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The effects of serum on the toxicity of manufactured nanoparticles

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ABSTRACT

The aim of the present study was to assess the effects of the presence and absence of serum in NP suspension media in relation to their cytotoxicity, as well as their potential to cause oxidative stress and stimulate pro-inflammatory cytokine release from J774.A1 murine 'macrophage-like' cells. Different sized (20 nm and 200 nm) carboxylated, fluorescent, model polystyrene beads (PBs) at concentrations from 12.5 μg ml^{-1} to 100 μg ml^{-1} were used. Both 20 nm and 200 nm PBs, independent of the suspension media, were observed to cause limited, yet significant ($p < 0.05$) cytotoxicity over 48 h up to 100 μg ml^{-1}. Significant differences ($p > 0.05$) were also found between NP size and serum content of the suspension media used. The smaller sized PBs were found to affect intracellular glutathione (GSH) levels, causing a significant loss ($p < 0.05$) in GSH when suspended in the presence of serum. Subsequent analysis also showed significant ($p < 0.05$) increases in tumour necrosis factor-α production after 48 h when the 20 nm PBs were suspended in both the presence and absence of serum, compared to the affects observed by the larger, 200 nm sized PBs. In conclusion, the results of the present study show that the interaction of NPs with serum can significantly affect their resultant toxicity *in vitro*.

1. Introduction

Over the past decade, substantial evidence has demonstrated that nanoparticles (NPs), defined as nano-objects with all three external dimensions in the nanoscale (1–100 nm) (ISO TS 27687:2008), made from low solubility materials, such as polystyrene, generate a greater toxic response when compared to larger particles at the same mass dose (Oberdorster et al., 2005a, 2007). The heightened toxicity associated with NPs has been linked to their small size, relatively larger surface area per unit mass and increased surface reactivity compared to larger particles (Brown et al., 2001; Duffin et al., 2002, 2007). Due to the constantly escalating production of NP based products for consumer and medical applications, such as cosmetics and drug delivery, human exposure to NPs is inevitable. It is imperative therefore, to investigate and understand the interaction of NPs with biological systems, such as the human body, in order to ensure that their development and use is safe and sustainable (Maynard et al., 2006; Maynard, 2007).

In addition to the NP characteristics highlighted as being responsible for their resultant toxicity (Oberdorster et al., 2005a,b, 2007), it has also been reported that the interaction of proteins with NPs could potentially affect the entry and intracellular localisation of NPs with cells, and thus affect their potential toxicity (Cedervall et al., 2007). Recently therefore, the NP–protein–cell interaction has formed the basis for increased research. Interestingly, the form of serum, or protein supplementation contained within the NP suspension media has been shown to have an effect upon their dispersion and aggregation status within cell culture media. Foucaud et al. (2007) showed that the use of bovine serum albumin (BSA) in the cell culture medium significantly reduces the size of ultrafine carbon black (ufCB) aggregates and subsequently alters the ROS response from the Monomac-6 cell-line in comparison to exposure of ufCB suspended in cell culture media without BSA present. Similarly, Johnston et al. (2010) reported negatively surface charged polystyrene bead microspheres (20 nm and 200 nm) (PBs) to interact differently with the hepatocyte cell-lines C3A and HepG2, as well as primary rat hepatocyte cells, when suspended in foetal calf serum (FCS) containing cell culture media, compared to cell culture media only (i.e. no FCS present). Despite the difference in serum used (i.e. BSA vs. FCS), Johnston et al. (2010) supported the conclusion of Foucaud et al. (2007), which highlighted that the presence of serum in NP suspension media can cause a reduction in the size of NP aggregates. In the study of Johnston et al. (2010) however, this was only true for the smaller, 20 nm PBs and not for the 200 nm PBs. It was found that the impact of FCS on the 200 nm PBs aggregation status was not significant, with both suspension scenarios resulting

