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Preparation, Characterization and Ecotoxicological evaluation of Four Environmentally Relevant Species of *N-isopropylacrylamide* and *N-isopropylacrylamide-co- N-tert-butylacrylamide* Copolymer Nanoparticles.

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Abstract

Poly *N*-isopropylacrylamide and *N*-isopropylacrylamide / *N*-tert-butylacrylamide copolymer nanoparticles of 50 to 70 nm were prepared by free radical polymerisation. The particle sizes of the copolymer nanoparticles were measured in the test media Milli-Q water, Algae Media, Daphnia Media and Microtox Diluent as a function of temperature. Whereas in Milli-Q water the particle size was seen to decrease above the lower critical solution temperature of the thermoresponsive polymer, in the test media it was seen to increase significantly, indicative of aggregation. At the temperatures employed for the ecotoxicological studies all particles, with the exception of the 50:50 copolymer existed as nanoparticles, however. The zeta potential of Poly *N*-isopropylacrylamide and *N*-isopropylacrylamide / *N*-tert-butylacrylamide copolymer particles measured in the different media was seen to correlate well with the ratio of *N*-tert-butylacrylamide monomer and therefore the hydrophobicity of the particles. Ecotoxicological studies of the copolymer nanoparticles was performed using four test species *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Daphnia magna*, *Thamnocephalus platyurus* and the cytotoxicity of the 100% Poly *N*-isopropylacrylamide and 85:15 *N*-isopropylacrylamide / *N*-tert-butylacrylamide copolymer nanoparticles was evaluated using a salmonid cell line. Although no significant cytotoxicological response was recorded, significant ecotoxicological response was observed at particle concentrations of up to 1000 mg l⁻¹. The ecotoxicological response was seen to correlate well with the ratio of *N*-tert-butylacrylamide monomer and therefore with the zeta potential of the nanoparticles. The toxic response in Daphnia Magna was seen to further correlate with the reduction in zeta potential pointing towards a contribution of secondary

effects due to modification of the medium. No correlation with particle size was observed. The sensitivity of the test species was seen to vary depending on copolymer composition. The relevance of the derived structure activity relationships is discussed.

Keywords *N*-isopropylacrylamide (NIPAM); *N*-tert-butylacrylamide (BAM); Ecotoxicity; Nanoparticles; Zeta potential; structure-activity relationships.

1. Introduction

Polymeric nanoparticles are widely used in different aspects of the medical field in terms of diagnosis, tissue engineering and as drug delivery devices (Storrie and Mooney, 2006). Several polymeric micro- and nanoparticles have been used for the delivery of drugs and therapeutic proteins (Silva et al., 2006; Blasi et al., 2007). In particular polylactic-*co*-glycolic acid (PLGA) and poly-lactic acid (PLA) polymers, which are biodegradable in nature, show great potential and have been widely used in drug delivery systems (Ito et al., 2008; Naha et al., 2008a,b; Sun et al., 2008). Currently four other nanoparticles namely, polymer coated iron oxide nanoparticles, PEGylated liposomes, dendritic fullerenes, and fullerene derivative nanoparticles are being investigated in clinical trials for their anticancer activity (Dobrovolskaia and McNeil, 2007).

Poly *N*-isopropylacrylamide (PNIPAM) is a well known thermoresponsive polymer (Hsiue et al., 2002). It exhibits a lower critical solution temperature (LCST) of about 32°C in an aqueous medium (Xu et al., 2006). Copolymerisation of NIPAM with the more hydrophobic monomer *N*-tert-butylacrylamide (BAM) reduces the LCST of the resultant co-polymer compared to that of PNIPAM, increasingly so as the ratio of the BAM monomer in the co-polymer is increased. As the ratio of BAM increases, the amount of N-H groups exposed at the surface decreases, and the amount of $-\text{C}-(\text{CH}_3)_3$ groups increases, reducing the hydrophilicity of the resulting copolymer. For example LCSTs for the polymers with monomer ratios NIPAM/BAM 85:15, NIPAM/BAM 65:35, NIPAM/BAM 50:50 are 25°C, 17°C and 12°C respectively (Lynch et al., 2005). 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 (Xu et al., 2004; Zhang et al., 2005; Kavanagh, 2005; Xu et al., 2006). PNIPAM has also been developed and proposed for controlled release of ophthalmic drops for glaucoma therapy (Hsiue et al., 2002, 2003). Recently the effect of NIPAM/BAM copolymer nanoparticles of varying size and varying copolymer ratios on adsorption of proteins from plasma and the potential implications for biological interactions has been reported (Cedervall et al., 2007).

