$ . The upper values are well above the proposed nano reference value or exposure limit value for clays ( $40,000 \text{ particles/cm}^3$ ) set as a precautionary approach to control workplace concentrations of nanomaterials (van Broekhuizen and Dorbeck-Jung 2013). When used in a nanocomposite packaging, the clays will be embedded in the polymer matrix. Therefore, from a consumer perspective, the clays will only pose a potential risk following migration from the packaging (e.g. into the cosmetic). Accordingly, migration of the clays from the packaging may lead to exposure of the; skin, gastrointestinal tract (via hand to mouth contact) or secondary target sites (e.g. the liver, following translocation into the circulation) ((Wu et al. 2009; Schleh et al. 2012). The potential for

nanomaterials to migrate from packaging materials into food has been reported previously (Echegoyen et al. 2016). However, exposure concentrations for consumers are likely to be orders of magnitude lower than would be experienced by workers handling clays in a manufacturing setting.

Despite this, investigations into the toxic potential of clays are limited. Although they have been recognised by the OECD as a group of manufactured nanomaterials with high priority for testing, studies on their impact on human health are currently limited (OECD 2015). Early reports of cytotoxic effects of aluminium silicate clays (bentonite and montmorillonite) towards certain cell types (e.g. human umbilical vein endothelial cells and primary murine spinal cord neurons) were made by Murphy et al. (1993a, b). Subsequent studies also have evidenced cytotoxic effects associated with exposure of lung cells (A549 cells; Verma et al. 2012), and liver cells (HepG2 cells; Loordan et al. 2011; Baek and Lee 2012) *in vitro* as well as recent evidence of acute liver injury *in vivo* in mice following administration via the tail vein (Isoda et al. 2017). In fact, in a comparative study clays were ranked as the most cytotoxic compared to other commonly used nanofillers (e.g. silica, silver, ZnO and calcium carbonate) (NanoSafepack 2012; Nones et al. 2015). However, the lack of concurrent data on clay toxicity makes it challenging to reach a definitive conclusion regarding their hazard potential. This leads to uncertainties surrounding the risk they pose to human health (Maisanaba et al. 2015). Accordingly, it is prudent to compare the toxicity of clays to different cell types *in vitro* in one study.

Clays are commonly modified to improve their functionality in products. However, very little consideration has been given to what effects modification may have on the physico-chemical properties and/or toxicity of organoclays in the context of a safety assessment. The organic modifiers hexadecyl trimethyl ammonium bromide (HDTA) and octadecyl trimethyl ammonium chloride (TMSA), were used in this study to make the clays more compatible with the polymer matrix of a novel cosmetic packaging being developed. Both modifiers belong to a group of quaternary ammonium compounds (QACs) commonly used in cation exchange reactions to modify clays for



polymer-clay nanocomposites. While permitted for use as additives in personal care products (European Commission 2009), certain QACs have been shown to stimulate cytotoxic effects *in vitro* (Madine-Darby canine kidney (MDCK) II cells; Inacio et al. 2013), impair reproductive health in mice (Melin et al. 2014) and cause skin and respiratory irritation *in vivo* at higher concentrations (Lin and Hemming 1996; Larsen et al. 2012). Adverse effects are thought to be related to the alkyl chain length, cationic nature and structure of the specific QAC (Rhein et al. 1986, Lin and Hemming 1996). Toxicological data for the QACs used in this study (HDTA and TMSA) are lacking. However, the potential role of organic modifiers in the toxicity of organoclays has been eluded to in previous studies (Janer et al. 2014; Sharma et al. 2010; Sharma et al. 2014). Presently, organically-modified clays have been designated with individual chemical abstracts service (CAS) numbers. However, they are exempt from registration under REACH, as despite their surface modification the structure of the clay mineral does not change and therefore it is not considered a new chemical entity. Instead registration of the organic modifier itself will suffice (Article 3(39), Article 3(40) and Annex V (7) of REACH (European Commission 2006)). However, it is essential to investigate if exposure to both the clay and organic modifier will result in an increased hazard. Such information will feed into the regulation of organoclays to ensure their safe use and can be used to inform the safe design of future generations of organoclays.

The main aim of this study was to provide information on the potential cytotoxic and inflammatory I hazards (from occupational and consumer perspectives) of organoclays for their safe use as nanofillers in polymer-clay nanocomposite packaging materials. Any potential adverse effects associated with exposure to workers and end users was assessed using relevant and representative *in vitro* cellular models for target organs (skin, liver, lungs), namely HaCaT human keratinocytes, C3A liver cells and J774.1 macrophage-like cells. These cells were selected as keratinocytes are the main cell type of the human dermis and epidermis forming the skin barrier, macrophages play a key role in the clearance of inhaled nanomaterials (Geiser et al. 2008), and nanomaterials have been demonstrated to accumulate primarily in the liver following exposure via several routes (e.g. Choi et

al. 2010; Wang et al. 2007). The use of *in vitro* systems improves the alignment of nanotoxicology studies with the 3Rs principles (Replacement, Reduction and Refinement), in order to reduce the reliance placed on animal testing (Burden et al. 2017; Johnston et al. 2018) and allows a quick and cheap screen of organoclay toxicity to be performed. A robust (3-in-1) cytotoxicity screening assay system (Connolly et al. 2015) was applied to compare the toxicity of the parent and modified clays in order to rank their potency and to compare the sensitivity of the different cell types being investigated. Both unmodified (N116) and organically modified clays (N116\_HDTA and N116\_TMSA) were included, as well as an assessment of the cytotoxic potential of the organic modifiers alone to elucidate the contribution of organic modifier to any adverse effects observed. The ability of the clays to elicit inflammatory responses (TNF- $\alpha$ ) in J774.1 macrophage-like cells was assessed and studies on clay and J774.1 cellular interaction were performed using microscopy.

## **2. Materials and methods**

### **2.1. Clay modification**

A commercially available natural sodium bentonite clay (Nanofil®116 (N116) (CAS no: 1302-78-9)) was purchased from BYK Additives & Instruments, Wesel, Germany. The manufacturers reported clay particle size was between 1-5  $\mu\text{m}$  and it had a high cation exchange capacity (CEC): 116 mEq/100g (BYK Additives & Instruments, 2016). The organic modifiers hexadecyl trimethyl ammonium bromide (HDTA) (CAS no. 57-09-0) and octadecyl trimethyl ammonium chloride (TMSA) (CAS no. 112-03-8) were supplied by CymitQuimica S.L., Barcelona, Spain. The clay was organically modified using a cationic exchange reaction, whereby sodium cations present in the clay were exchanged for ammonium cations of the respective organic modifiers.. The final amount intercalated according to thermogravimetric analysis (TGA) results were: HDTA (27.3 g/100 g clay) and TMSA (29.5 g/100 g clay). The organically modified clays (organoclays) were termed N116\_HDTA and N116\_TMSA respectively throughout this study, according to the modifier used.

### **2.2. Organoclay characterisation**

The organoclays were characterised using thermogravimetric analysis (TGA), Fourier transform infrared (FTIR) spectrophotometry and X-ray diffraction (XRD). FTIR spectrophotometry was used to determine the surface structure of the clay minerals and modifiers and to qualitatively determine the presence of the organic modifiers in the organoclays. A Tensor 27 model Infrared spectrophotometer with an attenuated total reflectance (ATR) module, with monolithic diamond crystal for full spectral range from 4000 - 500  $\text{cm}^{-1}$ , was used (Bruker, Bremen, Germany).

A wide-angle X-ray powder Diffractometer (D8 Advance A25 (Bruker, Bremen, Germany)) was used to measure the distance between the individual layers of the clay when the organic modifiers are intercalated between them. The interlayer spacing (D001) was calculated from the XRD spectrum according to the  $2\theta$  angles corresponding to the diffraction peaks of the organoclay by the Bragg's Law, whose formula is:

$$n\lambda = 2d \sin\theta$$

Where,  $n$  is the order of diffraction,  $\lambda$  is the wave length,  $\theta$  is the angle of diffraction and  $d$ - inter planar distance. According to Bragg's law, the  $d$ -spacing is inversely proportional to the scattering angle. Therefore, any shifts of the peaks to lower scattering angles corresponds to larger  $d$ -spacing and hence larger distances between the silicate layers will be found.

Thermogravimetric analysis (TGA) was performed on clay powders using a TA Q5000IR thermobalance (TA instruments, Newcastle, USA) to quantify the total amount of modifier in each organoclay. A total of 10.0 mg of sample (clay powders) was heated from 25 to 900°C at a heating rate of 20°C per min in a nitrogen atmosphere.

### **2.3. Preparation and Characterisation of clay suspensions**

Suspensions of organoclays (N116\_HDTA, N116\_TMSA) and the parent sodium bentonite clay (N116) were prepared according to the ENPRA protocol which was developed for preparation of dispersions of engineered nanomaterials for toxicity testing (Jacobsen, 2010). According to this protocol

nanomaterials are coated with serum prior to dispersion in culture medium. This has been shown to reduce clay/medium interactions (e.g. ion exchange reactions) in *in vitro* cell culture testing platforms (Katic et al. 2006) and thus was considered an appropriate methodology. Aqueous dispersions of clays (1 mg/mL) were first prepared in 2% (v/v) bovine serum albumin (BSA) (in water) and then sonicated (water bath sonication, 400 W power (VWR, Leicestershire, UK) for 16 min. Clay dispersions were then diluted immediately, in cell culture medium (37°C) according to the requirements of the different cell types. Test suspension concentrations ranged from 0.3-100 µg/mL (equivalent to 0.24-31.25 µg/cm<sup>2</sup>).

