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Intracellular Localisation, Geno- and Cytotoxic Response of Poly N-isopropylacrylamide (PNIPAM) Nanoparticles to Human Keratinocyte (HaCaT) and Colon Cells (SW 480)

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Intracellular localisation, Geno- and Cytotoxic response of Poly *N*-isopropylacrylamide (PNIPAM) nanoparticles to human keratinocyte (HaCaT) and colon cells (SW 480)

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Abstract

PNIPAM nanoparticles, with and without a covalently-linked fluorescent label, were prepared by a free radical polymerisation technique. The cyto- and genotoxicity of PNIPAM nanoparticles were analysed in two representative mammalian cell lines, SW480, a colon, and HaCaT, a dermal cell line. Physical characterisation in terms of particle size and zeta potential of the PNIPAM nanoparticles was carried out both in aqueous solution and in the appropriate cell culture media. Uptake and co-localisation of fluorescently labelled PNIPAM nanoparticles was monitored in both cell lines using confocal laser scanning microscopy. Genotoxicity analysis using the Comet assay was performed in both cell lines to evaluate any DNA damage. It was observed that the PNIPAM nanoparticles were internalized and localised in lysosomes within 24 hrs. No significant cytotoxic response ($p \leq 0.05$) was observed in either cell line over concentration ranges from 25 mg/l to 1000 mg/l for all exposure time periods. Furthermore, no significant genotoxic response ($p \leq 0.05$) was observed in either cell line over concentration ranges from 12.5 mg/l to 800 mg/l for all exposure time periods. The results suggest that the PNIPAM nanoparticles show excellent biocompatibility *in vitro*.

Key words: Nanotoxicology, Aggregation, PNIPAM, HaCaT, SW 480, Lysosomes, Genotoxicity, *In Vitro*.

Introduction

Nanomaterials are widely used in a range of biomedical applications such as drug delivery, diagnostics (as MRI contrast agents) and tissue engineering. Poly *N*-isopropylacrylamide (PNIPAM) is a well known thermoresponsive polymer (Hsiue et al. 2002) which exhibits a lower critical solution temperature (LCST) of about 32°C in aqueous media (Xu et al., 2006). Adjustment of the LCST to near body temperature (Zhang and Misra 2007) is essential, particularly for ‘smart’ drug delivery applications (Peppas et al. 2000; Lin and Metters 2006). Because of this reversible phase transition, PNIPAM has been widely used in the preparation of stimuli responsive systems for biomedical applications, such as in the controlled release of drugs and in tissue engineering (Kavanagh et al. 2005; Xu et al., 2006; Xu et al. 2004; Zhang et al., 2005). PNIPAM has also been developed and proposed for controlled release of ophthalmic drops for glaucoma therapy (Hsiue et al. 2003; Hsiue et al. 2002). NIPAM/BAM copolymer nanoparticles of varying size and copolymer ratios have been observed to adsorb plasma proteins on to their surface with potential implications for selective biological interactions (Cedervall et al. 2007). Recently, NIPAM/BAM copolymer particles have been shown to retard or even reverse the fibrillation of amyloid- β , the protein involved in Alzheimer’s disease (Cabaleiro-Lago et al. 2008).

In particulate form (both micro and nano), several polymeric systems have been used for the delivery of drugs and therapeutic proteins (Blasi et al. 2007; Silva et al. 2006; Ito et al. 2008; Naha et al. 2008 and 2009a). Given their potential widespread use, there is an increasing need for information regarding the human health and environmental implications of these polymeric nanomaterials. To date the human health impacts of nanomaterials have received the greatest attention, and it has been demonstrated through both *in vivo* and *in vitro* studies with mammalian test systems that the properties that make nanomaterials so attractive from a commercial application viewpoint (*e.g.* nanoparticle size and increased surface area) can also potentially be responsible for undesirable health effects (Meng et al. 2007; Oberdorster et al., 2005; Papageorgiou et al. 2007; Poland et al. 2008; Singh et al. 2007). However, to date, no conclusive links between engineered nanoparticles and a biological or health impact have been observed, and insufficient data exists to make generalisations about the biocompatibility or safety of nanomaterials in general, or even about a specific nanoparticle type.