As nanomaterials are currently being widely used in modern technology, there is an increasing need for information regarding the human health and environmental implications of these 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 (Oberdörster et al., 2005, Meng et al., 2007; Papageorgiou et al., 2007; Singh et al., 2007; Poland et al., 2008). Recently, several nanoparticles, including the NIPAM/BAM copolymer nanoparticles used here, were found to enhance the rate of protein fibrillation of β -2-microglobulin, which is implicated in dialysis related amyloidogenesis, in solution experiments at pH 2 by a surface catalysis type mechanism (Linse et al., 2007). More recently, the 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), also in solution experiments, indicating that such processes are complex and depend on

the nature of both the particle and the protein. However, to our knowledge there have been no reports on the environmental impact of these copolymer particles.

The assessment of environmental effects requires an understanding of their mobility, reactivity, ecotoxicity and persistency (Nowack and Buchelli, 2007). Recently reports on the ecotoxicity of various nanomaterials have started to emerge in the literature. Of the studies conducted, the majority have focused on the carbon based materials (Lovern and Klaper, 2006; Zhu et al., 2006; Cheng et al., 2007; Lin and Xing, 2007; Smith et al., 2007; Baun et al., 2008; Lovern et al., 2007) but there have also been recent reports on the ecotoxicological evaluation of various metal based nanomaterials (Lovern et al., 2007; Federici et al., 2007; Heinlaan et al., 2008; Gagné et al., 2008; Mortimer et al., 2008) and dendrimers (Mortimer et al., 2008). These studies have looked at various aquatic species representing the different trophic levels (decomposer, primary producer, invertebrates and vertebrates).

The aim of the present investigation was therefore the preparation, characterisation and ecotoxicological assessment of PNIPAM and NIPAM/BAM copolymer nanoparticles. Variation of the co-polymerisation ratio from 100% NIPAM through NIPAM/BAM 85:15, NIPAM/BAM 65:35 and NIPAM/AM 50:50 allowed a systematic variation of the surface chemistry which manifested as changes in the Zeta potential, facilitating the establishment of structure-activity relationships. Since these particles are thermosensitive, the particle size was measured as a function of temperature. The surface area of the particles was measured by BET. Investigation of the acute ecotoxicological effects of PNIPAM and NIPAM/BAM copolymer particles was conducted using a battery of bioassays representing different trophic levels. The tests employed included a bacterial species, a

unicellular algae species and two crustaceans. In addition, the cytotoxicity of PNIPAM and NIPAM/BAM (85:15) copolymer particles was investigated in a salmonid fish cell line.

2. Materials and methods

2.1 Test compounds

Poly *N*-isopropylacrylamide (PNIPAM) and *N*-isopropylacrylamide-co-*N*-tert-butylacrylamide (NIPAM/BAM) copolymer particles with three different ratios of the comonomers (85:15, 65:35, and 50:50 NIPAM/BAM) were synthesised by free radical polymerisation. The procedure for the synthesis was as follows: 2.8g monomers (in the appropriate wt/wt ratio), and 0.28g crosslinker (N,N-methylenebisacrylamide) was dissolved in 190 ml MilliQ water [MQ] with 0.8 g SDS and 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, until no traces of monomers, crosslinker, initiator or SDS could be detected by proton NMR (spectra acquired in D₂O using a 500 MHz Varian Inova spectrometer). Particles were freeze-dried and stored in the fridge until used. Transmission Electron Microscopy measurements confirmed the as produced particles to be of size 30-50 nm in the dry state [based on I. Lynch, personal communication].

Due to the inverse solubility of PNIPAM and NIPAM/BAM particles, 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 favourable than polymer-polymer contacts resulting in uptake of water and swelling of the particles), before gradually warming them to the test conditions.

Phenol (CAS No. 108-95-2) and potassium dichromate (CAS No. 7778-50-9) were purchased from Sigma-Aldrich (Ireland) and employed as reference toxicants (positive controls) to validate the test procedures.

2.2. Particle Characterisation

2.2.1 Particle size measurement

The particle size distributions of all particles in the appropriate assay media were analyzed using a Zeta sizer (Malvern Instruments, UK). For a typical experiment approximately 1.5 ml of a 1000 µg/ml concentration suspension of nanoparticles in MQ water and the respective assay media (*i.e.* algal medium [AM], *Daphnia* medium [DM] and Microtox[®] diluent [MD]) were analysed as a function of temperature from 0°C to 30°C with an interval of 5°C.

2.2.2 Zeta potential measurement

The Zeta potential of all particles in MQ water and the respective assay media was measured using the Zeta sizer (Malvern Instruments, UK). Although the zeta potential are also affected by temperature (size), measurements were conducted at 20 °C only, using a concentration of 1000 µg/ml.

2.2.3 Surface Area measurement

BET surface area measurements were performed using a Gemini series surface area analyser (Micromeritics, USA). Approximately 0.5 g particles of each of the materials were degassed with nitrogen gas at a constant temperature of 25 °C for two hours prior to surface area measurements being recorded.