Dynamic light scattering (DLS) was used to characterise the hydrodynamic size distribution of the clays in cell culture medium, at the highest test suspension concentration (100 µg/ml) and to monitor if the dispersion was stable over the exposure period (T0-T24 h) when incubated at 37°C/5% CO<sub>2</sub>. A Zetasizer Nano-ZS (Malvern Panalytical, Malvern, UK) was used to take measurements directly after preparation and after 24 h. Test suspensions were agitated/resuspended prior to taking measurements. Z-average values and polydispersity indices (PDI) (mean ± standard deviation) have been used as a measure of the average size and width of the particle size distribution in test suspensions (Zetasizer Software version 6.34 (Malvern Panalytical, Malvern, UK)).

Suspensions of each clay (in cell culture medium; 100 µg/mL) were also visualised using an inverted light microscope (Axiovert 40C (Carl Zeiss, Cambridge, UK)) equipped with a Canon EOS 1000D (Canon, Surrey, UK). The clay suspensions were also deposited onto copper grids and viewed in a JEOL JEM-1400 Plus transmission electron microscope (JOEL Inc., Peabody, MA USA) operating at an accelerating voltage of 200 kV. Micrographs were taken using a GATAN OneView camera (GATAN, Pleasanton, CA, USA).

#### **2.4. Cell culture and maintenance**

The murine macrophage-like cell line, J774.1, the human keratinocyte skin cell line, HaCaT, and the human hepatocellular liver carcinoma cell line, C3A, were all used. J774.1 cells (ECACC, Porton

Down, UK) were cultured in RPMI 1640 medium (Thermo Fischer Scientific, Cramlington, UK) supplemented with 1 mM sodium pyruvate and 1% non-essential amino acids. C3A cells (ATCC; Manassas, VA, USA) were cultured in minimum essential medium supplemented with L-Alanyl-Glutamine (MEM, GlutaMAX™ (Sigma Aldrich, Dorset UK)). HaCaT cells (CLS cell lines service, Eppelheim, Germany) were cultured in dulbecco's modified eagle's medium (DMEM) with a high glucose content (4.5 g/L) (Thermo Fisher Scientific, Cramlington, UK) supplemented with 2mM L-glutamine.

All media were supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Thermo Fisher Scientific, Cramlington, UK, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco, USA) and cells were maintained in 75 cm<sup>2</sup> culture flasks (Corning, USA) at 37°C with 5% CO<sub>2</sub>, 95% air in a humidified incubator.

## 2.5. Clay exposures

Cells were seeded into 96 well plates at a density of 5 x 10<sup>5</sup> cells/mL, 4 x 10<sup>5</sup> cells/ml and 2.5 x 10<sup>5</sup> cells/mL (100ul/well) for J774.1, C3A, and the HaCaT cells, respectively and incubated at 37°C/ 5% CO<sub>2</sub> for 24 h. Cells were then exposed (in triplicate) to the clays (N116 clay and modified N116\_HDTA and N116\_TMSA organoclays) at a concentration range of 0.3-100 µg/ml (100 µl/well) (equivalent to 0.24 to 31.25 µg/cm<sup>2</sup>) for 24 h. Cells were also exposed to the organic modifier HDTA and TMSA (0.049-12.5 µg/mL (100ul/well))(0.015-3.9 µg/cm<sup>2</sup>). The modifiers were first solubilised in ethanol (20 mg/mL) and subsequently diluted in cell culture medium. Cells exposed to cell culture medium only served as negative controls. 5 % ethanol was used as a positive control. Assays showing cellular viability of between 15-20 % following 24 h exposure to the positive control were considered valid. Vehicle controls (0.2% v/v ethanol and 0.2% (v/v) aqueous BSA solution were also included.

The concentration range was selected by taking into consideration the potential levels of exposure from use of a cosmetic cream (30 mL) that has been packaged using a polymer-nanocomposite packaging (4 % w/w clay). The concentration range incorporates a worst-case and a theoretical low

exposure scenario in which all (100%) or a small fraction (10%) of organoclays present in a polymer-nanocomposite packaging is released into a cosmetic formulation and a subsequent single application ( $2\text{mg}/\text{cm}^2$ ) to the skin. The theoretical worst case and low exposure scenario levels to organoclays are 8 and  $0.8\ \mu\text{g}/\text{cm}^2$ , respectively (Additional file 1, figure 1). An exposure scenario has also been performed for the organic modifiers only with worst case scenario concentrations of 2.2 or  $2\ \mu\text{g}/\text{cm}^2$  and lower concentrations 0.19 and  $0.17\ \mu\text{g}/\text{cm}^2$  for HDTA and TMSA, respectively (Additional file 1, figure 2).

## 2.6. Cytotoxicity Assessment

A 3 in 1 fluorometric-based assay system was used to assess cytotoxicity following exposure of the skin, liver and immune cells to test suspensions. This system facilitates the application of three different assay reagents (namely alamarBlue® (AB), a 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) probe and a neutral red (NR) dye) simultaneously to the same set of cells to monitor different endpoints of toxicity (loss in metabolic activity, plasma membrane disruption, and/or lysosomal dysfunction, respectively) (Dayeh et al. 2005), and it has been adapted for use with mammalian cell lines (Lammel et al. 2013). Using the same methodology as has previously been outlined in Connolly et al. (2015) the cytotoxicity of the organic modifiers HDTA and TMSA, N116 parent clay and organoclays N116\_HDTA and N116\_TMSA were assessed. As well as providing mechanistic information such a testing platform increases reliability by identifying any assay specific interactions leading to false positive results. Any interferences of clays/organoclays with specific assays reagents was fully assessed.

## 2.7. Assessment of contribution of organic modifier to cytotoxicity

In the N116\_HDTA and N116\_TMSA organoclays tested there is a proportion of modifier exchanged and a proportion that is free (not exchanged) that equates to the total amount of modifier used in the cation exchange process. To investigate the contribution of organic modifier to cytotoxicity and the extent to which the free and exchanged modifier contributes to the effect, dose response curves

obtained for the known amount of modifier used (total) and not exchanged (free) in the modification process were compared with those obtained for modified clays.

### **2.8. Cytokine Analysis (J774.1 cells)**

Samples for cytokine analysis were taken from supernatants which were generated in cytotoxicity tests from immune cells which were exposed for 24 h to clays and organoclays and frozen at  $-80^{\circ}\text{C}$ . Supernatants from cells exposed to sublethal concentrations ( $3.91\ \mu\text{g}/\text{cm}^2$ ) of N116 and organoclays were analysed for levels of the pro-inflammatory cytokine, TNF- $\alpha$ . TNF- $\alpha$  levels were measured by ELISA according to the manufacturer's instructions (DuoSet<sup>®</sup> ELISA, R&D Systems Inc. Minneapolis, MN, USA). Culture medium from cells exposed to lipopolysaccharide (LPS) ( $10\ \text{ng}/\text{mL}$ ) was included as a positive control. Samples were analysed in duplicate from three independent experiments.

### **2.9. Clay-cellular interaction and uptake (J774.1 cells)**

Clay-cellular interaction was investigated through cytological staining techniques and cellular ultrastructural analysis. A romanowsky-type stain variant (Diff-Quik<sup>®</sup> Stain Kit (TCS Bioscience Ltd, Buckingham, UK)) was used for cellular nuclear and cytoplasmic staining of the J774.1 immune cells following exposure to clays to monitor the physical state of the cells and to differentiate or identify specific clay-cellular interactions. Cells were seeded on 10 mm glass coverslips (Nunc<sup>™</sup> Thermanox<sup>™</sup> plastic, Thermo Fisher Scientific, Cramlington, UK) at a density of  $1.5 \times 10^6$  cells/mL in 24 well plates. Following 30 min, 2 h, 4 h and 24 h exposure to sublethal concentrations of clay ( $7.82\ \mu\text{g}/\text{cm}^2$ ) and a washing step (PBS,  $150\ \mu\text{l}$ ), coverslips were dipped into a fixative solution followed by staining with a thiazine methylene blue dye and red Eosin G dye in phosphate buffer for nuclear and cytoplasmic staining, respectively. Stained coverslips were mounted on slides and viewed using a OLYMPUS IX70 polarizing microscope (OLYMPUS, Southend-on-Sea, UK) equipped with a AxioCamERc 5s digital camera and filters for light polarization.

To investigate cellular uptake of clays, coverslips with J774.1 cells exposed to sublethal concentrations ( $3.91 \mu\text{g}/\text{cm}^2$ ) of N116 clays and N116\_HDTA/TMSA for 24 h were prepared for TEM analysis. Exposed cells were fixed (3% glutaraldehyde for 2 h in sodium cacodylate buffer (0.1 M, pH7.3, room temperature)), washed (sodium cacodylate buffer x3), post fixed (1% osmium tetroxide for 45 mins in sodium cacodylate buffer), dehydrated in increasing concentrations of ethanol (50%, 70%, 90% and 100% (15 minutes for each)), 10 minutes changes of propylene oxide. Processed samples were embedded in 812 resin (TAAB, Reading, UK) and ultrathin sections (600-1000 nm thick) were cut on a Leica Ultramicrotome (Leica Microsystems, Milton Keynes, UK). Sections were stained in uranyl acetate and lead citrate and then viewed in a JEOL JEM-1400 Plus TEM and micrographs taken using a GATAN OneView camera (GATAN, Pleasanton, CA, USA) (Institute of Molecular Plant Sciences Electron Microscope facility, University of Edinburgh).