Nanomaterials such as polyamidoamine dendrimers (generation 3.5) have been shown to contribute to the reduction of trans-membrane potential and to hinder the influx of Ca^{2+} ions in the mitochondria of rat liver cells (Labieniec and Gabryelak 2008). Mitochondrial Ca^{2+} overload in combination with oxidative stress and ATP depletion induces mitochondrial permeability which results in ischemia reperfusion, oxidative stress, and apoptosis (Vergun and Reynolds 2005). In a recent study, it has been shown that nano-sized PAMAM dendrimers G-4, G-5 and G-6, of diameter 4.5nm, 5.4 nm and 6.7nm respectively, produce toxic and inflammatory-like responses via an oxidative stress pathway in J774A.1 cells (Naha et al., 2010a). A known route to toxicity both *in vitro* and *in vivo*, which has been shown for combustion derived pollution particulates, and more recently identified for several nanomaterials, starts with the induction of oxidative stress by free radical formation at the particle surface (Donaldson et al. 2006; Lanone and Boczkowski 2006; Nel et al. 2006; Oberdorster et al., 2005). In excess, free radical formation causes damage to biological components through oxidation of lipids, proteins and DNA (Singh et al. 2009).

The aim of the present investigation, therefore, is the measurement, characterisation and assessment of the mammalian cytotoxicity and genotoxicity of PNIPAM nanoparticles in immortalised non-cancerous human keratinocyte (HaCaT) and a primary adenocarcinoma of colon (SW480) cell line, as dermal and intestinal models respectively, these being considered two of the potential routes of exposure to nanomaterials. Physico-chemical characterisation of PNIPAM nanoparticles was performed in terms of measurement of hydrodynamic diameter and zeta potential in the appropriate cell culture media. As the particles are thermo-responsive in nature, the particle size was measured as a function of temperature, because all exposures were performed at 37 °C. Cellular uptake and co-localisation studies were carried out with fluorescently-labelled PNIPAM nanoparticles, synthesised in the presence of a fluorescent co-monomer, using Confocal Laser Scanning Microscopy (CLSM).

2. Materials and methods

2.1 Test compounds

Poly *N*-isopropylacrylamide (PNIPAM) nanoparticles were synthesised by free radical polymerisation. Prior to the reaction, the monomer was re-crystallised twice from n-hexane, by heating until dissolved, and then cooling slowly, vacuum filtering and air-drying. Once dried it was stored at 4° C in the dark until used. The procedure

for the synthesis was as follows: 2.8g monomers, and 0.28g crosslinker (N,N-methylenebisacrylamide) were dissolved in 190 ml MilliQ water [MQ] with 0.8 g SDS and the solution was degassed by bubbling with nitrogen gas for 30 minutes.

Polymerisation was induced by adding 0.095g ammonium persulfate initiator in 10 ml MQ water and heating at 70°C for 4 hours. Particles were extensively dialysed against MQ water for several weeks, changing the water daily, to remove all traces of monomer, crosslinker, initiator and SDS. Particles were freeze-dried and stored in the fridge until used.

Fluorescently tagged PNIPAM nanoparticles with nominally 500 fluorescent labels per particle were synthesized as follows. 0.1 g of SDS was mixed with 0.0044 g of methacryloxyethyl thiocarbamoyl Rhodamine B in 10 ml of Milli Q water and then sonicated using the Covaris S2 at a frequency of 450 kHz for 500 seconds until most of the dye was visibly dissolved. The solution was transferred into a falcon tube adding an additional 10 mL Milli Q water together with the rest the SDS (0.3 g) and then sonicated using a ultrasonic bath (Branson 1510) at a frequency of 42 kHz for 5 hours continuously until the dye was completely dissolved in the SDS. The monomers (1.4 g of NIPAM, 0.14 g of cross linker) were added to this solution with 75 ml of Milli Q water, stirred for 30 minutes under nitrogen flow to remove dissolved O₂, heated at 70°C and then the synthesis was performed by adding a degassed solution composed of 0.0475 g of initiator diluted in 5 ml of Milli Q water. The reaction was carried out for 12 hours at 70°C and under nitrogen flow. The labelled particles were dialysed against ethanol for 6 days and then extensively dialysed in ultrapure water, freeze dried and stored at 4°C.

