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Characterisation and cytotoxic screening of metal oxide nanoparticles putative of interest to oral healthcare formulations in non-keratinised human oral mucosa cells *in vitro*

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ABSTRACT

Nanoparticles are increasingly being utilised in the innovation of consumer product formulations to improve their characteristics; however, established links between their properties, dose and cytotoxicity are not well defined. The purpose of this study was to screen four different nanomaterials of interest to oral care product development in the absence of stabilisers, alongside their respective bulk equivalents, within a non-keratinised oral epithelial cell model (H376). Particle morphology and size were characterised using scanning electron microscopy (SEM) and dynamic light scattering (DLS). The H376 model showed that zinc oxide (ZnO) was the most cytotoxic material at concentrations exceeding 0.031% w/v, as assessed using the lactate dehydrogenase (LDH) and dimethylthiazolyl-diphenyl-tetrazolium-bromide (MTT) assays. ZnO cytotoxicity does not appear to be dependent upon size of the particle; a result supported by SEM of cell–particle interactions. Differences in cytotoxicity were observed between the bulk and nanomaterial forms of hydroxyapatite and silica (SiO_2); titanium dioxide (TiO_2) was well tolerated in both forms at the doses tested. Overall, nano-size effects have some impact on the cytotoxicity of a material; however, these may not be as significant as chemical composition or surface properties. Our data highlights the complexities involved at the nano-scale, in both the characterisation of materials and in relation to cytotoxic properties exerted on oral epithelial cells.

1. Introduction

The European health burden of oral disease has been attributed principally to dental caries, periodontal diseases and oral cancers. Oral diseases not only impact individuals through pain and discomfort, but affect the wider community through the health system and related economic costs (Patel, 2012). Frequent exposure to fluoride, regular brushing, a healthy diet and routine oral care regimes all contribute to improved oral healthcare outcomes and a reduction in oral care inequality (Petersen, 2003). Thus, personal oral healthcare products form an important part of any oral hygiene routine and include the use of toothpastes, flossing agents and mouthwash products that can help conditions such as dentine hypersensitivity, tooth decay, gum disease, halitosis and xerostomia.

Developers of next-generation oral care product formulations are increasingly researching new materials that can enhance the efficacy or characteristics of existing products. Nanomaterials have the potential to exploit the difference in physiochemical properties that result as particle size is reduced to the nanoscale (generally defined as a particle with one or more dimensions between 1 and 100 nm in size, but also including groups of nanoparticles in aggregates). Nanosize confers unique properties upon a material due to a vastly increased surface area to volume ratio when compared with non-nano (bulk) counterparts (Borm et al., 2006; Thomas et al., 2006). This has led to nanomaterials formulated as the active ingredient in cosmetics, as well as delivery vehicles (or nanocarrier) or as formulation aids (Mihranyan et al., 2012). Currently, carbon, gold, silver, silica, titanium and zinc are present as nanomaterials in marketed cosmetic products (SCCP, 2007; Aitken et al., 2006; Mihranyan et al., 2012; Nohynek et al., 2010; Raj et al., 2012; Thomas et al., 2006).

Although nanomaterials have the potential to enhance formulations, their increased surface reactivity has also driven concern towards their *in vivo* toxicity and potential safety (Nel et al., 2006; Oberdörster, 2010). Currently, many gaps remain within the literature as to the fate of nanomaterials and their interactions with different cells, and/or subcellular structures. In addition, no established screening procedures

Abbreviations: DLS, dynamic light scattering; LDH, lactate dehydrogenase; MTT, dimethylthiazolyl-diphenyl-tetrazolium-bromide; NTA, nanoparticle tracking analysis; PRF, Phenol red-free; PBS, phosphate-buffered saline, SiO_2, silica; TiO_2, titanium dioxide; SD, standard deviation; SEM, scanning electron microscopy; ZnO, zinc oxide.

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are yet able to adequately test the cytotoxic potential that may arise in a material upon reduction in its particle size (<100 nm). Nanoparticles have been found to induce cytotoxic responses in the nasal (Hackenberg et al., 2011), bronchial (Magdolenova et al., 2012; Park et al., 2007; Wang et al., 2014) and gut mucosae (Gerloff et al., 2009; McCracken et al., 2013; Piret et al., 2012). However, few studies have been undertaken to document any interactions that might exist between nanoparticles and the tissues of the oral mucosae, particularly in the absence of stabilisers. This is an important consideration relating to formulation design of particulate delivery to the oral mucosa, where the flow of saliva presents a real challenge towards effective administration (Brading and Marsh, 2003). The regional imbalances in saliva volume and its flow rate can cause dilution, pH changes and enzymatic degradation (Vivien Castioni et al., 1998) resulting in non-uniform residual exposure of particulates (Wen & Park, 2011).

This study aims, for the first time, to utilise a fully characterised oral epithelial cell line to screen nanomaterials of interest as oral healthcare excipients. This is to be undertaken at concentrations representative of high residual exposure (compared to similar metal oxide particles already present in toothpaste formulations), following delivery in personal care products (Hernández-Sierra et al., 2008; Weir et al., 2012), and in their native form free from stabilisers. The investigation utilises a non-keratinised oral epithelial cell model, due to their increased sensitivity to toxic stimuli (Squier et al., 1991; Squier and Kremer, 2001; Wertz et al., 1993), and two common chemical assays combined with morphological evaluation of cytotoxicity. Any cytotoxic effects identified will be linked to common properties established during nanomaterial characterisation (and in comparison to bulk reference materials) to evaluate the initial risk posed from nanomaterial exposure, in a representative *in vitro* environment to oral healthcare product applications.