### **2.10. Statistical analysis**

Data points presented in dose response curves are mean values ( $\pm$  standard error of the mean (SEM)) of at least three independent experiments. Significant differences among treatments and controls were determined by one-way analysis of variance (ANOVA,  $p < 0.05$ ) followed by a Dunnett's Post hoc test using GraphPad Prism version 6 software. Dose response curves were generated using the same software and applying four-parameter nonlinear regression to calculate the concentration of test compound (i.e. clay, organoclay or organic modifier) that reduced cell viability by half (median inhibition concentration, IC<sub>50</sub> values).

## **3. Results**

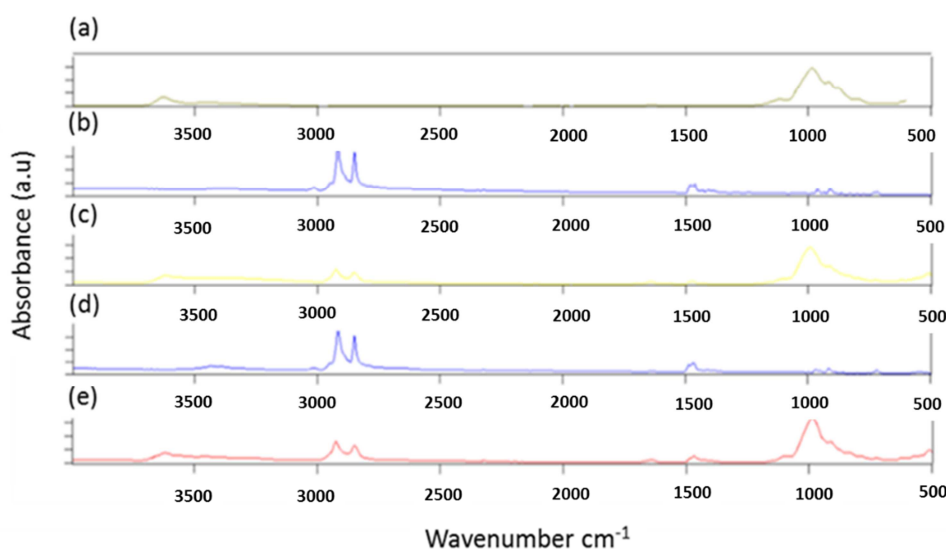
### **3.1. Clay organic modification**

#### **3.1.1. Fourier transform infrared (FT-IR) spectroscopy characterisation**

FTIR was used to confirm the presence of the HDTA and TMSA organic modifiers between and/or surrounding the clay layers. The attenuated total reflection (ATR) spectra of the unmodified clay



(N116), QAC organic modifiers HDTA and TMSA as well as the clays modified with HDTA (N116\_HDTA) and TMSA (N116\_TMSA) are presented in figure 2. The spectra of the clay shows a peak around  $1200\text{ cm}^{-1}$ , which corresponds to Si-O absorptions and OH bands that are typical of clay minerals (figure 2 (a)). Inner hydroxyl groups, lying between the tetrahedral and octahedral sheets, give the absorption peaks near  $3620\text{ cm}^{-1}$  which are characteristic of bentonite clays (Ikhtiyarova et al. 2012). In the spectra of both the organic modifiers, stretching vibrations of the C-H bonds of the alkyl chain occur in the  $2800 - 2950\text{ cm}^{-1}$  region as well as a characteristic C-H bending vibration of the  $(\text{CH}_3)_4\text{N}^+$  cation at  $1487\text{ cm}^{-1}$  (figure (b) and (d)). The presence of both these peaks in the spectra of the organoclays confirms the presence of the modifiers in the organoclays and a successful modification process (figure 2 (c) and (e)). These peaks are absent in unmodified clays (figure 2(a)).



**Figure 2.** ATR-FTIR spectra of nanoclays, organic modifiers and organoclays: (a) the sodium bentonite nanoclay, (b) organic modifier HDTA, (c) N116\_HDTA organoclay, (d) organic modifier TMSA, (e) N116\_TMSA organoclay.

### 3.1.2. Thermogravimetric Analysis (TGA) and X-ray diffraction (XRD)

Following the modification process, it is expected that not all the modifier is incorporated into the structure of the clay (i.e. it does not intercalate). TGA was performed to calculate the percentage of intercalated or exchanged and non-intercalated free modifier in the organoclay preparations.

Thermogravimetric curves confirming the onset temperatures of degradation for organic modifiers were used to calculate the fraction of the weight loss coming from the non-intercalated (free) modifier and exchanged modifier in organoclay preparations. TGA curves are presented in additional file 2, showing the fraction of the weight loss that comes from the modifier non-intercalated and exchanged modifier that is decomposed at higher temperatures (upto 500°C). These values are presented as percentages of free and exchanged modifier in Table 1. Briefly, there is a similar level of incorporation of each modifier into the organoclay; 19.1% for HDTA, and 21.3% for TMSA (Table 1).

Any increases in the interlayer distance ( $d_{001}$ ) of the nanoclays were also calculated from X Ray diffractograms of the clays modified with HDTA and TMSA (as detailed in methods section) (diffractograms presented in Additional file 3, figure 1 and 2). The organic modification increased the interlayer distance of unmodified clays from 10.39 Å to 18.78 Å and 21.53 Å for HDTA and TMSA modified clays, respectively, suggesting that the modifiers were successfully integrated between the layers of the clay.

**Table 1** Content of organic modifiers in the organoclays and interlayer distances.

	<i>% Free</i>	<i>% exchanged</i>	<i>d<sub>001</sub> (Å)</i>
<b>N116_1HDTA</b>	8.2	19.1	18.78
<b>N116_1TMSA</b>	8.2	21.3	21.53

### 3.2. Physico-chemical characterisation of clay and organoclay suspensions

Test suspensions of clays and organoclays were characterised prior to performing *in vitro* studies. According to the ENPRA protocol developed for dispersing nanomaterials, the clays were first suspended in 2% fetal calf serum. Bath sonication (16 minutes) was used to improve dispersion and thereafter test suspensions were prepared in relevant cell culture medium (Jacobsen et al. 2010). A

detailed physico-chemical characterisation was performed on these test suspensions to gain information on the size and distribution of clay particles being tested under exposure conditions (24 h at 37°C / 5% CO<sub>2</sub> atmosphere).

The hydrodynamic diameter and stability of clay dispersions in test suspensions was analysed using dynamic light scattering. Measurements were taken directly after preparation (T0 h) and 24 h post incubation at 37 °C. Z-average values are presented for comparative purposes showing the mean cumulative particle size for all clay suspensions. At T0 h unmodified N116 clays suspensions had a hydrodynamic diameter of 2681±133 nm. The N116\_HDTA and N116\_TMSA organoclay suspensions both had very similar, but larger mean particles sizes (5115±223 and 5516±216 nm, respectively) at this timepoint. After 24 h there was no change in overall mean size distribution for any of the clay suspensions (Table 2). The polydispersity index (Pdl) measured indicated a non-uniform distribution of clay particles with a wide variety of sizes present in all suspensions (PDI range; 0.267-0.662). The largest PDI value was measured for N116\_TMSA organoclay suspensions following 24 h incubation.

There was no obvious difference in distribution between the distinct media (MEM GlutaMAX™, RPMI 1640, MEM high glucose) used to prepare suspensions for exposures to different cell types (data not shown).

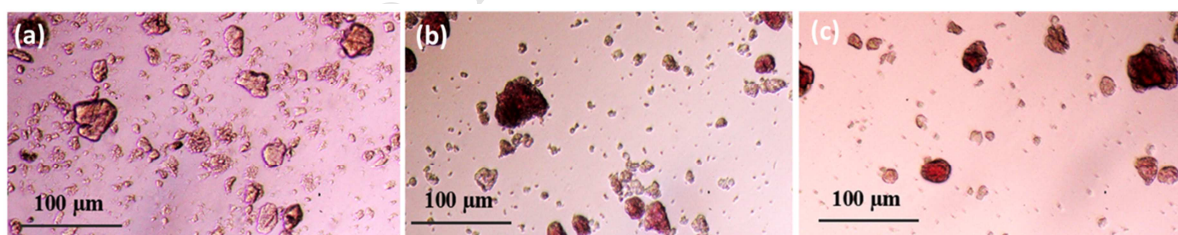
**Table 2.** Hydrodynamic diameter of unmodified (N116) and modified (N116\_HDTA or N116\_TMSA) clay particles in complete cell culture medium (100 µg/mL immediately after preparation (T0 h) and 24 h post incubation at 37 °C.

	<i>Pdl</i>	<i>Z-Av (d.nm ± sd) T0h</i>
		<i>T24 h</i>
<b>N116</b>	0.267	2681 ± 133
	0.304	3276 ± 177
<b>N116_1HDTA</b>	0.293	5515 ± 223
	0.402	5458 ± 274

<b>N116_1TMSA</b>	0.475	5516 ± 216
	0.662	4611 ± 204

Hydrodynamic size is represented as mean diameter ( $d.$ ) ± standard deviation ( $sd$ ) in nm and calculated from the average of three independent measurements. [%] represents the relative intensities of the different peaks. Pdl, polydispersity index; Z-Av, zeta-average.