2.2 Particle dispersions for uptake, and toxicity testing

Due to the inverse solubility of PNIPAM nanoparticles, solutions were prepared by dispersing the particles on ice to ensure good solubility of the particles (i.e. to ensure that the solutions are below the lower critical solution temperature of the particles and thus that polymer-water contacts are more favorable than polymer-polymer contacts resulting in uptake of water and swelling of the particles), before gradually warming them to the test conditions. Particle dispersions of different concentration were prepared in Dublecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM F-12) with 2mM L-glutamine supplemented with 5 % foetal bovine serum (FBS), 45 IU ml⁻¹ penicillin and 45 IU ml⁻¹ streptomycin.

2.3. Particle Characterisation

2.3.1 Particle size measurement

The hydrodynamic diameters of the PNIPAM nanoparticles, unlabelled and labelled, in the appropriate assay media were analyzed using a Zeta sizer (Malvern Instruments, UK). For a typical experiment, approximately 1.5 ml of a 100 and 1000 µg/ml concentration of nanoparticles in MQ water, DMEM and the supplemented cell culture media were measured over the temperature range from 30 °C to 38 °C with an interval of 2 °C.

Particle size was also determined by Electron Microscopy. Samples were prepared by negative-contrast staining as described previously (Gorelov et al 1997). Briefly, stock solutions of Tungstophosphoric acid (TPA, 200 mg / ml) (Fluka) and labelled NIPAM nanoparticles (5mg / ml) were prepared in water, and were left in a drying cabinet for about 2 hours at 55 °C. The mixing of the final solution and the sample preparation was done in the drying cabinet at a constant temperature of 55 °C. The final solution contained 20 mg / ml of TPA and 4.5 mg /ml of NIPAM nanoparticles, and was left in the drying cabinet for about 15 minutes together with the EM grids. A drop of this final solution was placed on the grid and immediately soaked with filter paper in order to leave on the grid a thin film of nanoparticles, in this way minimising the nanoparticle aggregation during the drop drying time. Samples were investigated in a TECNAI G² 12 TWIN TEM using an acceleration voltage of 120 kV and objective aperture of 20 µm. Digital images were recorded with a MegaView III (SIS) camera.

2.3.2 Zeta potential measurement

The zeta potential of the PNIPAM particles, both unlabelled and labelled, in MQ water, DMEM and the cell culture media was measured using the Zeta sizer (Malvern Instruments, UK). Although the zeta potential is also affected by temperature (size), measurements were conducted at 20 °C only, using a concentration of 100 µg/ml.

2.4 Reagents

Alamar Blue (AB) was purchased from Biosource (UK). LMP agarose. Ethyl-N-nitrosourea (ENU). Cell culture media and supplements were purchased from Sigma Aldrich (Ireland) and Bioscience (Dublin, Ireland). SYBR[®] Green and GelBond[®] films were purchased from Lonza (Rockland, USA). Lab- Tek[®] Chamber Slides[™]

were purchased from Nalge Nunc International, LysoTracker[®] Green DND-26 from Molecular probes, Invitrogen (UK).

2.5 Cell Culture

HaCaT cells, an **immortalized** non-cancerous human keratinocyte cell line (kindly provided by Prof. Dr. Boukamp, Heidelberg) and SW480 cells (ATCC, CCL-228) a primary adenocarcinoma colon cell line, were employed for testing. SW480 cells were cultured in Dublecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM 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. HaCaT cells were cultured in the same cell culture media under the same conditions with the addition of 1µg/ml hydrocortisone (Smola et al., 1993).

2.6 Cytotoxicity assay

For the cytotoxicity test, 96-well microplates (Nunc, Denmark) were plated with 100 µl of the following cell suspension concentrations: 1 × 10⁵ cells/ml for 24h exposure; 8 × 10⁴ cells/ml for 48h exposure; 6 × 10⁴ cells/ml for 72h exposure and 4 × 10⁴ cells/ml for 96h exposure. After 24 h **incubation**, plates were washed with 100 µl/well PBS and the cells were treated with increasing concentrations of PNIPAM particles prepared in 5% FBS containing media (concentrations tested were 25, 50, 100, 200, 400, 600, 800 and 1000 mg/L). 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. Note that, due to the nature of the assay, and the need for lower cell numbers for the longer duration exposure experiments (to allow sufficient room for the cells to proliferate), for each exposure concentration the ratio of particles:cells (and hence the exposure dose) was different for each time-point, with the dose per cell being a factor of 2.5 different between the 24 and 96 hour exposures initially, and the dose being distributed among the daughter cells during proliferation.

