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Reactive Oxygen Species induced cytokine production and cytotoxicity of PAMAM dendrimers in J774A.1 cells.

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Abstract

The immunotoxicity of three generation of polyamidoamine (PAMAM) dendrimers (G-4, G-5 and G-6) was evaluated in mouse macrophage cells in vitro. Using the Alamar blue and MTT assays, a generation dependent cytotoxicity of the PAMAM dendrimers was found whereby G-6 > G-5 > G-4. The toxic response of the PAMAM dendrimers correlated well with the number of surface primary amino groups, with increasing number resulting in an increase in toxic response. An assessment of intracellular ROS generation by the PAMAM dendrimers was performed by measuring the increased fluorescence as a result of intracellular oxidation of Carboxy H$_2$DCFDA to DCF using a plate reader as well as by confocal laser scanning microscopy. The inflammatory mediators Macrophage Inflammatory Protein-2 (MIP-2), Tumour Necrosis Factor -α (TNF-α) and Interleukin -6, (IL-6) were measured by the enzyme linked immunosorbant assay (ELISA) following exposure of mouse macrophage cells to PAMAM dendrimers. A generation dependent ROS and cytokine production was found, which correlated well with the cytotoxicological response and therefore number of surface amino groups. A clear time sequence of increased ROS generation (maximum at ~4hrs), TNF-α and IL-6 secretion (maximum at ~24hrs), MIP-2 levels and cell death (~72 hrs) was observed. The intracellular ROS generation and cytokine production induced cytotoxicity point towards the mechanistic pathway of cell death upon exposure to PAMAM dendrimers.

Key words : Nanotoxicology, PAMAM dendrimers, Reactive Oxygen Species, cytokine production, macrophage, in vitro.
Introduction

Dendrimers have a well-defined nanoscale architecture and potential novel applications in the biomedical field (Lee et al., 2005). Polyamidoamine (PAMAM) dendrimers contain a 2-carbon ethylenediamine core and primary amino groups on the surface (http://www.dendritech.com/pamam.html). The systematically variable structural architecture and the large internal free volume make these dendrimers an attractive option for drug delivery and other biomedical applications (Venuganti and Perumal 2008; Svenson and Tamalia 2005; Emanuele and Attwood 2005; Ducan and Izoo 2005). It is possible to passively target PAMAM dendrimers to a tumour because of the increased permeability of tumour vasculature to macromolecules and also due to the limited lymphatic drainage (Maeda and Matsumura 1986). The unique properties of dendrimers, as compared to linear polymers, render them of interest for intracellular drug delivery system for cancer therapy (Gillis and Frechet 2005). PAMAM dendrimers have been utilised for gene delivery (Yoo and Juliano 2000; Huang et al., 2007) and also as delivery agents for antisense and siRNA oligonucleotides (Kang et al., 2005). Dendrimers have been considered as additives in several routes of administration, including intravenous, oral and ocular (Cheng et al., 2008). Amino terminated PAMAM dendrimers result in enhanced anti-ovalbumin immunoglobulin-G and immunoglobulin-M levels and have also been used as adjuvants in vaccine delivery systems (Rajananthanan et al., 1999). In a recent study it has been shown that N-acetyl-D-glucosamine modified PAMAM dendrimers improve the immunogenicity by up-regulation of antibody formation via activation of natural killer cells (Hulikova et al., 2009) and the mannosylated form of PAMAM dendrimers potentiate the immunogenicity and have been proposed for vaccine delivery systems (Sheng et al., 2008).

The application of nanomaterials as intravenous drug delivery platforms may depend on avoiding rapid elimination from the systemic circulation by cells of the immune system. When nanoparticles enter into the bloodstream, they immediately encounter a complex environment of plasma proteins and immune cells. The interaction of nanoparticles with immune cells may occur both in the bloodstream via monocytes, platelets, leukocytes, and dendritic cells (DC) and in tissues by resident phagocytes, e.g., Kupffer cells in liver, DC in lymph nodes, macrophages and B cells in the spleen (Dobrovolskaia et al., 2008). As PAMAM dendrimers are potentially proposed for vaccine and intracellular gene delivery applications, and macrophages are the main target cells to produce/improve the immunogenicity of the different antigens, this study explores the understanding of the
interaction with and toxicity to macrophages cells of PAMAM (G-4, G-5 and G-6) dendrimers. J774A.1 cells, derived from the blood of female BALB/c mouse, are chosen for the in vitro model. ROS and subsequent cytokine production are monitored as indicators of oxidative stress and inflammatory response.