Light microscopy images of modified and unmodified clay suspensions prepared in complete culture medium also revealed a heterogeneous distribution of different sized clay particles (figure 3). The presence of large clay particles  $\leq 45 \mu\text{m}$  as well as smaller particles  $\leq 1 \mu\text{m}$  was evident in both N116 clay (figure 3 (a)) and organoclay suspensions (figure 3 (b) and (c)). Interestingly in the culture medium environment the organoclay appear red in colour and there is an obvious change in colour of the medium, suggesting that components of the medium may adsorb onto the clay surface (figure 3 (b) and (c)).



**Figure 3.** Light microscope images of clay N116 (a), organoclay N116\_HDTA (b), and organoclay N116\_TMSA (c) suspended in complete culture medium at a concentration of  $100 \mu\text{g/mL}$ , immediately after preparation.

### 3.3. Hazard Assessment

Interference checks were performed for each of the cytotoxicity assays (alamarBlue®, CFDA-AM and Neutral red uptake) prior to performing hazard assessments. A strong adsorption of the neutral red dye reagent to the organoclays and parent clay was observed (Additional file 4, figure 1 and 2). This led to a major interference of the clays with this assay and thus it was omitted from the cytotoxicity screen. No interference was detected with assay reagents or conversion products of the other two assays used (data not shown).

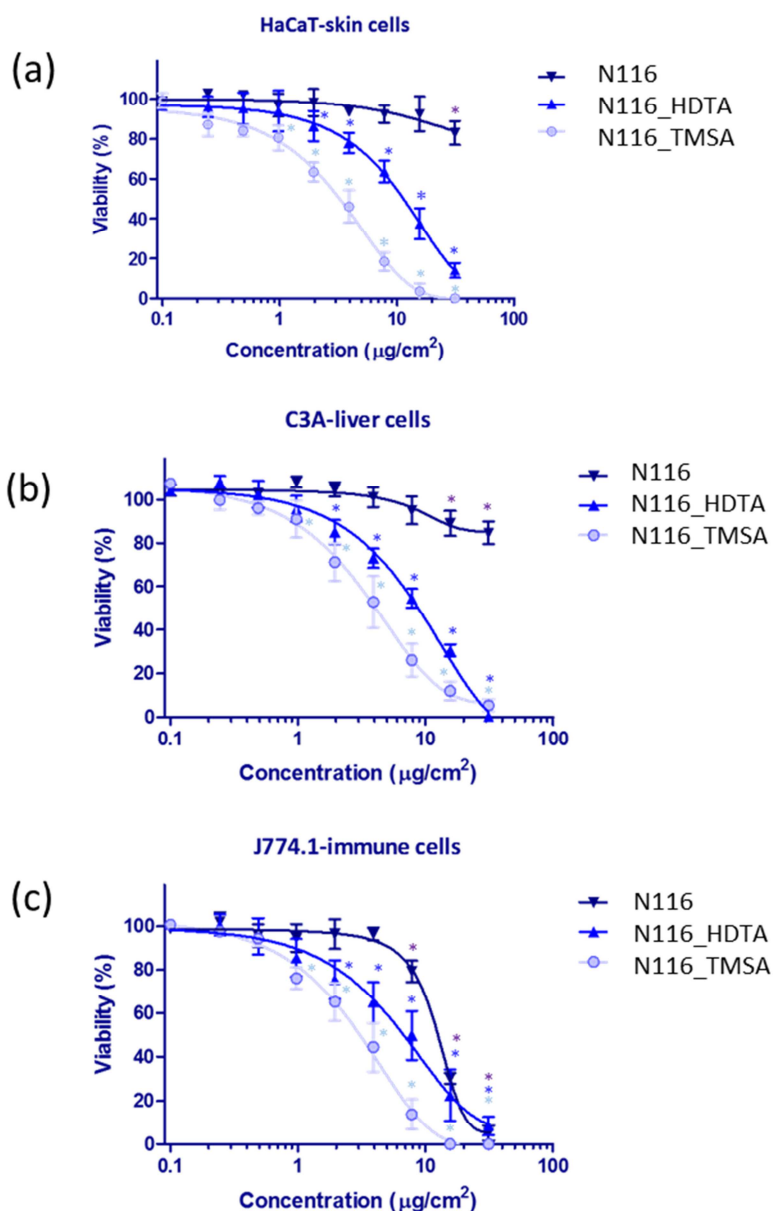
### 3.4. Cytotoxicity of unmodified clay and organoclays

Figure 4 shows the viability of HaCaT skin cells (a), C3A liver cells (b) and J774.1A immune cells (c) following exposure to the parent clay N116 and organoclays (N116\_TMSA and N116\_HDTA). Data obtained from the alamarBlue® assay are presented (figure 4) as it proved the most sensitive system for detecting cytotoxic effects of the clays, however similar trends were measured using the CFDA-AM assay (data not shown). Both organoclays caused a concentration-dependent reduction in viability in the three different cell types, in both assays. The N116\_TMSA organoclay was most cytotoxic to all cell types, causing a significant ( $p < 0.05$ ) reduction in cell viability at concentrations  $\geq 0.98 \mu\text{g}/\text{cm}^2$ , whilst N116\_HDTA significantly reduced cell viability at concentrations  $\geq 1.95 \mu\text{g}/\text{cm}^2$ . In contrast the parent (unmodified) N116 clay only elicited a concentration dependent decrease in cell viability in J774.1 macrophage-like cells, with a significant reduction of cell viability observed at concentrations  $\geq 7.81 \mu\text{g}/\text{cm}^2$ . In the skin and liver cells, viability remained above 80% following exposure to the highest concentration of the parent clay tested ( $31.25 \mu\text{g}/\text{cm}^2$ ) (figure 4 (a) and (b)).

The IC50 values calculated from the dose response curves of the alamarBlue® and CFDA-AM assay systems for unmodified and modified clays in each cell type are presented in Table 3, along with IC50 values calculated from exposures of cells to the modifiers alone (HDTA and TMSA). Due to the low levels of reduced viability measured in both assay systems IC50 values could not be calculated following exposure of HaCaT and C3A cells to the unmodified N116 parent clay. The IC50 values

calculated following exposure of J774.1 cells to unmodified N116 were 24.3 and 91.3  $\mu\text{g}/\text{cm}^2$  according to the alamarBlue® and CFDA-AM assays. The IC50 values calculated for N116\_HDTA and N116\_TMSA organoclays ranged from 8.3 – 93  $\mu\text{g}/\text{cm}^2$  and 3.2– 19.8  $\mu\text{g}/\text{cm}^2$  respectively, according to the cell type and assay system applied. Using the IC50 values for all cell types the clays can be ranked N116\_TMSA>N116\_HDTA>N116 in hazard potential. Lower IC50 values were calculated using the alamarBlue® assay in all cell types indicating its increased sensitivity, compared to the CFDA-AM assay. J774.1 cells were the most sensitive cell type with the lowest IC50 values calculated for all test substances will cell sensitivity ranked: J774.1>HaCaT>C3A.

The IC50 values calculated following exposures with organic modifiers only were lower, ranging from 0.46-18.75  $\mu\text{g}/\text{cm}^2$  and 0.47-16.9  $\mu\text{g}/\text{cm}^2$  for HDTA and TMSA respectively, again depending on the cell type and assay system applied. Similar IC50 values calculated for HDTA and TMSA indicate that the organic modifiers have a similar hazard potential however a distinct cell-type sensitivity order can be made as follows HaCaT> J774.1>C3A.



**Figure 4.** Levels of viability in HaCaT skin cells (a), C3A liver cells (b) and J774.1 macrophage-like cells (c) following 24 h exposure to a range of concentration (0.12- 31.25  $\mu\text{g}/\text{cm}^2$ ) of clay N116 and organoclays (N116\_HDTA, N116\_TMSA) (expressed as average % untreated control cells  $\pm$  SEM and measured according to the alamarBlue<sup>®</sup> assay). Significance differences compared to control untreated cells are represented by \*  $p \leq 0.05$  ( $n=3$ ).

**Table 3.** Comparison of IC50 values (in  $\mu\text{g}/\text{cm}^2$ ) calculated for modified and unmodified clays and the organic modifiers in HaCaT skin cells, C3A liver cells and J774.1A macrophage-like cells.