2. Materials and methods

2.1. Culture of H376 oral mucosa cell line

Oral epithelial keratinocytes were cultured in Dulbecco's MEM/Ham's F12 media without L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 2,500 IU/mL penicillin/streptomycin, 2 mM L-glutamine and 0.5 μg/mL hydrocortisone (PAA Laboratories, UK). Cells were seeded at a density of 8.0×10^3 cells/cm^2 and incubated at 37 °C/5% CO_2 for 48 h. Media was removed and wells washed with PBS before application of experimental treatments.

2.2. Chemicals

All bulk particle-sized materials were purchased as commercial powders from Sigma-Aldrich (UK). Hydroxyapatite (<200 nm, surface area 9.4 m^2/g) and SiO_2 (12 nm, surface area 175–225 m^2/g) nanomaterials were also purchased from Sigma-Aldrich (UK), as distinct nanopowders. ZnO nanomaterials were purchased as two separate commercial products from Alfa Aesar (Hersham, UK) in aqueous dispersions: 45009 and 45408 (70 nm and 20–30 nm particle sizes, respectively). TiO_2 nanomaterial (~21 nm) was an anatase–rutile mix (80:20) also purchased in a stabilised aqueous dispersion from Sigma Aldrich (UK).

2.3. Characterisation of nanoparticles

For SEM, dry powder samples were mounted on adhesive patches applied to aluminium SEM stubs. TiO_2 and ZnO dispersions were dried directly on aluminium stubs prior to analysis. All samples were analysed using a Zeiss Σigma™ field emission gun scanning transmission electron microscope at an accelerating voltage of 2 kV to acquire images at 300 K× magnification.

Using electron microscopy, mean particle size measurements were calculated using the Carl Zeiss Tiff Annotation Editor on $n = 90$ random individual particles: 10 particles from the field of view across 3 different images each across 3 magnifications (20 K, 50 K and 100 K× magnifications).

Size distributions and aggregation tendency of particles were assessed *in situ* in biological media (compared against both ethanol and dH_2O) using dynamic light scattering (DLS) (ZetaSizer ZS90, Malvern Instruments Ltd., UK). Nanoparticles were diluted from a 1% w/v stock concentration in un-supplemented DMEM-Ham's F-12 media to a 0.001% w/v final concentration. Samples were vortex-mixed before sonicating (Ultrawave U50H Sonic bath, Ultrawave Ltd., UK) for 10 min prior to characterisation. The surface zeta-potential of all nanoparticle dispersions, at pH 7.4, was assessed using the same ZetaSizer ZS90 instrument.

2.4. Cell treatment

Cells at 70% confluence were treated with bulk or nanoparticle material diluted in solutions of serum-free Dulbecco's MEM/Ham's F12 media without L-glutamine, at concentrations between 0.25% w/v and 0.03125% w/v. Serum-free Dulbecco's MEM/Ham's F12 media without L-glutamine served as a negative control with Triton™ X-100 included as a cytotoxic/lysis positive control. Cells were incubated with treatment solutions for 5 min at 37 °C/5% CO_2 to simulate a typical oral healthcare product exposure, before removal and washing three times with PBS. Subsequently, growth media was replaced, and plates incubated at 37 °C/5% CO_2 for a further 24 h.

2.5. Measurement of cytotoxicity and cell death

Nanoparticle effects on cells were measured using the LDH assay of cytotoxicity (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega UK) and MTT cell viability assay (Life Technologies, UK) simultaneously on the same treated (H376) cells. Absorbance was read on a plate reader (Thermo Multiskan Ascent 354) at 492 nm and 540 nm for LDH and MTT assays, respectively. LDH results were converted to a percent cytotoxicity value by calculating against the average absorbance of the negative control cell population, and fully lysed (with 1% v/v Triton™ X 100) cell populations. Formazan absorbance was converted to percent cell viability against the negative control.

2.6. Fixing cells for SEM imaging

Cell morphological changes in response to particle exposure were investigated using SEM. Cells were seeded on 13 mm diameter Thermanox™ coverslips (Agar Scientific Ltd.) within 6-well plates (Falcon™ 6-well Multiwell plate, Becton-Dickson UK) at a density of 8.0×10^3 cells/cm^2 for 48 h. Cells were exposed to particles as described earlier, at a concentration of 0.125% w/v. Following exposure with materials, cells were fixed using 5% glutaraldehyde 0.1 M sodium cacodylate buffer (pH 7.4) for 2.5 h at 4 °C, rinsed in 0.1 M sodium cacodylate before dehydrating using a graded alcohol series. Before analysis, samples were sputter coated with 4 nm platinum (Q150T ES; Quorum Technologies Ltd.). SEM was performed using the Zeiss sigma field emission gun scanning electron microscope at an extra-high tension (EHT) voltage of 5 kV to acquire images at 10 K× magnification.

2.7. Statistical analysis

Results are expressed as the mean ± standard deviation (SD) of six replicates of six independent experiments, unless otherwise stated. After checking data for normality, comparison of mean values between groups was assessed by analysis of variance test (ANOVA) and Tukey's multiple comparison test using the PASW 18 (v.18.0.0) statistics package (IBM SPSS software, USA). Significance was identified by a P value less than 0.05.