In addition to a study of the PAMAM dendrimer materials in their own right, the systematically varied molecular nanostructures potentially provides a route towards an understanding of the dependence of the interactions on the physico-chemical properties of nanomaterials. In a recent study, the toxicity of PAMAM dendrimers in mammalian cells has been demonstrated to be generation dependent (Mukherjee et al., 2009), potentially laying the foundation for structure-activity relationships underlining the mechanistic responses. Conjugation of poly (ethylene glycol) (PEG) to the surface of PAMAM dendrimers has been observed to improve their biocompatibility, reducing the cytotoxic response (Wang et al., 2009). Nanoparticle toxicity has been proposed to have origin in the induction of oxidative stress by free radical formation (Nel et al., 2006; Lanone et al., 2006; Donaldson et al., 2006; Obedoster et al., 2005). In excess, free radical formation causes damage to biological components through oxidation of lipids, proteins and DNA. Oxidative stress may have a role in the induction of inflammation through up regulation of redox sensitive transcription factors, NF-κB and activator protein-1 (AP-1), and kinases involved in inflammation (Lanone et al., 2006; Rahman, 2000; Rahman et al., 2005; Park and Park 2009).

The objective of the present work is an in vitro assessment of the immunotoxicological response of three generations of the cationic PAMAM dendrimers (G-4, G-4 and G-6) in mouse macrophage cells (J774A1). The systematically varied structure and size allows an evaluation of the dependence of the response on the physico–chemical properties. The PAMAM dendrimers were characterized in terms of particle size and zeta potential in cell culture media with and without protein (FBS). In accordance with the EU policy of Reduction, Replacement and Refinement (RRR) an in vitro rather than animal model is employed to assess the cytotoxic response and underlying mechanisms of these materials. (Directive 86/609/EEC on the protection of animals used for experimental and scientific purposes). Cytotoxicological effects of PAMAM dendrimers were evaluated using the Alamar blue and MTT [(3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide, a tetrazole)] assays. Intracellular reactive oxygen species (ROS) were measured following exposure of the macrophages to PAMAM dendrimers for time periods of 1h, 2h, 4h and 6h. The visualisation of ROS production by the cells was performed by confocal laser scanning
microscopy. The secretion of the cytokines and chemokines, Macrophage Inflammatory Protein-2 (MIP-2), Tumour Necrosis Factor -α (TNF-α) and Interleukin -6 (IL-6), following exposure of the macrophage cells to PAMAM dendrimers was measured quantitatively by enzyme linked immunosorbant assay (ELISA).

**MATERIALS AND METHODS**

**Test materials**

Polyamidoamine (PAMAM) dendrimers, G4, G5 and G6, were purchased from Sigma Aldrich Ltd. (Dublin, Ireland). All the particles have an ethylenediamine core and PAMAM G4, G5 and G6 have respectively 64, 128 and 256 functional primary amino groups on the surface. The molecular weights of G4, G5 and G6 are 14,215 Da, 28,826 Da and 58,048 Da respectively. The nominal diameters of the G4, G5 and G6 dendrimers are 4.5, 5.4 and 6.7 nm respectively [Dendritech, Sigma Aldrich Ltd., (Supporting information, Table S1)]. The diameter and zeta potential of the dendrimers were measured using a Malvern Zeta Sizer Nano ZS (Malvern Instruments, Worcestershire, UK). The measurements were performed on 20µM dendrimer solutions in J774A1 cell culture media at 24°C and compared to those in MilliQ water and DMEM cell culture medium, shown in table S2.

**Reagents**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2,2’-Azino-Bis(3-Ethylbenzthiazolin-6-Sulfonic acid), were purchased from Sigma Aldrich Ltd. (Dublin, Ireland). Alamar Blue (AB) was purchased from Biosource (UK). Cell culture media (DMEM, D5546) and supplements were purchased from Sigma Aldrich Ltd. (Dublin, Ireland) and Bioscience (Dublin, Ireland). 5-(and 6) Carboxy-2’, 7’-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen, 522406), IL-6 (900-K50), MIP-2 (900-K152), TNF-α (900-K52) ELISA kits were purchased from Peprotech (UK).

**Cell Culture**

J774A.1 is a mouse macrophage cell line, (ECACC, 91051511) derived from tumour in a female BALB/c mouse. J774A.1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 2mM L-glutamine supplemented with 10% fetal bovine serum (FBS), 45 IU ml⁻¹ penicillin and 45 IU ml⁻¹ streptomycin at 37°C in 5% CO₂.

**Preparation of Dendrimer solution**
Dendrimer test solutions were prepared in the cell culture media, under laminar flow and sterile conditions. They were soluble in the media at 37 °C and were dispersed uniformly by low speed vortex. The concentration ranges used for the cytotoxicity assays with G4, G5 and G6 were 0.08 µM to 6 µM; 0.03 µM to 2 µM; and 0.013 µM to 1 µM respectively, as determined from an initial range finding study.

Throughout this study, molar concentrations have been chosen as the unit of preference as they enable the response to be easily conceptualised in terms of number of particles, surface area and/or number of surface groups. For comparison, 1M – xxxg/l for G4, yyy g/l for G5 and zzzg/l for G6, respectively.

**Cytotoxicity assays**

Alamar blue (AB) and MTT assays were employed to assess the cytotoxicity of PAMAM dendrimers with the mouse macrophages (J774A.1) cells.