IC <sub>50</sub> [ $\mu\text{g}/\text{cm}^2$ ]	N116		N116_HDTA		N116_TMSA		HDTA		TMSA	
	AB	CFDA-AM	AB	CFDA-AM	AB	CFDA-AM	AB	CFDA-AM	AB	CFDA-AM
HaCaT	-	-	17.8	37.7	3.6	19.8	0.46	1.4	0.47	1.66
C3A	-	-	18.3	55.8	6.1	18.3	1.00	18.75	0.94	16.9
J774.1A	24.3	91.3	8.3	93	3.2	16.9	0.80	11.51	0.78	13.86

(-) Indicates that the IC<sub>50</sub> could not be calculated, AB, alamarBlue® assay, CFDA-AM, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) probe

### 3.5. Contribution of organic modifier to organoclays cytotoxicity

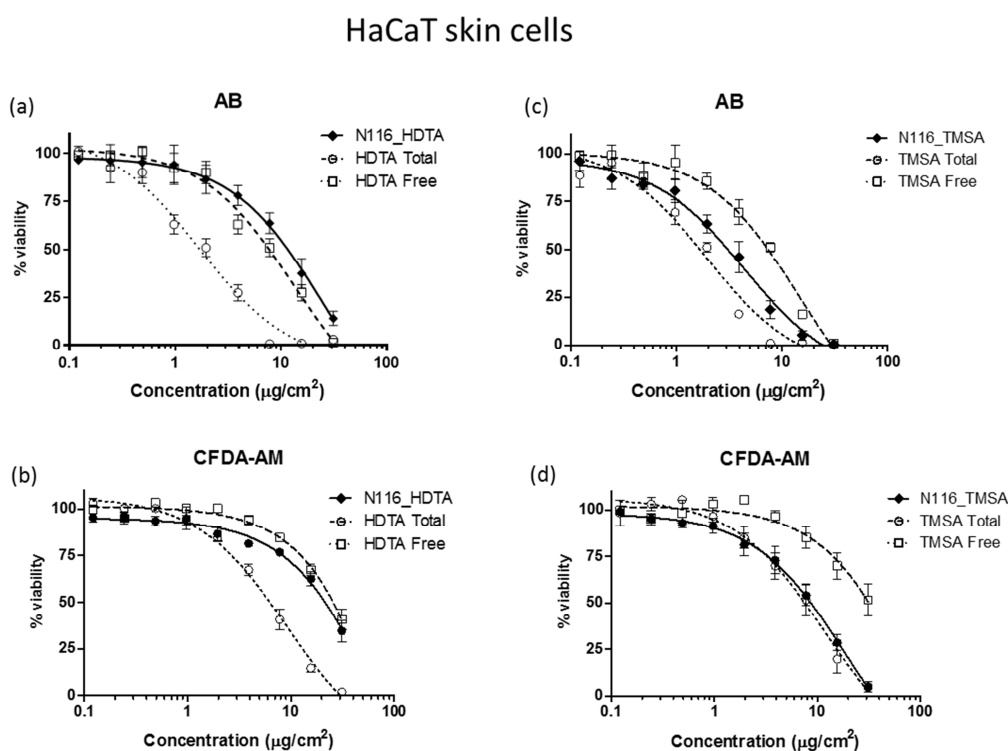
Based on knowledge about how much of the modifier was incorporated into the organoclay and remained free (from TGA and XRD analysis), and the known responses following exposure to modifier alone, the contribution of intercalated and/or free concentrations of modifier to organoclay cytotoxicity could be assessed. In figure 5, to identify the role of organic modifier in HaCaT cell cytotoxicity, concentration response curves for cell viability were compared to expected viability levels when the concentration of the modifier was expressed as total and free concentration.

The concentration response curve for N116\_HDTA organoclay corresponds to that of the free modifier for the HaCaT cells (figure 5). This is evident in both assay systems employed (figure 5 (a) and (b)) and suggests that the free modifier is responsible for the toxicity of N116\_HDTA toxicity. In contrast the N116\_TMSA concentration response curve lies between that generated for the total and free modifier dose response according to the alamarBlue® (figure 5(c)) and almost on top of the total modifier dose response curve according to the CFDA-AM assay (figure 5(d)). Therefore, for N116\_TMSA both the free and exchanged modifier (total) are likely to contribute to the toxicity observed to HaCaT skin cells.

Accordingly, despite similar levels of incorporation into the clay (according to TGA analysis) (27.3% and 29.5% (w/w) for N116\_HDTA and N116\_TMSA, respectively), as well as free modifier content (8.2 % (w/w)), there are different contributions from organic modifiers to the cytotoxicity of the organoclays.

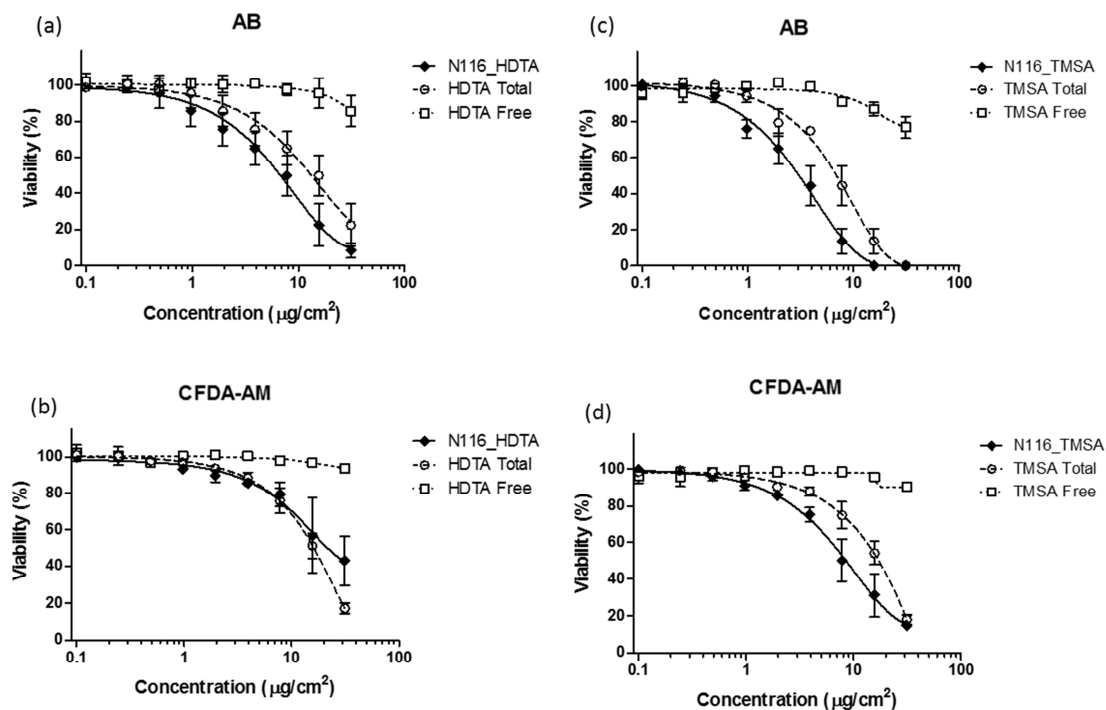


In figure 6 the contribution of organic modifier (total or free concentrations) to organoclay cytotoxicity to J774.1 cells was assessed. In contrast to the distinct contribution from free (N116\_HDTA) and total modifier (N116\_TMSA) seen in HaCaT cells (figure 5) dose response curves generated from organoclay exposure to J774.1 cells either lie directly on top of curves for total modifier contribution or show a steeper hill slope (figure 6 (b) and (a)(c)(d) respectively). This would indicate a unique contribution from both the clay and organic modifier in both N116\_HDTA and N116\_TMSA cytotoxicity in J774.1 cells and a possible additive or synergistic effect.



**Figure 5.** Comparing the contribution of total concentration of organic modifier and free (non-exchanged) modifier to N116\_HDTA (a)(b) and N116\_TMSA (c)(d) cytotoxicity to HaCaT skin cells. Bold solid lines denote the observed response from organoclay exposure ( $n=3$ ). Dashed lines represent the calculated effects (extrapolated from dose responses) from known concentration of total modifier (white circles) and only the free portion (non-exchanged) (white squares).

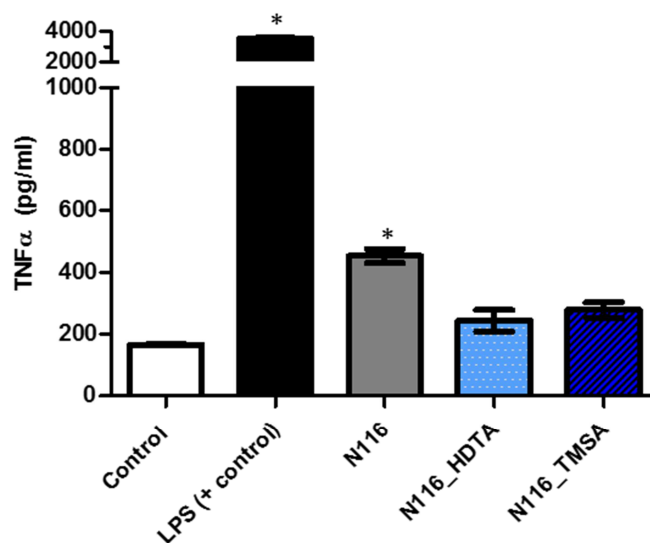
## J774.1 immune cells



**Figure 6.** Comparing the contribution of total concentration of organic modifier and free (non-exchanged) modifier to N116\_HDTA (a)(b) and N116\_TMSA (c)(d) cytotoxicity to J774.1 macrophage-like cells. Bold solid lines denote the observed response from organoclay exposure ( $n=3$ ). Dashed lines represent the calculated effects (extrapolated from dose responses) from known concentration of total modifier (white circles) and only the free portion (non-exchanged) (white squares).