Alamar Blue (AB) uptake was used as a cytotoxicity assay. The assay was carried out according to the manufacturer's instructions. Briefly, control media or test exposures were removed; the cells were rinsed once 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 of incubation, AB fluorescence was measured at the excitation and emission wavelengths of 540 nm and 595 nm respectively, in a microplate reader (TECAN GENios, Grodig, Austria). In order to ensure that the presence of PNIPAM nanoparticles did not influence the assay readout, the fluorescence intensity of AB media in the absence and presence of PNIPAM particles was compared, and no significant difference was observed, suggesting that the particles do not interact with the AB.

2.7 Alkaline Comet assay

The genotoxicity of PNIPAM nanoparticles was assessed using the micro-comet assay technique in both the cell lines (HaCaT and SW480 cells). For a typical experiment, 100 μ l of 1×10^5 cells/ml for 24h; 8×10^4 cells/ml for 48h; 6×10^4 cells/ml for 72h exposure of nanoparticles were plated in 96 well microplate and incubated at 37°C in 5% CO₂ for 24 hours to ensure cell attachment. The cell monolayers were then washed with PBS and exposed to varying particle concentrations (12.5 mg/l, 25 mg/l, 100 mg/l, 200 mg/l, 400 mg/l, and 800 mg/l) for different time intervals (24h, 48h and 72h). After the appropriate exposure time, cells were washed once with PBS, trypsinized and suspended in low melting point agarose and cast onto a gel bond film fixed with chamber slides. After the agarose solidified, it was suspended in freshly prepared and pre-cooled cell lysis buffer overnight. The following day, electrophoresis was conducted in alkaline electrophoresis buffer (pH 12.7) for 12 mins (conditions: 300 mA, 1.5 V/cm at 4°C). After completion of the electrophoresis run time, the Gelbond™ film was treated with neutralisation buffer (pH 7.5) for 30 mins to neutralise the DNA embedded gels and then dehydrated in absolute ethanol for 2 h. Gels were stored in the dark overnight at 4°C, allowed to dry completely, and were then stained with SYBR-Green nucleic acid stain. Image analysis was performed using Komet 5.5 software (ANDOR™, UK) and a Nikon Eclipse E600 microscope attached to a CCD camera. Values of the olive tail moment (OTM) and percentage of tail DNA were automatically calculated by the software. Ethyl Nitrosourea (ENU) was used as a positive control to validate the experimental protocol.

2.8 Internalisation study of fluorescently labelled PNIPAM nanoparticles

Methacryloxyethyl thiocarbamoyl rhodamine B labelled PNIPAM nanoparticles were used for the uptake study in the HaCaT and SW480 cells. HaCaT and SW480 cells were seeded at a density of 25,000 in glass bottom petri dishes. The petri dishes were kept in a CO₂ incubator at 37 °C for 24h. After attachment, the cells were exposed to different concentrations of fluorescent nanoparticles and after a 24 hour exposure the monolayer of cells was washed with PBS to remove external particles. The particles in the cells were visualised by excitation at 543 nm and fluorescence emission was collected above 560 nm 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.

2.9 Co-localisation study of the fluorescently labelled PNIPAM nanoparticles

Co-localisation studies of the labelled PNIPAM nanoparticles were performed in the HaCaT cells by using lysotracker green. HaCaT cells were seeded at a density of 25,000 in glass bottom **Petri dishes**. The **Petri dishes** were kept in a CO₂ incubator at 37 °C for 24h to attach the cells on the glass surface. After attachment, the cells were exposed to different concentrations (30, 50, and 100 mg/l) of fluorescent nanoparticles and after 24 hour exposure the monolayer of cells was washed with PBS. The cells were then incubated for 30 minutes with **75 nM** concentration of lysotracker in a CO₂ incubator at 37 °C. The particles in the cells were visualised using excitation at 543 nm and fluorescence emission was collected above 560 nm, whereas fluorescence from lysosomes was recorded using 488 nm excitation, emission being measured through a 505-530 nm in both cases using a confocal microscope (LSM 510 META, Zeiss, Germany). Fluorescence and phase contrast images were recorded from a minimum of 3 areas per sample.