*Alamar blue (AB) assay*

For the cytotoxicity test, wells are plated with 100 µl of the following cell suspension concentration: 1× 10^5 cells/ml for 6, 12, and 24 h exposure; 8× 10^4 cells/ml for 48 h exposure and 6× 10^4 cells/ml for 72 h exposure in 96-well microplates (Nunc, Denmark). After 24h of cell attachment, plates were washed with 100 µl/well PBS and the cells were treated with increasing concentrations of each generation of dendrimer prepared in 5% FBS containing media. All incubations were performed at 37°C in a 5% CO₂ humidified incubator. Six replicate wells were used for each control and test concentrations per microplate.

The assays were carried out according to the manufacturer’s instruction. Briefly, control media or test exposures were removed; the cells were rinsed with PBS and 100µl of AB medium (5% v/v solution of AB) prepared in fresh media (without FBS or supplements) were added to each well. After 3h incubation, AB fluorescence was measured at the respective excitation and emission wavelength of 540 nm and 595 nm in a microplate reader (TECAN GENios, Grodig, Austria).

*MTT assay*
A parallel set of plates was set up for the MTT assay and seeded and exposed in an identical manner to that described in AB assay. After 24h of PAMAM dendrimer exposure, the control medium or test exposures was removed, the cells were washed with PBS and 100 µl of freshly prepared MTT in media (5 mg/ml of MTT in un-supplemented media) were added to each well. After 3h incubation, the medium was discarded and the cells were rinsed with PBS and 100 µl of DMSO were added to each well to extract the dye. The plates were shaken at 240 rpm for 10 min and the absorbance was measured at 595 nm in a microplate reader (TECAN GENios, Grodig, Austria).

**Intracellular Reactive Oxygen Species (ROS)**

Intracellular reactive oxygen species were measured by a fluorimetric assay using Carboxy H$_2$DCFDA as the probe. Carboxy H$_2$DCFDA was used because it carries an additional negative charge that improves its retention compared to non-carboxylated forms (http://probes.invitrogen.com/media/pis/g002.pdf). Intracellular oxidation of Carboxy H$_2$DCFDA to DCF was monitored according to the increase in fluorescence as measured by a plate reader and using confocal fluorescence microscopy. In brief, the assay was performed in black 96 well microplates (Nunc, Denmark). The J774A.1 cells were seeded in 100 µl of cell suspension in each well at a density of 4 x 10$^5$ cells/ml. After 24 h of cell attachment, plates were washed with 100 µl/well PBS and the cells were treated with increasing concentrations of each generation of dendrimer prepared in 5% FBS containing media. Hydrogen peroxide (400 µM) was used as positive control to validate the protocol. All incubations were performed at 37°C in a 5% CO$_2$ humidified incubator. Six replicate wells were used for each control and test concentrations per 96 well microplate. After the specified incubation time period (1, 2, 4 and 6 h) the plates were washed with 100 µl/well PBS and then 100 µl/well of 10 µM Carboxy H$_2$DCFDA was added to each well. The plates were incubated at 37°C for a period of 40 minutes. The fluorescence was quantified using a plate reader, which provides an average of the statistically variable response of individual cells (Elbekai and El-Kadi, 2005). Fluorescence was measured using an excitation of 485 nm and emission of 530nm, in a TECAN GENios (Grodig, Austria) microplate reader. For visualisation of the intracellular fluorescence, carboxy H$_2$DCFDA was excited at 488 nm and fluorescence emission at 520 nm (with a 505 nm long pass filter) was recorded using a confocal laser scanning microscope (LSM 510 META, Zeiss, Germany). Fluorescence and phase contrast images were recorded from a minimum of 3 areas per sample.
Cytokines assay