### 3.6. TNF- $\alpha$ cytokine release from J774 cells

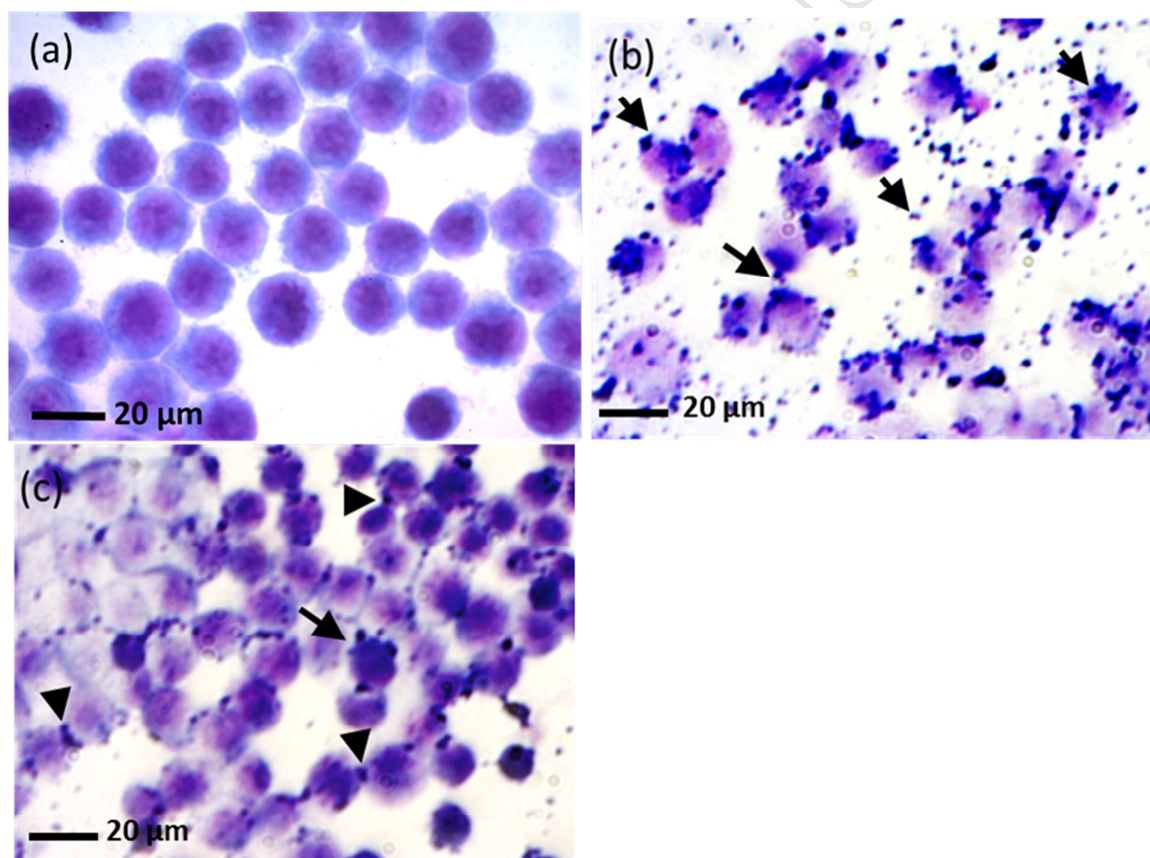
J774.1 cells were most sensitive to the toxicity of the parent and modified clays when cytotoxicity was used as an indicator of toxicity. To further investigate toxicity to these cells, the production of the pro-inflammatory cytokine TNF- $\alpha$  was assessed following exposure to sublethal concentrations of the clays (figure 7). Interestingly a significant ( $p < 0.05$ ) increase in TNF- $\alpha$  levels ( $454 \pm 13$  pg/mL) was measured in the supernatants of the parent N116 clay exposed cells, while no significant increase in TNF- $\alpha$  levels were detected in both N116\_HDTA and N116\_TMSA cell supernatants ( $241 \pm 20$  pg/mL and  $276 \pm 14$  pg/mL, respectively) compared to control untreated cells ( $164 \pm 2$  pg/mL).



**Figure 7.** Levels of tumor necrosis factor (TNF)-  $\alpha$  secretion from J774.1 cells following 24 h exposure to N116 clay and organoclays N116\_HDTA and N116\_TMSA ( $3.91 \mu\text{g}/\text{cm}^2$ ). Levels of production from cells exposed to cell culture medium (control) and exposed to lipopolysaccharide (LPS) (10 ng/ml) (+ control) are also shown. Data is expressed as mean  $\pm$  SEM ( $n=3$ ). Significance differences compared to control untreated cells are represented by \*  $p \leq 0.05$ .

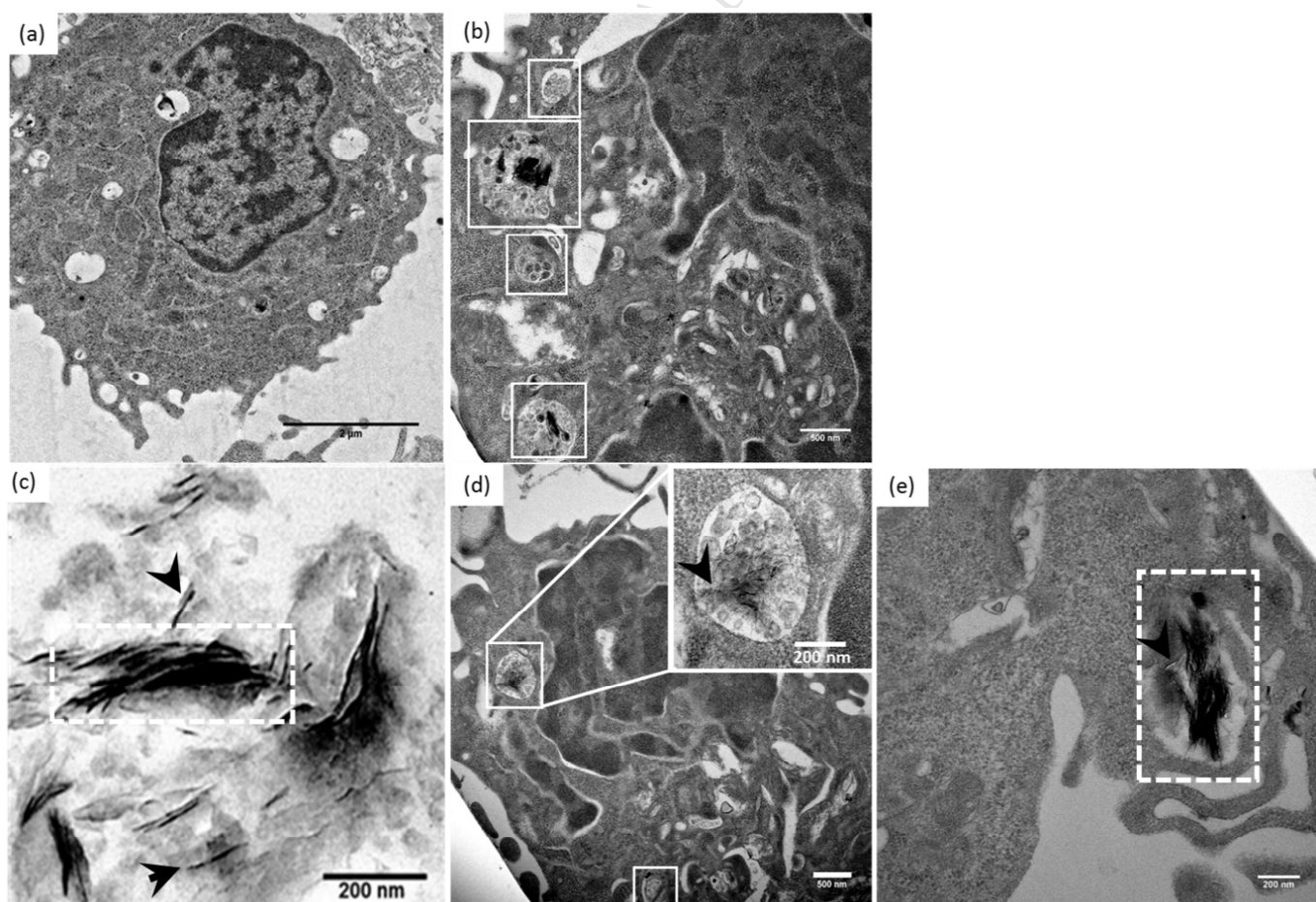
### 3.7. Clay-cellular interactions and uptake

The interaction of the clays with J774.1 cells was studied by light microscopy. Images of J774.1 cells stained using the commercial Diff-Quik® staining kit are presented in figure 8. For N116 exposed cells, the clays can be clearly identified and distinguished from cells as smaller dark spherical assemblages. A high level of clay-cellular interaction was seen even after 2 h exposure (figure 8 (b)). After 4 h clays are visible at the cell membrane and junctions between individual cells. Clays also appear to be internalised (figure 8 (c)). However, in order to confirm the uptake of clays by cells TEM was used as it is challenging to distinguish particles associated with the cell surface with those that are internalised using light microscopy.



**Figure 8.** Cytological examination of J774.1 macrophages unexposed (control cells) (a) and when exposed to N116 clays ( $7.82 \mu\text{g}/\text{cm}^2$ ) for 2 h (b) 4 h (c). Black arrows indicate stained clays and their interaction with cell surface.