2.10 Statistical analysis

All experiments were conducted in at least triplicate (three independent experiments) and the genotoxicity was **performed** twice in duplicate. Fluorescence (AB assays) as fluorescent units (FUs) was quantified using a microplate reader (TECAN GENios, Grödig, Austria). Raw data from cytotoxicity assays were collated and analyzed using Microsoft Excel[®] (Microsoft Corporation, Redmond, WA). Cytotoxicity was expressed as the mean percentage inhibition relative to the unexposed control $\pm$ standard deviation (SD). Genotoxicity was expressed in terms of percentage tail DNA

and OTM as the mean percentage $\pm$ 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.

3. Results and Discussion

3.1 Characterisation of PNIPAM nanoparticles

The average particle sizes of PNIPAM nanoparticles were measured as a function of increasing temperature due to its thermoresponsive nature. When the temperature is raised above the LCST, the polymer undergoes a phase transition and the random coil structure (hydrophilic state) collapses to form a globular structure (hydrophobic state) (Xu et al., 2006). This behaviour of decreasing particle size with increasing temperature is demonstrated well by the temperature dependence of the nanoparticles when prepared in MQ water (Table 1). In our experiments, the measured particle size of PNIPAM nanoparticles was found to increase with increasing temperature when they were prepared in cell culture media (Table 1), although in MQ water the particle size decreases with increasing temperature. This dramatic increase of particle size is characteristic of agglomeration (Naha et al., 2009b) and at the exposure temperature of 36°C - 38 °C, the particles in the supplemented media have sizes ~0.5- 1 μm . Similar results were obtained using the fluorescently-labelled particles, where the presence of the covalently-linked rhodamine B did not affect the particle size, transition temperature or aggregation behaviour significantly, confirming that labelled particles are representative of unlabelled ones, so that direct comparisons of their behaviour can be made. Thus, for the rhodamine-labelled NIPAM particles, the particle hydrodynamic diameter was confirmed by DLS as 76nm at 25°C with a polydispersity index (PDI) around 0.239. Transmission Electron Microscopy confirmed the as produced particles to have a dry size of 40 ± 10 nm, with a representative TEM image being shown in Figure 1.

The BET surface area of the unlabelled PNIPAM nanoparticles was found previously to be 5.77 ± 0.07 m²/g (Naha et al., 2009b). Zeta potentials of the PNIPAM nanoparticles were measured in MQ water, DMEM and supplemented cell culture media (Table 2). An increasingly negative zeta potential value was observed when the nanoparticles were suspended in the protein supplemented cell culture media,

although it must be noted that the values are always very low, which explains the agglomeration behaviour observed, as zeta potential values of at least ± 30 mV are considered necessary for charge stabilisation to be prominent. This increase may therefore be due to the interaction / adsorption of proteins on to the surface of the nanoparticles yielding a protein corona (Lynch et al., 2007). Such an increase in zeta potential towards negative values is attributable to shielding of the surface charge due to adsorption of and/or interaction with components of the medium by the nanoparticles, supporting the conclusions of the particle size measurements.

3.2 Intracellular uptake study

An intracellular uptake study was performed with the fluorescently labelled PNIPAM nanoparticles, the uptake and localisation of the particles in HaCaT and SW480 cells being visualised by confocal laser scanning microscopy after 24 hours of exposure. The internalisation of the particles is illustrated in Figure 2 and optical sections (z-sections) of the HaCaT and SW 480 cells are shown in Figure 3. Each optical section represents a particular plane of focus and it is clear that the nanoparticles have been internalised completely, rather than being accumulated in or at the cell membrane. Washing of the cells prior to observation in the confocal microscope also ensured that non-internalised particles were removed. The results demonstrate that the PNIPAM nanoparticles were taken up and were distributed throughout the cell within 24 hrs of exposure. Furthermore, the particles appear to be localised in specific sub-cellular organelles. The results of the co-localisation study, in which lysosomes were co-stained using lysotracker green, confirms that the nanoparticles are specifically localised in lysosomes (Figure 4). No evidence of localisation within the cell nuclei was observed. This is not unexpected, given the large size of the agglomerates in the cell culture medium. From the images of Figures 2 and 4, it appears that further particle agglomeration may be occurring intracellularly, or that particles are being really accumulated into the lysosomes. **Lysosomes have also been shown to be the final destination for polystyrene and silica nanoparticles following uptake by a range of cell types like A549 (carcinomic human alveolar basal epithelial) (Salvati et al., 2010), and also with HeLa (cervical cancer) (Salvati et al., unpublished) cells.** Additionally, once the particles reach the lysosomes they do not exit over at least a 24 hour time period, (Salvati et al., 2010) and this is likely also the case for the PNIPAM particles.