An enzyme linked immunosorbant assay (ELISA) was performed to quantify the proinflammatory mediators (IL-6, TNF-\(\alpha\) and MIP-2) after the exposure of the J774A.1 cells to PAMAM dendrimers. The low cost, routine technique is widely used to monitor and quantify the production of cytokines and is favoured over the relatively time consuming and costly techniques of qRT PCR (Osuchowski et al., 2005, 2006, Batista Jr. et al. 2006). LPS (lipopolysaccharide) was used as positive control to stimulate the TNF-\(\alpha\) and MIP-2 and validate the ELISA protocol. The principle of the ELISA is based on the sandwich technique, in which the capture antibody (primary antibody) at concentrations of 1\(\mu\)g/ml (TNF-\(\alpha\)), 2 \(\mu\)g/ml (IL-6) and 0.5 \(\mu\)g/ml (MIP-2) in PBS (pH -7.4), was coated in the 96 well plate (Nunc-immuno plate, Denmark). The plates were incubated over night at room temperature. The wells were aspirated to remove the liquid and the plates were washed four times with PBS-T (phosphate buffer saline with 0.05% of Tween 20) and then blocked with 1% BSA solution at room temperature for 1 hour. The plates were again washed with PBS-T four times and 100 \(\mu\)l of different dilutions of supernatant were added to the respective wells and standards of IL-6, TNF-\(\alpha\) and MIP-2 at a concentration from 10 to 800 pg/ml in duplicate were added to the first two columns of the 96 well plates and incubated for 2h at room temperature. The plates were aspirated and washed four times, whereupon 100\(\mu\)l of the detection antibody against the respective marker (secondary antibody) were added to the 96 well ELISA plate at a concentration of 0.25\(\mu\)g/ml (for TNF-\(\alpha\) and MIP-2), or 0.5 \(\mu\)g/ml (IL-6) and the plates were incubated at room temperature for 2h. The plates were aspirated and washed four times, 100 \(\mu\)l of avidine-HRP (1:2000 dilutions in blocking buffer) were added to each well and the plates were incubated for 30 minutes at room temperature. The plates were washed four times with washing buffer and 100 \(\mu\)l of substrate solution (2,2’-Azino-Bis(3-Ethylbenzthiazolin-6-Sulfonic acid)) were added to each well and the plates were incubated at room temperature to develop the colour. The colour development time was optimised to be 15 minutes for each assay using the standards and the absorbance was measured at 405 nm in a VICTOR\(^3\)V\(^\text{TM}\) 1420 Multilabel Counter plate reader (Perkin Elmer, USA).

Statistics
All experiments were conducted in at least triplicate (three independent experiments). Fluorescence as fluorescent units (FUs) of all of the assays was quantified using a microplate reader (TECAN GENios, Grödig, Austria). Raw data from cell cytotoxicity assays, all the ELISA and ROS data were collected and analyzed using Microsoft Excel® (Microsoft Corporation, Redmond, WA). Cytotoxicity and ROS data were expressed as mean percentage viability relative to the unexposed control (100%) ± standard deviation (SD). MIP-2, IL-6 and TNF-α data were calculated from their respective standards and were expressed in mean (pg/ml) ± standard deviation (SD). Statistical analyses were carried out using one-way analyses of variance (ANOVA) followed by Dunnett’s multiple comparison tests. Statistical significance was accepted at $P \leq 0.05$ for all tests. Toxicity data was fitted to a sigmoidal curve and a four parameter logistic model was used to calculate EC$_{50}$ values. This analysis was performed using Xlfit3™ a curve fitting add-in for Microsoft Excel (ID Business Solutions, UK).

**RESULTS**

**Characterisation of PAMAM dendrimers**

The PAMAM dendrimers (G-4, G-5 and G-6) were characterised in terms of particle size, and zeta potential. From our previous study, the hydrodynamic diameter correlated well with that quoted by the manufactures (Naha et al., 2009). In the J774A.1 cell culture media, the hydrodynamic diameter of G-4, G-5 and G-6 was 6.2 nm ± 0.3 nm; 7.5 nm ± 0.3 nm and 10.3 nm ± 0.4 nm respectively. The slight increase in size in cell culture media may be due to the interaction with proteins or other components of the cell culture media producing a ‘protein corona’ as has been observed with other nanoparticles (Lynch et al., 2007).

In Milli Q water and DMEM cell culture medium, the zeta potential of the PAMAM dendrimers was observed to be positive, due to the cationic surface amino group. However, the surface charge was observed to be negative in the J774A1 cell culture medium containing 5% FBS due to the interaction of proteins with the surface amino group, as observed in our previous study (Naha et al., 2009). The zeta potential of G-4, G-5 and G-6 was found to be -2.9 ± 1.1; -3.1 ± 0.9; and -3.9 ± 0.4 mV respectively (Table 1). This is a clear indication of the interaction of the protein with the PAMAM dendrimers as shown previously (Naha et al., 2009).

**Cytotoxicity assay**
The cytotoxicity of PAMAM dendrimers was determined using two different assays for an exposure time of 24h and the results are shown in Figure 1, with the EC$_{50}$ values shown in Table 2. The toxic response observed in both assays is well matched and there is no significant difference between the calculated EC$_{50}$ from both the assays in J774A1 cells.

A time and generation dependent toxic response was furthermore observed as shown in Figure 2a. Maximum cell death occurs at 72 h exposure of all the three generation of PAMAM dendrimers. The trend of toxic response was G-6 > G-5 > G-4, as observed previously for aquatic species and fish cell lines (Naha et al., 2009) as well as mammalian cell lines (Mukherjee et al., 2009). Notably, the toxic response in terms of inverse EC$_{50}$ (Ragnvaldsson et al., 2007) is linearly correlated with the number of surface primary amino group present in the PAMAM dendrimers shown in Figure 2.b for the case of 24 h exposure, demonstrating a clear structure-activity relationship. In order to further explore the mechanisms of cell death, the induced oxidative stress and inflammatory response were explored.