Further investigations were performed using TEM analysis to positively identify uptake of clays by J774.1 cells. Figure 9 (a) and (b) compare the cellular ultrastructure of unexposed cells and cells exposed to N116\_HDTA organoclays, respectively. The presence of clay particles in multiple intracellular vesicles within the exposed cell cytoplasm is evident. A number of clay particles are present in each vesicle, with vesicles measuring from 500-1000 nm in diameter. Depending on the clay particles orientation they appear as sheets 100-200 nm in diameter or as thin fibres ~1 nm thick. A micrograph showing the clay particles sheet-like structure and the presence of individual high aspect ratio sheets is presented in figure 9c. Individual layers stacked to form clay particles can be viewed as well as single individual layers with high aspect ratio. Further micrographs of exposed J774.1 cells (figure 9 (d) and (e)) show the presence of both clay particles and individual clay sheets contained within membrane vesicles.



**Figure 9.** TEM micrographs of unexposed J774.1 cells (a), J774.1 cells exposed to organoclays (N116\_HDTA) (b), showing structure of clays under acellular conditions (N116\_HDTA) (c) and when present in intracellular vesicles of J774.1 cells (d and e). Solid white boxes were used to highlight the presence of clay particles in intracellular vesicles. Black arrows have been used to indicate individual clay sheets while larger layered clay particles are inside dashed white box. Inserts also show clay particles in vesicles at higher magnification.

#### 4. Discussion

It is important to consider both safety and functionality in the rational design of clays for application in consumer products. Organic modification of clays renders them compatible with polymer matrices, so they can be used as nanofillers in polymer-clay nanocomposite materials for the packaging industry. However, the organic modification may change the safety profile of the resulting organoclay. In this study two organoclays were produced (N116\_HDTA and N116\_TMSA) from a commercially available parent sodium bentonite clay by performing a cation exchange reaction with the QACs HDTA and TMSA. These organoclays are being investigated for their use in nanocomposite packaging materials for the cosmetic industry. The hazard of these organoclays was assessed *in vitro* using a panel of relevant cell types (skin, liver, and macrophage-like cells) and a battery of cytotoxicity assay systems (alamarBlue<sup>®</sup> and CFDA-AM assays) were used to compare the potency of the organoclays and identify differences in cell sensitivity to the organoclays. The use of *in vitro* models to screen particle toxicity has recently been proposed as a key component of intelligent testing strategies to allow risk evaluation of nanomaterials (Farcas et al. 2015; Stone et al. 2014), and to improve alignment of nanotoxicology testing with the 3Rs principles (Burden et al., 2017; Johnston et al., 2018).

##### 4.1. Effect of organic modification on the physico-chemical properties of the clays

FT-IR analysis confirmed the exchange of Na<sup>+</sup> cations on the surface of clay layers with alkylammonium ions of the HDTA and TMSA organic modifiers. The interlayer distance measured

increased from 10.39 Å in the parent clay to 18.78 Å and 21.53 Å in the N116\_HDTA and TMSA organoclays, respectively. This increase in interlayer distance will facilitate polymer intercalation between individual clay layers during polymer nanocomposite production (Jang et al. 2005). The concentration of modifier present within, or surrounding, the clay layers was identified to be 27.3% HDTA (w/w) and 29.5% TMSA (w/w). However, the total content of organic modifier in the organoclays consisted of both an exchanged (i.e. intercalated) and free (non-intercalated) portion (approx. 8%) that likely contributed to the adverse effects observed.

In the cytotoxicity studies performed the parent clay and organoclays were first suspended in aqueous suspensions (2% FCS) and subsequently test suspensions were prepared in cell culture media for application to cells. These clays have swelling properties and therefore an expansion of interlayer spacing would be expected in these aqueous suspensions. The degree to which expansion would take place would dictate whether the clay particles remained as tactoids (stacks of parallel clay sheets with equal interlayer spacing) or became delaminated (broken down into individual clay sheets). Images taken of the clay suspensions showed the presence of large clay particle tactoids (1.6-4.4 µm) as well as smaller populations of clay particle tactoids (300-900 nm) in both parent and organoclay suspensions. When viewed at higher magnification using transmission electron microscopy, there was evidence of both tactoids and individual high aspect ratio delaminated clay sheets. Hydrodynamic size measurements taken directly following preparation and after incubation at 37 °C did not reveal any significant changes in clay particle sizes in biological medium. The clay particles under investigation were between 2-6 µm in diameter. Larger z-average values were measured in organoclay suspensions suggesting a greater tendency to form larger clay particles and flocculate in aqueous suspension. This is most likely due to the hydrophobic properties of these clays following modification.

The use of a dispersant such as serum is known to create a steric stabilisation effect (Moore et al. 2015) and thus was used to help create homogenous suspensions of the clays for testing. Also, in

this particular case, the clays have strong adsorption properties and complexation abilities and thus the addition of a serum coating step could reduce potential clay-media interactions that may have a direct influence on physico-chemical properties and the hazard assessment (Katic et al. 2006). Hydrodynamic size measurements point to the overall stability of clay/organoclay suspensions in biological media. However, imaging of the clay suspensions using light microscopy revealed that organoclay particles appeared opaque and red. The findings from this study suggest that the organoclays have a higher capacity to adsorb media components than the parent, unmodified clay. The strong affinity of clays for cationic and anionic dyes has been reported previously and attributed to the negatively charged surface lattices found in clays (Ramakrishna and Viraraghavan 1997). In this study the organic modification has markedly increased the clays uptake of the organic dye phenol red, a common constituent in culture medium used as a pH indicator. A strong interaction of the cationic surfactant HDTA with anionic phenol red has also been reported previously (Nong et al. 2014). Research performed into this phenomenon has shown that adsorption occurs due to electrostatic attraction and hydrogen bonding between the amine on the organoclay and the dye and that this can actually be tuned for potential applications (Chen et al. 2017).

However, in the context of this study the high adsorption properties of the clays have also led to interference with one of the assay systems employed. More specifically, the clays strongly adsorbed to the neutral red cationic dye used in the cytotoxicity screening to assess possible lysosomal dysfunction in the Neutral red assay. The importance of assessing for interference when testing nanomaterials using *in vitro* systems is well known (Stone et al. 2009; Ong et al. 2014) but has not been reported for clays previously. As many of the *in vitro* assays commonly applied to assess cell function make use of dyes, careful consideration needs to be taken and a thorough interference check performed when testing the toxicity of clays. Indeed, the neutral red uptake assay was excluded from this study due to interference issues.

#### **4.2. Effect of organic modification on clays toxicological/safety profile**



Both of the organoclays tested produced higher and distinct cytotoxic effects relative to the parent nanoclay in all cell types. This highlights an increased hazard from organoclays that should be acknowledged within a risk assessment framework. This is consistent with reports in a number of other studies assessing clay toxicity using different organic modifiers and cell types (e.g. Janer et al. 2014). For example, the commercially available organoclay, Cloisite®30B which is modified with methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium (CAS no. 91721-84-5) produced distinct cytotoxic effects in both the human intestinal epithelial cell Line, Caco-2 (24 h EC50 value was  $79 \pm 4 \mu\text{g/ml}$  ( $24.68 \pm 1 \mu\text{g/cm}^2$ ) (Maisanaba et al. 2014) and the human bronchial epithelial cell line, BEAS-2B (Wagner et al. 2017).

In this study a significant reduction in cellular metabolic activity (alamarBlue® Assay) was measured following administered doses of  $\geq 1.95 \mu\text{g/cm}^2$  and  $0.98 \mu\text{g/cm}^2$  for N116\_HDTA and N116\_TMSA, respectively. These LOEC values are only slightly higher than theoretical exposure levels calculated following the use of a cosmetic packaging incorporating 4% (w/w) organoclay nanofiller ( $0.8 \mu\text{g/cm}^2$ ) (Additional file 1, figure 1). Therefore, it would be important in the packaging design process to minimise the amount of organoclays that are incorporated into the packaging and ensure low migration from the packaging into the cosmetic in order to mitigate potential risks to the consumer from exposure. A study investigating actual migration levels from nanocomposites incorporating N116\_HDTA and N116\_TMSA is currently being performed and this exposure information along with the toxicological information generated in this study will contribute to a complete risk assessment for the packaging being developed (Connolly et al, manuscript in preparation).

The identification of specific modes/mechanisms of action that lead to adverse effects is key to a better understanding of hazard potential. A recent comprehensive investigation, that included a range of modified montmorillonite clays with different modifiers, showed that the chemical composition and structure of the modifiers played a major role in the adverse effects of organoclays (Wagner et al. 2017). In this study we have investigated the specific contribution of organic modifier

to cytotoxicity and confirmed the role of free HDTA and TMSA modifiers in the toxicity observed. These QACs belong to a class of cationic surfactants commonly used in household cleaning products, disinfectants and personal care products. They have positively charged head groups and therefore have a strong tendency to adsorb to negatively charged surfaces (e.g. cell membranes). Also, as a group, QACs are known skin and respiratory irritants with different degrees of irritation depending on the alkyl chain length and structure of the molecule (Rhein et al. 1986, Lin and Hemming 1996). Therefore, the concentration of organic modifiers such as HDTA or TMSA present on or intercalated with the clay is likely to dictate toxic potency.