3.3 Cytotoxicity

The cytotoxicity of the unlabelled PNIPAM nanoparticles was studied in the HaCaT and SW480 cell lines. The assay was carried out by analysing the uptake of Alamar blue (AB), a water-soluble dye that has been previously used for quantifying *in vitro* viability of various cells (Fields and Lancaster, 1993; Ahmed et al., 1994). When added to cell cultures, the oxidized form of the AB enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity, accepting electrons from NADPH, FADH, FMNH, and NADH as well as from the cytochromes. This redox reaction is accompanied by a shift in colour from indigo blue to fluorescent pink, which can be easily measured by colorimetric or fluorometric analysis (Al-Nasiry et al., 2007). The percentage of cell viability was determined by comparison with cells which were not exposed to nanoparticles i.e. the control group. Although the PNIPAM nanoparticles are clearly internalised in the mammalian cells and predominantly localised in the lysosomes, no statistically significant cytotoxicity was observed over a broad concentration range from 25 to 1000 $\mu\text{g/ml}$ in either cell line (Figure 5 and 6).

Some nanomaterials such as single wall carbon nanotubes have been shown to produce toxic responses through medium depletion even without being internalised into the cells (Davoren et al., 2007). More commonly, engineered nanoparticles of size ranges ≤ 100 nm such as titanium dioxide and silicon dioxide (crystalline), produce toxic effects by entering into cells causing stress, inflammation, genotoxicity and finally cell death (Nel et al., 2006; Donaldson et al., 2006; Oberdorster et al., 2005). In this study, however, although PNIPAM nanoparticles (~ 50 to 60 nm at 37°C in MQ water) and are clearly internalised and localised specifically in lysosomes, no cytotoxicological response is observed for up to 96 hours of exposure.

Cationic nanoparticles such as polystyrene nanospheres have been shown to induce lysosomal rupture in RAW 264.7 cells (Xia et al., 2008). Similarly charged PAMAM dendrimers have been shown to increase the lysosomal pH and cytotoxicity as a function of the number of surface amino groups in KB cells, derived from a human carcinoma of the nasopharynx (Thomas et al., 2009). Recent studies of cationic amino terminated PAMAM dendrimers have demonstrated that they become localised in the mitochondria of human lung cells (WI-26 VA4, Lee et al. 2009), and that in J774A.1 mouse macrophages the generation of intracellular ROS is the primary toxic

mechanism, leading to inflammation and apoptosis (Naha et al., 2010a). A similar response has been observed with cationic PAMAM dendrimers in HaCaT and SW480 cells (Mukherjee et al., 2010). However, PNIPAM nanoparticles, even at very high concentrations, do not elicit a cytotoxic response in either HaCaT or SW480 cells. Upon internalisation, the particles are engulfed in lysosomes, rendering them effectively harmless. Polylactic-co-glycolic acid (50:50) and Eudragit RS100 nanoparticles of size 200 nm have similarly been shown to elicit no cytotoxicological response in SW480 cells, although internalised, suggesting their potential as nano-carriers of drugs for delivery systems (Naha et al., 2010b). In our previously reported ecotoxicological analysis of PNIPAM nanoparticles, they were found to be similarly non-cytotoxic to fish cells, although a systematic increase in the ecotoxicity response was observed with increasing concentration of a more hydrophobic co-monomer, N-tert-butylacrylamide (BAM), in NIPAM/BAM co-polymer nanoparticles (Naha et al., 2009b).

3.4 Genotoxicity

The Comet assay is widely accepted as a simple, sensitive, and rapid tool for assessing DNA damage in different test models and is extensively used for chemical testing (Dhawan et al., 2009). The genotoxicity of the PNIPAM nanoparticles in the concentration range from 12.5 µg/ml to 800 µg/ml was analysed in both HaCaT and SW480 cells for different concentrations and different exposure times. DNA damage was estimated by analyzing the OTM (Olive Tail Moment) and tail percentage DNA. At all concentrations and exposure times tested, no statistically significant DNA damage ($p \leq 0.5$) was observed, as shown in Figures 7a and b; and 8a and b for the case of HaCaT and SW480 cells respectively. This is an important result as although no cytotoxicological response is observed up to 1000 µg/ml, the absence of DNA damage is important for biocompatibility. Although titanium dioxide nanoparticles show no cytotoxic response in V79 cells (Chinese hamster lung fibroblasts), significant genotoxicity has been observed. The micronucleus assay indicated both chromosome breakage as well as aneuploidy (Bhattacharya et al., 2008) and further indications of genotoxicity via induction of sister chromatid exchange and micronucleus formation in human white blood cells have been reported (Turkez et al., 2007). However, in the current study, no significant genotoxic response is observed, indicating excellent biocompatibility of PNIPAM particles with mammalian cells over