**Intracellular Reactive oxygen species (ROS)**

The intracellular ROS study was performed at different time points (1, 2, 4 and 6h) and with different exposure concentrations of each dendrimers (G-4, G-5 and G-6). For all generations, the exposure time points and the concentration of PAMAM dendrimer (0.031 µM to 3 µM) used were the same, in order to correlate the increased ROS production by the three generations of PAMAM dendrimers and also to enable comparison with the cytotoxic and inflammatory response. Intracellular ROS production in the macrophage cells upon the exposure to PAMAM dendrimers was easily visualised using confocal fluorescence microscopy, as shown in figure 3 for the case of 2 h exposure of each dendrimer at a concentration of 1 µM. The fluorescence was quantified using a plate reader, which provides an average of the statistically variable response of individual cells (Elbekai and El-Kadi, 2005). This in situ method was favoured over flow cytometry as J7741.A cells are strongly adherent and do not detach easily from the flask after trypsinisation. The concentration dependent increase in ROS production for four different time points for the three dendrimer generations was monitored and the results are shown in the supporting information (Figure S1a-d). Increased ROS production was found to be dendrimer generation and time dependent (Figure 4a). In a previous study of poly (propyleneimine) dendrimers (PPI), a similar generation dependent intracellular ROS production and reduction of mitochondrial membrane
potential was observed in macrophages (Kuo et al., 2007). The generation dependence follows the trend of G-6 > G-5 > G-4, as was seen for the cytotoxic response. Similar to the case of the cytotoxic response, the generation dependent ROS levels appear to be correlated with the increase of surface primary amino group with increasing generation, as shown in figure 4b for the case of 1 h exposure.

Cytokines production

Macrophage inflammatory protein-2 (MIP-2)

The level of macrophage inflammatory protein-2 (MIP-2) secretion by the J774A.1 cells following exposure to PAMAM dendrimers was analysed from the cell culture supernatant after exposure to 5 different concentrations of each dendrimer and also at 5 different exposure time periods (6, 12, 24, 48 and 72h). MIP-2 levels at different exposure time periods were calculated from the standard curve of the MIP-2 standards (ranging from 10 to 800 pg/ml). The secretion of MIP-2 level was found to be time, PAMAM generation and concentration dependent, as shown in Figures 5a-c and 6. Maximum levels of MIP-2 were generated at all the time points at concentrations of G-4 (1.2 µM); G-5 (0.4µM) and G-6 (0.2 µM) and The secretion of MIP-2 protein from the macrophage cell was found to be generation dependent, G-6 producing the highest level and G-4 the lowest, (G-6 > G-5 > G-4) which is well correlated with the cytotoxic response and the ROS generation. Although a linear dependence is not obvious, the MIP-2 protein secretion levels increase monotonically with the number of surface primary amino group of the PAMAM dendrimer, as shown in Figure 7a.

Interleukin-6 (IL-6)

The levels of IL-6 in after different exposure time periods (6, 12, 24, and 48 h) were calculated from the standard curve of IL-6 standards (ranging from 10 to 800 pg/ml). In the case of G-4 (0.6 µM); G-5 (0.4 µM) and G-6 (0.2 µM), the maximum levels of IL-6 were observed after 24 h exposure. A generation dependent IL-6 secretion from the macrophage cells was observed upon the exposure to PAMAM dendrimers as shown in Figure 6, G-6 producing the highest levels and G-4 the lowest (G-6 > G-5 > G-4) which is well correlated with the cytotoxic response. Again the level of IL-6 secretion by the J774A.1 cells increases monotonically with the surface area of the each dendrimer generation, as shown in Figure 7 b. The secretion of IL-6 was time dependent, generation dependent and concentration of PAMAM dependent shown in supporting information (Figure S2a-c).
Tumour necrosis factor-α (TNF-α)

The TNF-α levels at different exposure times (6, 12, 24, and 48 h) were calculated from the standard curve of TNF-α standards (ranging from 10 to 800 pg/ml). Maximum levels of TNF-α were generated at concentrations of G-4 (1.2 µM); G-5 (0.8 µM) and G-6 (0.2 µM) after 24h exposure. A generation dependent TNF-α secretion from the macrophage cells was observed upon the exposure to the dendrimers, G-6 producing the highest levels and G-4 the lowest, (G-6 > G-5 > G-4) (Figure 6) which is well correlated with the cytotoxic response. Again the levels of TNF-α secretion by the J774A.1 cells increase monotonically with the number of surface primary amino group of the PAMAM dendrimer, shown in Figure 7c. The secretion of TNF-α was found to be time dependent, PAMAM generation and concentration dependent shown in supporting information (Figure S3a-c).

DISCUSSION

The cytotoxic response of J774A.1 cells to the PAMAM dendrimers, with varying numbers of –NH₂ surface group (G-4, G-5 and G-6), was evaluated using the Alamar blue (AB) and MTT assays. The EC₅₀ was calculated and no significant difference was observed between the assays after 24 h exposure (Figure 1). Alamar blue is a water-soluble dye and the oxidized form enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from the cytochromes (Al-Nasiry et al., 2007). Mitochondrial activity was determined by the MTT assay. MTT is reduced to the purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. MTT is completely reduced by mitochondrial enzyme. The close agreement of the EC₅₀ as determined by the two assays is an indication of significant mitochondrial injury as origin of the cytotoxic response.