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in similar NP aggregate size and NP–cell interaction; both 200 nm PB suspensions were observed to have a slower entry process than the 20 nm PBs. It was subsequently concluded that NP size may affect NP–protein interactions. The observation that NP size may influence NP–protein interactions was similar to that of Lundqvist et al. (2008). Lundqvist and colleagues investigated the specific protein attachments to the particle surface of a series of different surface charged PBs (plain, as well as negatively and positively charged), of different sizes (50 nm and 100 nm) in their specific NP suspension media. Although it was observed that numerous different proteins attached to different areas of the surface of the plain (no charge) PBs, it was also reported that the 100 nm negatively and positively charged PBs associated specifically with a group of immunoglobulin proteins that were not observed on the surface of their smaller (50 nm) counterparts. In addition to the differences in NP–protein interactions in respect to their surface charge/coating, the finding that different proteins attach to surface of different sized NPs provides further evidence to the knowledge regarding the effects of NP size in relation to NP–cell interactions (Geiser et al., 2005).

It has been shown that the specific NP–cell interaction (i.e. the NP-entry mechanism) and intracellular localisation is directly associated with their potential toxicity (Lovric et al., 2005). Currently however, research into the NP–protein–cell interaction has mainly focussed on the NP cellular entry and protein attachment to the particle surface. Information regarding the effects of proteins, or serum upon the toxicity of NPs is not fully understood. The aim of this study therefore, was to examine the potential adverse effects of different sized PBs when suspended in either the presence or absence of serum on a macrophage cell-line. It is hypothesised that NPs suspended in serum will elicit a lower cytotoxicity, intracellular oxidative stress environment and pro-inflammatory response than NPs suspended in the absence of serum.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma–Aldrich UK, unless otherwise stated.

2.2. Cell culture

J774.A1 murine 'macrophage-like' cells were cultured in RPMI 1640 medium containing phenol red, L-glutamine (L-G) (5 ml at 200 mM), penicillin (5 ml at 100 U ml^{-1})/streptomycin (5 ml at 0.1 $\mu g\,ml^{-1}$) (P/S) and 10% FCS (hereafter known as '*complete medium*') as previously described in Clift et al. (2008).

2.3. Particles and particle preparation

Fluorescent, carboxylate (COOH)-modified microsphere PBs at a size (Ø) of 20 nm and 200 nm (Molecular Probes, USA) were used (excitation λ 581 nm/emission λ 605 nm). Both sized particles were used at concentrations of 12.5 $\mu g\,ml^{-1}$, 25 $\mu g\,ml^{-1}$, 50 $\mu g\,ml^{-1}$ and 100 $\mu g\,ml^{-1}$. Prior to use, PB stock solutions were vortexed for a total of 1 min in order to aid dispersal, and were then suspended in either complete medium or RPMI 1640 medium containing L-G and P/S in the absence of 10% FCS. The resultant particle suspensions were then vortexed for 1 min immediately prior to cellular exposure or experimental analysis.

2.4. Particle characterisation

Both the 20 nm and 200 nm PBs used have previously been assessed for their size (Clift et al., 2008) and zeta potential (Clift et al., under review) over time in the specific cell culture media used in this study at 50 $\mu g\,ml^{-1}$. No significant differences were observed in the size of both the 20 nm and 200 nm PBs over a 30 min period, as assessed by dynamic light scattering (90Plus/BI-MAS Multi Angle Particle Sizer, Brookhaven Instruments Corporation, New York (Clift et al., 2008)). The 20 nm PBs were found to alter their zeta potential in suspension media with FCS absent after both 60 and 120 min (Clift et al., under review). In contrast, the zeta potential of the 200 nm PBs was only observed to significantly alter following 120 min in suspension media containing FCS (Clift et al., under review).