72 hours of exposure to concentrations up to 800 $\mu\text{g/mL}$. In a recent study of the genotoxicity of 34 nm amorphous silica nanoparticles, no genotoxic effects were observed via the Comet assay at concentrations up to 400 $\mu\text{g/ml}$ (Barnes et al., 2008). The fact that the uptake and co-localisation studies suggested that the particles are localised in lysosomes and did not reach the nucleus would also suggest that DNA damage should be limited. However, direct contact between nanoparticles and DNA is not necessary in order to induce DNA damage, which can also result from inflammatory responses to nanoparticles, or oxidative stress (Singh et al., 2009). Amongst the polymeric nanoparticles used and proposed for various applications such as drug delivery, only a few have been analysed for genotoxicity (He et al., 2009). The chemical composition of nanomaterials has been shown to influence the mechanism of uptake, transport and toxic responses. For example the cationic $-\text{NH}_2$ surface coated PAMAM dendrimers have been shown to localise in mitochondria (Lee et al., 2009), eliciting a toxic response via production of reactive oxygen species, inflammation and apoptosis in mouse macrophage cells (Naha et al., 2010a). Neutral pegylated quantum dots have, however, been observed to be localised in lysosomes of HaCaT cells (Meade et al, 2009). The studies presented here indicate cellular uptake and localisation of PNIPAM particles in lysosomes. However, no adverse geno or cytotoxicological responses are observed up to the highest doses in either HaCaT or SW 480 cell-lines, indicating excellent biocompatibility. It is of course noted that, although as synthesised and dispersed in MilliQ water the PNIPAM particles are of a size classified as nano, in the cell culture medium, agglomeration and interaction with the medium components results in an effective average size of the order 0.5-1 μm being presented to cells. Nevertheless, in terms of adverse toxicological effects, it can be stated that the as produced nanoparticles show negligible effects.

4. Conclusion

PNIPAM nanoparticles are well known thermoresponsive particles, but to date there is little mammalian toxicity data available. Fluorescently labelled PNIPAM particles are clearly seen to be internalised by HaCaT and SW480 cells after 24hrs, and are specifically localised in lysosomes. Equivalent unlabelled PNIPAM nanoparticles are shown to elicit no significant cytotoxic response in HaCaT and SW480 cells, indicating that these particles are biocompatible in nature. No significant difference in

the cell viability upon exposure of either cell type to PNIPAM nanoparticles was found after 24h, 48h, 72h and 96h of exposure at concentrations ranging from 25 to 1000 mg/l. The biocompatibility of the unlabelled PNIPAM nanoparticles is further confirmed by the genotoxicity results, as there is no significant difference in the % tail DNA and olive tail moment (OTM) in either the HaCaT and SW 480 cells upon exposure of the particles.

The observed interaction of the PNIPAM nanoparticles with the two different mammalian cell lines and the interpretation of the consequences of the particle fate and behaviour within the cells is an indication of the biocompatibility of these polymer particles. Addition to this, from our previous study it has been observed that PNIPAM nanoparticles has no ecotoxicological response, hence it is also an eco-friendly polymer particle. The data presented here would suggest that these particles have significant potential as drug delivery agents in the form of hydrogels or as scaffolds in the field of tissue engineering.

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Legends to Tables

Table 1. Hydrodynamic diameter (nm) of NIPAM nanoparticles with increasing temperature in different media

Table 2. Zeta potential of NIPAM nanoparticles in different media

Legends to Figures

Figure 1. Representative TEM image of the labelled PNIPAM nanoparticles. Scale bar is 100 nm.

Figure 2. Confocal laser scanning micrograph (CLSM) of internalisation of fluorescently labelled NIPAM nanoparticles in HaCaT and SW 480 cells after 24h of exposure. Figure 2.1 represents the uptake study in the HaCaT cells and Figure 2.2 represents the uptake study in SW 480 cells. In each figure Panel A represents the untreated control and Panels B, C and D, represent the nanoparticle exposure concentrations of 30, 50 and 100 mg/l respectively. Scale bar is 10 μ m.