The toxic response was seen to increase with dendrimer generation and therefore size. A similar generation dependent toxicological response was previously seen with fish cells (Naha et al., 2009) and also mammalian cells lines (Mukherjee et al., 2009). A toxic response
which increases with nanoparticle diameter would appear to go against the commonly accepted trends of increased toxicity with decreased nanoparticle size, but can be understood in terms of the linear correlation with the number of surface amino groups of PAMAM dendrimer as shown in Figure 2 b.

The surface primary amino groups render the dendrimers cationic and as the generation of PAMAM dendrimer increases, there is a linear increase in the number of surface amino groups (Dendritech, Inc. http://www.dendritech.com/pamam.html). The observed generation dependence suggests that the toxic response has origin in the actions of the surface charge (Figure 2b). Cationic particles have been shown to localise in mitochondria and produce a cytotoxic response via the mitochondrial injury pathway (Xia et al 2008 and 2006), generating ROS as a result (Nel et al., 2006; Lee et al., 2003).

In this study, intracellular ROS generation by PAMAM dendrimers is clearly one of the toxic pathways and a clear generation dependence of intracellular increased ROS production is shown in Figure 4a. The sequence of increased ROS generation is G-6> G-5 > G-4. This response is also well correlated with the number of surface primary amino group of PAMAM dendrimers, as shown in Figure 4b. This indicates that the cationic surface amino groups play a direct role in the production of ROS. Increased intracellular ROS generation suggests that the PAMAM dendrimers can lead to disruption of the mitochondrial electron transduction chain which leads to additional $O_2^-$ production (Donaldson et al., 2005) and perturbs the mitochondrial permeability transition pore, which leads to release of pro-apoptotic factors and programmed cell death (Oberdorster et al., 2005).

Intracellular ROS production can lead to inflammation, as demonstrated for a number of different nanoparticles (Li et al., 2008; Stone et al., 2007; Lubos et al., 2008; Driscoll, 2000). Oxidative stress activates the MAPK signalling pathway, inducing transcription factors such as NFkB and AP-1, and these transcription factors induce mRNA expression of pro-inflammatory mediators and finally cause inflammation (Park and Park 2009). Macrophage inflammatory protein-2 (MIP-2) plays a major role in mediating the neutrophilic inflammatory response to nanoparticles (Driscoll et al., 1995). It is a potent neutrophil chemotactant and epithelial cell mitogen and is involved in acute pulmonary inflammation and mediates tissue damage (Walley et al., 1997; Chung et al., 2003). It is induced by LPS (Lipopolysaccharide), oxidative stress in a wide range of cells including alveolar
macrophages, mast cells, peritoneal macrophages, epithelial cells and fibroblasts (Monteiller et al., 2007).

Inflammatory response induced by nanoparticles is thus a further toxic mechanism (Monteiller et al., 2007), and in this work we have highlighted the inflammatory mediators (MIP-2, TNF-α and IL-6) induced by PAMAM dendrimers. The level of secretion of all three inflammatory mediators, MIP-2, TNF-α and IL-6 following exposure of macrophage cells to PAMAM dendrimers is dependent on the concentration, time of exposure and the generation of PAMAM dendrimers, as well as the cytotoxic response. A generation dependent cytokine production was found in all cases and the trend was G-6 > G-5 > G-4. Thus the ROS production, inflammatory response and cytotoxicity all show similar trends in terms of dendrimer structure. The pathway of the toxic response induced by PAMAM dendrimers may therefore be one of localisation in the mitochondria (Lee et al., 2009) leading to disruption of the mitochondrial electron transduction chain, and additional O$_2^-$ production (Donaldson et al., 2005) resulting in oxidative stress (Figure 3 and Figure 4) The systematic mechanistic pathways of cell death due to PAMAM dendrimers is presented in Figure 8a, and the sequence of responses as a function of time is shown in Figure 8b. Within the resolution of the measurements performed, the timing sequence is identical for all three dendrimer generations.

Although the toxic responses can be well correlated with the dendrimer generation structures, it should be noted that the particle size was seen to increase in the cell culture medium and furthermore that the zeta potential of the PAMAM dendrimers changed polarity from positive to negative. These observations point towards the interaction of proteins and/or other molecular components of the medium with the particle surface. In our previous study, we have seen spectroscopically the interaction of FBS with the PAMAM dendrimers (Naha et al., 2009), as has been documented for other nanoparticles, leading to the formation of a protein corona (Lynch et al., 2007). In the case of carbon nanotubes, such interactions have been shown to result in medium depletion and a secondary or indirect toxicity (Casey et al., 2008) even though the carbon nanotubes are not seen to be taken up by the cells (Davoren et al., 2007). From this point of view it is important to understand whether toxic responses originate from the interaction of particles external or internal to the cells.