2.5. Cytotoxicity

2.5.1. MTT assay

J774.A1 cell viability and proliferation status was determined at 2 h, 4 h, 24 h and 48 h as previously described in Clift et al. (2008). The period of time that particle exposed cells were treated with the MTT reagent (2,5-diphenyltetrazolium bromide) differed however, from the previously described protocol. Cells were treated with the MTT reagent for 3 h instead of 4 h (as used in Clift et al., 2008). Investigation into the effects of the shortened treatment using both (i) complete medium and (ii) 20 nm PBs (50 $\mu g\,ml^{-1}$), either in the presence or absence of 10% FCS for a period of 2 h, found no significant difference ($p > 0.05$) in the absorbance measured between 3 h and 4 h of treatment with the MTT reagent (data not shown). From these results, it was concluded that the shorter incubation time of 3 h with the MTT reagent was a reliable and consistent measure of J774.A1 cell viability/proliferation. Cell supernatants were collected and then used for subsequent analysis of lactate dehydrogenase (LDH) release from J774.A1 cells. The effects of PBs on cell viability/proliferation were assessed a total of five times ($n = 5$).

2.5.2. Lactate dehydrogenase release and adsorption

The ability for particles to cause the release of LDH from J774.A1 cells, an indicator of cell membrane permeability, was assessed at 2 h, 4 h, 24 h and 48 h using the protocol previously described in Brown et al. (2001). The potential for PBs to cause LDH release from J774.A1 cells was investigated a total of five times ($n = 5$).

The potential for the LDH enzyme to adsorb to the surface of the different sized PBs, and therefore result in a false negative toxicity was also assessed as previously described in Clift et al. (2008). The potential for the LDH enzyme to adsorb to the surface of each sized PB was assessed a total of three times ($n = 3$).

2.6. Oxidative stress and inflammation

2.6.1. Assessment of intracellular glutathione production

The effects of both sized PBs on J774.A1 intracellular glutathione (GSH) levels were assessed at 2 h, 4 h, 6 h and 24 h using the protocol previously described in Clift et al. (2010). The effects of PBs on J774.A1 intracellular GSH levels were investigated a total of three times ($n = 3$).

2.6.2. Tumour necrosis factor-alpha production

The ability for particulate treated cells to produce the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) was assessed at 2 h, 4 h, 6 h and 24 h exposure to NPs via the use of an enzyme-linked immunosorbant assay (ELISA) kit (BioSource Diagnostics, Oxford, UK). Assessment of the level of TNF-α stimulation by particulate treated J774.A1 macrophage cells was repeated a total of three times ($n = 3$).

2.7. Statistical analysis

All results are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined via a parametric one-way analysis of variance (ANOVA), followed by, when appropriate, a Tukey's pairwise comparisons *post hoc* test (MINITAB®, version 15.1, MINITAB Inc., 2006). The result was considered significant if $p \leq 0.05$.

3. Results

3.1. Cytotoxicity

3.1.1. MTT assay

J774.A1 cell metabolic activity was found to significantly decrease following treatment with 20 nm PBs at 25 $\mu g\,ml^{-1}$ ($p < 0.05$), 50 $\mu g\,ml^{-1}$ ($p < 0.001$) and 100 $\mu g\,ml^{-1}$ ($p < 0.001$) after 2 h, in both the presence and absence of 10% FCS (Fig. 1A). Subsequent analysis at both 4 h and 24 h, also demonstrated exposure to 20 nm PBs to significantly decrease J774.A1 cell metabolic activity in the presence of 10% FCS at the highest concentration (100 $\mu g\,ml^{-1}$) ($p < 0.05$) (Fig. 1B and C). Analysis after 48 h also showed 20 nm PBs, in the presence of 10% FCS, to cause a significant decrease in J774.A1 cell metabolic activity at all concentrations tested ($p < 0.01$) (Fig. 1D). Examination of the effects of 20 nm PBs in the absence of 10% FCS found no effects after 4 h (Fig. 1B), although analysis at both 24 h (Fig. 1C) and 48 h (Fig. 1D) demonstrated a significant decline in the level of J774.A1 mitochondrial succinate dehydrogenase activity following treatment with 50 $\mu g\,ml^{-1}$ and 100 $\mu g\,ml^{-1}$ ($p < 0.01$).

A significant decrease in J774.A1 cell metabolic activity was observed following treatment with 200 nm PBs for 2 h at 25 $\mu g\,ml^{-1}$