Interestingly, in this study TMSA modified clays were more cytotoxic compared to HDTA modified clays despite both organic modifiers producing similar cytotoxic effects when tested alone (IC<sub>50</sub> 0.46 vs 0.47 µg/cm<sup>2</sup>). To better understand this, the toxic contribution of both the known free, as well as exchanged, concentrations of HDTA and TMSA in organoclay exposure suspensions was determined. There was a positive correlation between total organic modifier contributions and N116\_TMSA exposure response curves not seen in the case of N116\_HDTA (positive correlation with free modifier concentration). Such a finding may point to the TMSA modifier becoming displaced from the clay surface in the cell culture medium environment (during preparation or during the exposure period) and mediating the toxicity observed. Such a phenomenon has been witnessed by Sharma and colleagues when dispersing a natural montmorillonite modified with the quaternary ammonium salt methyl, tallow, bis-2-hydroxyethyl (Cloisite® 30B) in DMEM cell culture medium (Sharma et al. 2014). Interestingly the authors observed no release of organic modifier in water. Cations in the culture medium (e.g. Ca<sup>2+</sup>, Na<sup>+</sup>) may be participating in exchange reactions with the clays and causing the displacement of the ammonium cations of the organic modifiers. This exchange process has been shown to be influenced by free energies of hydration, differences in head groups of organic modifiers as well as the strength of van der Waals forces (Teppen et al. 2007). The release of surfactants from the Nanomer1.44P and Cloisite 93A organoclays in food simulants (ethanol (100%), 50% ethanol (ethanol/water = 1:1, v/v) and water (100%)) has been studied and found to be

influenced by temperature, the affinity between the surfactant and the solvent, with the highest release measured in 100% ethanol (Xia et al. 2015). As well as explaining the increased cytotoxicity of TMSA modified organoclays seen in our study, it is possible that the increased cytotoxicity reported by other authors for organically modified clays using *in vitro* cell culture test systems may be explained by similar displacement (e.g. Wagner et al. 2017; Cloisite 30B and BEAS-2B cells) (Sharma et al. 2010; Cloisite® 30B and Caco-2 cells)). The results obtained can be used to inform the safe development of future generations of modified clays (i.e. by informing the selection of the modifier and ensuring stability of modification) and also has implications for the safe use of polymer nanocomposites made using organically modified clays.

While experimental conditions, in this case, has influenced results, this might also mimic what may occur in a biological environment *in-vivo* (e.g. following ingestion in the gastrointestinal tract of rats because of chemical reaction with proteins or amino acids or displacement caused by salts).

Macrophages were observed to be the most sensitive cell type to both the parent clay and organoclays tested. This may arise as a consequence of the phagocytosis of organoclays into lysosomes, which is likely to cause displacement of the organic modifier and the elicitation of cytotoxicity through organic modifier mediated mechanism of toxicity. Macrophages are professional phagocytes, and the ability of macrophages to take up such large particles ( $\geq 1\mu\text{m}$ ) via phagocytosis/micropinocytosis has been demonstrated previously (Kuhn et al. 2014). Furthermore, non-phagocytic cell types (e.g. hepatocytes, epithelial cells) can also internalise particles (Panzarini et al. 2018). A lysosomal-enhanced trojan horse type mechanism of cytotoxicity has been proposed for a variety of metal containing nanomaterials that are susceptible to dissolution processes (e.g. Sabella et al. 2014). The uptake of organoclays in lysosomes that have acidic environments is likely to affect clay-modifier interactions. For example, using liposomes with lipid bilayers as a model to study the mechanism of toxicity of surfactants at the cellular level Hamada and colleagues (Hamada et al. 2012) showed a distinct mechanism of membrane solubilization induced by various

surfactants. Further studies would need to be performed to investigate the increased sensitivity of macrophages (and how this relates to particle uptake) and potential modifier displacements in such intracellular environments and subsequent surfactant effects on lysosomal membranes.

#### **4.3. Cell-type specific and organoclay-specific effects**

In this study, the toxicity of clay and organoclay test suspensions were tested across different cell models (skin, liver and macrophage-like cells). J774.1 macrophage-like cells were the most sensitive cell type to the parent sodium bentonite clay tested in this study. Cytotoxic effects were measured at concentrations  $\geq 7.81 \mu\text{g}/\text{cm}^2$  and a complete loss in viability was measured following exposure to the highest concentration tested ( $31.25 \mu\text{g}/\text{cm}^2$ ). In contrast, the HaCaT skin epithelial and C3A hepatocyte cells only exhibited slight reductions (5-10%) in viability even at the highest concentration tested. Other authors have also observed the increased sensitivity of macrophages to cytotoxic effects of aluminium silicate clay minerals (Bowman et al. 2011; RAW murine macrophage cells), amorphous silica (Costantini et al. 2011; mouse alveolar macrophage (MH-S) cells) as well as other nanomaterials (Lanone et al. 2009; Brown et al., 2018). These cells are highly phagocytic in nature and thus the increased susceptibility of this cell line to the clay may be related to an increased intracellular uptake. The physiological role of macrophages in the clearance of foreign material may result in a more rapid phagocytic uptake of clays and a higher exposure concentration to cells (Malugin et al. 2011). TEM analysis has confirmed the uptake of numerous laminated clays in membrane bound vesicles. While hepatocytes and keratinocytes have been shown to internalise particles (Johnston et al. 2010; Li et al. 2016), a detailed comparative and quantitative study would need to be performed to confirm clay uptake across the cell types. Histocytological staining however has shown clay-cellular interactions with clays accumulation at the macrophage cell surface observed. This together with early reports of cytotoxic effects of clays involving rapid association of clays with the outer membrane of cells and complete cell lysis (Murphy et al. 1993) may render J774.1 cells more sensitive to the clays than the other cell types investigated. The release of TNF- $\alpha$

following clay exposure only further supports a distinct clay-induced mechanism of toxic action in the J774.1 macrophage-like cells that warrants further investigation. Clays have been shown to stimulate antigen presenting cell maturation, so much so that they have been highlighted as potential nano-adjuvants for enhancing immune responses (Chen et al. 2017). This distinct effect could be related to the reported silica sensitivity in macrophages as the silica content of bentonite clays can range from 1-20% (Gozal et al. 2002). More specifically, TNF- $\alpha$  has been identified as a major determinant in the development and progression of silicosis (Piguet et al. 1990). Bentonite clays have been shown to be more cytotoxic to human B lymphoblast cells than even DQ-12 crystalline silica, a substance commonly used in studies as a positive control for its fibrogenic properties (Zhang et al. 2010).

The presence of the organic modifier increased the cytotoxic effects of the parent clay suggesting that both modifier and particle effects contributed to the observed toxicity. By comparing concentration-response curves for organoclay responses and total concentrations of modifier present, a specific contribution of clay particles to cytotoxic effects is evident. Increased TNF- $\alpha$  levels were only measured following exposure to the unmodified clay. Modified clays show increased absorptive properties which may lead to interferences with cytokine levels through cytokine-clay interactions (Brown et al. 2010). Also, potential endotoxin contamination on unmodified clays that lack biocidal properties may explain such contrasting results. However, it may also indicate a distinct inflammatory response. Distinct mechanisms of cytotoxicity for unmodified clays involving reactive oxygen species (ROS) production (Lordan et al. 2011) and modified clays producing specific genotoxic effects (Sharma et al. 2010) have been reported previously. Also, genetic damage and lipid peroxidation was evidenced in workers occupationally exposed to bentonite clays modified with organic amine (including alkyl quaternary ammonium salt and methyl chloride) in a controlled experiment by Huang et al. (2013). Taken together the adverse effects reported in this *in vitro* study, and likely high exposure levels of workers to the clays and organoclays through inhalation suggests that they could represent a health risk. Appropriate risk management strategies (e.g. ventilation and

enclosures) incorporated during nanocomposite compounding have been shown to reduce airborne clay particle concentrations and thus minimize the health risks to workers (Tsai et al. 2012).

## 5. Conclusions

Organic modification of a sodium bentonite clay with the QACs HDTA or TMSA increased the hazard of this material, in a consumer and occupational setting. Cytotoxic effects were observed in all of the cell types tested (skin, liver and macrophage-like cells) for organoclays, and were dictated by the concentration of free or displaced modifier. Thus, the results suggest that the concentration of modifier used, as well as the stability of the exchange/intercalation must be considered in the context of a safety assessment for modified clays. The enhanced toxicity of modified clays may present a safety concern as the use of organoclays in polymer nanocomposites for packaging applications in different sectors (e.g. food and beverage) becomes more widespread. The parent N116 clay also produced cytotoxic effects at much lower concentrations compared to the organoclays in J774.1 macrophage-like cells, and stimulated TNF- $\alpha$  release. This may point to particular occupational hazards associated with the inhalation of such materials with a high dustiness factor and subsequent contact with alveolar macrophages in the lungs. However, it is important to distinguish between a non-adverse, physiological immune response stimulated by clays and a pathological inflammatory response. *In vivo* studies with clays are scarce (Warheit et al. 2010) and will need to be performed to assess how the results obtained from an *in vitro* study translate to possible effects *in vivo*, particularly following inhalation. This will enable an occupational risk assessment for the packaging to be performed. The *in vitro* toxicological information obtained in this study can be used to inform what measures need to be taken along the polymer-nanocomposite production chain to mitigate risk both to the consumer and workers in a safe by design concept and responsible development approach to sustainable nanotechnologies.

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## Highlights

- Organic modification increases hazard of clays *in vitro*
- Macrophages were most sensitive to the toxicity of clays
- Modification stability of clays should be considered when testing *in vitro*

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