Significant ROS generation has also been seen from carbon nanotubes external to the cells (Herzog et al., 2009). Thus it is conceivable that the ROS, cytokines production, cytotoxicity
cascade could be initiated from external stress (Herzog et al., 2009). However, a recent study has clearly demonstrated the internalisation of PAMAM dendrimers and their localisation in the mitochondria (Lee et al., 2009). Thus, although there may be external stress leading to some degree of indirect toxic response, it is proposed that the principal response is a direct result of internalisation of the nanomaterials.

**CONCLUSIONS**

PAMAM dendrimers show a significant cytotoxic response in mouse macrophage cells (J774A.1) *in vitro* at a concentration of 0.013 to 6 µM. The generation dependence (G-6 > G-5 > G-4) of the production of increased intracellular ROS, inflammatory mediators and the cytotoxicity indicates the direct effects of the positively charged surface amino groups. The mechanism of the toxic response is proposed to be one of localisation of the cationic particles in the mitochondria, leading to significant increase in ROS generation, induction of cytokines production and ultimately cell death. The generation dependent intracellular ROS levels, cytokines production and cytotoxicity of PAMAM dendrimers point towards the basis of structure activity relationships.

The choice of concentration ranges of the PAMAM dendrimer exposure for each study was guided by the manifestation of a generation dependent response. These concentrations are moreover of relevance for proposed therapeutic applications (Kukowska-Latallo et al., 2005). Based on the OECD Guidelines for acute oral toxicity (De Jong, 2009) and using an approximate guideline that *in vitro*, 1 mg/ml may be considered equivalent to 1g/kg (http://probes.invitrogen.com/media/pis/g002.pdf), the toxicity may be classified as the lowest Category, 5. As the toxicity may therefore be considered acceptable and based on the improvement in the cytokine production (TNF-α, MIP-2 and IL-6), PAMAM dendrimers may potentially be useful, for example as a vaccine delivering agent.

**Acknowledgement** : This work was conducted under the framework of the INSPIRE programme, funded by the Irish Government's Programme for Research in Third Level Institutions, Cycle 4, National Development Plan 2007-2013, supported by the European Union Structural Fund.

**Reference**


http://probes.invitrogen.com/media/pis/g002.pdf


Legends of Tables

Table 1. EC<sub>50</sub> data of PAMAM dendrimer G-4, G-5 and G-6 in Alamar blue (AB) and MTT assay for 24 hour exposure in J774A.1 cells.

Table 2. Zeta potential of PAMAM dendrimers in different media.

Legends of Figures

Figure 1. Comparison of cytotoxicity assay between AB and MTT of (a) G-4, (b) G-5 and (c) G-6 PAMAM dendrimers in J774A.1 cells after 24h exposure. The data shown in mean ± SD (n=3).

Figure 2. (a) Time and generation dependence of toxic response of PAMAM dendrimers (G-4, G-5, G-6). (b) Correlation between number of surface amino group and the toxic response of PAMAM dendrimers at 24h exposure (AB assay). All the data are expressed in mean ± SD (n=3).

Figure 3. Confocal fluorescence and phase contrast micrographs of intracellular ROS generation following exposure to (a) negative control, (b) positive control (H<sub>2</sub>O<sub>2</sub>), (c) PAMAM G-4, (d) PAMAM G-5 and (e) PAMAM G-6. The data are shown after 2h exposure of PAMAM dendrimers.

Figure 4. (a) generation dependent intracellular reactive oxygen species (ROS) production following exposure to PAMAM dendrimers (G-4, G-5 and G-6) in J774A.1 cells at an exposure concentration of 1µM. The data are presented in mean ± SD (n=3), (b) correlation between the production of intracellular ROS and number of surface primary amino groups.
The data is presented upon the exposure of different concentration of PAMAM dendrimers with mean ± SD.

**Figure 5.** The level of MIP-2 secreted after exposure of J774A.1 cells to (a) G-4, (b) G-5 and (c) G-6 PAMAM dendrimers for 6h, 12h, 24h, 48h and 72h. These data are presented in mean ± SD (n=3).

**Figure 6.** Generation dependent MIP-2, IL-6 and TNF-α secretion after 24h exposure of PAMAM dendrimers (G-4, G-5 and G-6) in J774A.1 cells. The data are shown as mean ± SD (n=3).

**Figure 7.** Correlation between inflammatory mediator (a) MIP-2, (b) IL-6 and (c) TNF-α response and the surface area of PAMAM dendrimers (G-4, G-5 and G-6). All the data are presented in mean ± SD (n=3).

**Figure 8.** (a) Schematic of the systematic sequence of events in J774A.1 cells following exposure to PAMAM dendrimers. (b) represents the schematic of different responses as a function of time.
Table 1.