Figure 3. CLSM optical sections (z-sections) of the HaCaT and SW 480 cells showing the localisation of the nanoparticles at different sections. Each optical section represents a particular plane of focus and nanoparticles have a different plane of focus indicating that they are internalised completely. Figure 3.1 represents the HaCaT cells (optical section thickness is 0.41 μm) with exposure concentration of 50 mg/l and Figure 3.2 represents the SW 480 cells (optical section thickness is 1.0 μm). Panels A and B of Figure 3.2 represent the different concentrations of NIPAM nanoparticles presented to the cells, i.e. 10 and 50 mg/l respectively. Scale bar is 10 μm .

Figure 4. CLSM images of colocalisation of NIPAM nanoparticles with lysosomes, by co-staining the HaCaT cells with lysotracker (green). Figures 4.1 and 4.2 are the 24 hour exposure to labelled PNIPAM nanoparticles at concentrations of 30 and 50 mg/l respectively. In each figure Panel A represents the brightfield image; Panel B shows the green fluorescence channel showing the localisation of the lysotracker dye and indicating the lysosomes; Panel C is the red fluorescence channel and shows the localisation of the PNIPAM nanoparticles; and Panel D is the overlay of Panels B and C (the green and red channels) and shows the co-localisation of the nanoparticles with the lysosomes. Scale bar is 10 μm .

Figure 5. Cytotoxicological response of HaCaT cells after 24, 48, 72 and 96 hours of exposure to increasing concentrations of NIPAM nanoparticles.

Figure 6. Cytotoxicological response of SW 480 cells after 24, 48, 72 and 96 hours of exposure to increasing concentrations of NIPAM nanoparticles.

Figure 7. Genotoxicity response of NIPAM nanoparticles to HaCaT cells. Panel A represents the % tail DNA and Panel B represents the olive tail moment after exposure of the particles at three different time points.

Figure 8. Genotoxicity response of NIPAM nanoparticles to SW 480 cells. Panel A represents the % tail DNA and Panel B represents the olive tail moment after exposure of the particles at three different time points.

Figure 1.

Figure 2.1

Figure 2.2

Figure 3.1

Figure 3.2

Figure 4.1

Figure 4.2

Figure 5.

Figure 6.

Figure 7. a.

Figure 7.b.

Figure 8. a.

Figure 8.b.

Table 1.

Conc. of Nanoparticles	Type of Media	Size in nm (30 °C)	Size (nm) (32 °C)	Size (nm) (34 °C)	Size (nm) (36 °C)	Size (nm) (38 °C)
1000 mg/l	Water	90.6 ± 4.5	84.3 ± 2.4	74.7 ± 3.1	57.36 ± 2.3	52.72 ± 1.1
100 mg/l	Water	88.9 ± 3.6	82.2 ± 1.9	75.3 ± 3.4	55.3 ± 2.5	49.2 ± 1.1
1000 mg/l	DMEM	90.9 ± 3.6	343 ± 32	1324 ± 179	1781 ± 86	2936 ± 150
100 mg/l	DMEM	85.7 ± .66	88.1 ± 4.9	708 ± 65	1424 ± 97	1497 ± 77
1000 mg/l	DMEM with 5% FBS	88.1 ± 2.9	422 ± 45	1129 ± 140	1266 ± 36	1259 ± 93
100 mg/l	DMEM with 5% FBS	83.4 ± 4.3	81.5 ± 6.5	407.8 ± 16.1	563.2 ± 11.9	641.5 ± 82.9
1000 mg/l	DMEM with 10 % FBS	89.9 ± 7.9	368.9 ± 31.1	879.4 ± 34.6	950.6 ± 55.7	853.1 ± 11.5
100 mg/l	DMEM with 10 % FBS	86.8 ± 6.5	71.3 ± 17.4	285.8 ± 77.6	461.8 ± 34.43	473.3 ± 51.4

Table 2.

Type of media	Zeta potential in mV
Milli-Q Water	-5.38 $\pm$ 0.52
DMEM	-1.33 $\pm$ 0.66
DMEM with 5% FBS	-7.09 $\pm$ 0.34
DMEM with 10% FBS	-7.93 $\pm$ 0.63