<table>
<thead>
<tr>
<th>Different media</th>
<th>PAMAM G-4 in mV</th>
<th>PAMAM G-5 in mV</th>
<th>PAMAM G-6 in mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q water</td>
<td>8.3 ± 5.5</td>
<td>21.5 ± 8.7</td>
<td>26.5 ± 4.7</td>
</tr>
<tr>
<td>DMEM</td>
<td>6.7 ± 3.7</td>
<td>18.0 ± 2.1</td>
<td>14.4 ± 3.0</td>
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<td>5% J774A.1 Cell culture media</td>
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<td>-3.1 ± 0.9</td>
<td>-3.9 ± 0.4</td>
</tr>
<tr>
<td>PAMAM Dendrimer</td>
<td>EC$_{50}$ of AB assay in (µM)</td>
<td>EC$_{50}$ of MTT assay in (µM)</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td>G-4</td>
<td>1.6 ± (0.3)</td>
<td>1.4 ± (0.3)</td>
<td></td>
</tr>
<tr>
<td>G-5</td>
<td>0.5 ± (0.2)</td>
<td>0.5 ± (0.2)</td>
<td></td>
</tr>
<tr>
<td>G-6</td>
<td>0.3 ± (0.1)</td>
<td>0.2 ± (0.1)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. a.

Concentration of PAMAM (G-4) dendrimer in µM

% Cell Viability

b.

Concentration of PAMAM (G-5) dendrimer in µM

% Cell viability
Concentration of PAMAM (G-6) dendrimer in µM

% Cell Viability

AB
MTT
Figure 2. a.

![Bar graph showing EC50 in µM for different time points (24H, 48H, 72H) and groups (G4, G5, G6).]

b.

![Line graph showing the inverse of EC50 in µM for different no. of surface amino groups (64, 128, 256) and groups (G4, G5, G6).]
Figure 3.
Figure 4b.
Figure 5. a.

Concentration of PAMAM dendrimer in µM

MIP-2 Level in pg/ml

6H 12H 24H 48H 72H

b.

Concentration of PAMAM dendrimer in µM

MIP-2 Level in pg/ml

6H 12H 24H 48H 72H
Concentration of PAMAM dendrimer in µM

MIP-2 Level in pg/ml

- 6H
- 12H
- 24H
- 48H
- 72H
Figure 6.

The graph illustrates the level of TNF-α, MIP-2, and IL-6 in pg/ml for different generations of PAMAM dendrimers (G-4, G-5, G-6). The bars represent the mean with standard deviation error bars. The levels of each cytokine are quantified and compared across the different generations of dendrimers.
Figure 7. a.

![Graph showing Level of MIP-2 in pg/ml against Number of surface primary amino groups for different time points (6H, 12H, 24H, 48H, 72H).]

b.

![Graph showing Level of IL-6 in pg/ml against Number of surface primary amino groups for different time points (6H, 12H, 24H, 48H).]
Figure 8a.

Cellular uptake

PAMAM dendrimers (G-4, G-5 and G-6)

Intracellular ROS

Cytokines, Chemokines,

IL-6

TNF-α

MIP-2

Cytotoxicity

Lee et al.
Supporting information for

Reactive Oxygen Species Induced cytokines production and cytotoxicity of PAMAM dendrimers in J774A.1 cells

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Figure S.1.a.

(a) % of cell fluorescence as compared to control vs. Conc of PAMAM dendrimers in µM

(b) % of cell fluorescence as compared to control vs. Conc of PAMAM dendrimers in µM

Key:
- G4-1h
- G5-1h
- G6-1h
- G4-2h
- G5-2h
- G6-2h
d.

Figure S.2. a
Concentration of PAMAM (G-4) dendrimer in µM

IL-6 level in pg/ml

6H
12H
24H
48H

Concentration of PAMAM (G-5) dendrimer in µM

IL-6 Level in pg/ml

6H
12H
24H
48H
Concentration of PAMAM (G-5) dendrimer in µM

IL-6 Level in pg/ml

6H
12H
24H
48H
Figure S.3. a

Concentration of PAMAM (G-4) dendrimer in μM

TNF-α level in pg/ml

6H  12H  24H  48H

b.

Concentration of PAMAM (G-5) dendrimer in μM

TNF-α Level in pg/ml

6H  12H  24H  48H
Concentration of PAMAM (G-6) dendrimer in µM

TNF-α Level in pg/ml

- 6H
- 12H
- 24H
- 48H
Legends of Figures

**Figure S1.** Concentration and time dependent intracellular ROS generation by G-4, G-5 and G-6 at (a) 1, (b) 2, (c) 4 and (d) 6 h exposure time points. The data are presented as mean ± SD (n=3).

**Figure S2.** Secretion of IL-6 following exposure of (a) G-4, (b) G-5 and (c) G-6 in J774A.1 cells at 6, 12, 24 and 48 h time points. The data are presented as mean ± SD (n=3).

**Figure S3.** Secretion of TNF-α upon the exposure of (a) G-4, (b) G-5 and (c) G-6 in J774A.1 cells at 6, 12, 24 and 48 h time points. The data are presented as mean ± SD (n=3).