2.3 Ecotoxicity tests

Each ecotoxicity test was performed in two stages. A preliminary or range finding test was conducted which determined the range of concentrations of interest for the definitive test. The definitive test used a concentration range (at least five concentrations) in which effects were likely to occur thereby permitting the calculation of the respective Effective Concentrations (EC₁₀, EC₅₀) or Lethal Concentrations (LC₁₀, LC₅₀), No Observed Effect Concentration (NOEC), and Lowest Observed Effect Concentration (LOEC). The acute toxicity of PNIPAM and each of the NIPAM/BAM copolymer particles was investigated in four test systems representing different trophic levels. The cytotoxicity of PNIPAM and PNIPAM/BAM 85:15 was also evaluated in the RTG-2 salmonid fish cell line.

2.3.1 Microtox[®] test

The acute toxicity of PNIPAM and each of the PNIPAM/BAM copolymers to the marine bacterium *Vibrio fischeri* was determined using the 90% basic test for aqueous extract protocol (Azur Environmental Ltd, 1998). Lyophilised *Vibrio fischeri* bacteria (NRRL B-11177) and all Microtox[®] reagents were obtained from SDI Europe, Hampshire, UK. Exposure was carried out at 15°C in Microtox[®] diluent. Phenol was used as a reference chemical and a basic test for phenol was run for every fresh vial of bacteria to ensure the validity of all tests. Readings of bioluminescent response were measured using a Microtox[®] Model 500 analyser and the acute toxicity data was obtained and analysed using the MicrotoxOmni software (SDI Europe, Hampshire, UK). Five, fifteen and thirty minute EC₅₀ tests were performed.

2.3.2 Microalgae growth inhibition assay

Assessment of the acute toxicity of the materials to the freshwater algae *Pseudokirchneriella subcapitata* was conducted in accordance with OECD Guideline 201 (2002). *Pseudokirchneriella subcapitata* CCAP 278/4 was obtained from the Culture Collection of Algae and Protozoa (CCAP) Argyll, Scotland. All microalgae growth inhibition tests were conducted at 20 ± 1°C with continuous shaking at 100 rpm and continuous illumination of 10,000 lx. The initial algal density of all flasks was 1x10⁴ cell ml⁻¹ in a final volume of 20 ml. Negative controls were incorporated for each test containing only algal growth media and algal inoculum. The cell density of each replicate was measured after 72 h using a Neubauer Improved (Bright-Line) chamber (Brand, Germany). Average specific growth rate (μ) and percentage inhibition of average specific growth rate (%Ir) relative to controls were calculated for

each concentration. The reference chemical potassium dichromate was employed as a positive control to ensure validity of the test method.

2.3.3. *Thamnotoxkit FTM*

The acute toxicity of the materials was also evaluated using the freshwater shrimp *Thamnocephalus platyurus*. This toxicity test was purchased in kit form from SDI Europe (Hampshire, UK) and the test was performed according to manufacturer's instructions (*Thamnotoxkit FTM*, 1995). Briefly, the test is a 24 h LC₅₀ bioassay, which is performed in a 24-well test plate using instars II–III larvae of the shrimp, which are hatched from cysts. Hatching was initiated 24 h prior to the start of the test. Upon hatching, shrimp were exposed to various concentrations of PNIPAM and each NIPAM/BAM copolymer nanoparticle and incubated at 25 °C for 24 h in the dark. The test endpoint was mortality. The number of dead shrimp for each concentration was recorded and the respective LC₅₀ was determined. Potassium dichromate was used as a positive control.

2.3.4. *Daphnia magna acute immobilisation assay.*

Acute toxicity immobilization tests were performed on each of the nanoparticles according to the British standard (BS EN ISO 6341, 1996). *Daphnia magna* were originally obtained from TNO laboratories (the Netherlands) and were cultured in static conditions at 20 ± 1°C and under 16 h/8 h light/dark photoperiod. Daphnid sensitivity was verified by determining the 24 h EC₅₀ using potassium dichromate. Acute toxicity tests were performed on *Daphnia magna* neonates that were less than

24 h old. Four replicates were tested for each test concentration and five neonates were used in each replicate. There was no feeding during the tests. Immobilisation (no independent movement after gentle agitation of the test liquid for 15 s) was determined visually after 24 and 48 h.

2.3.5. Cell culture

RTG-2 cells (Catalogue no. 90102529), a rainbow trout gonadal cell line, were used for the cytotoxicity testing of PNIPAM and NIPAM/BAM 85:15 only. These cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained in Dulbecco's Modified Medium Nutrient Mixture/F-12 Ham (DMEM) supplemented with 10% foetal calf serum (FCS) and 45 IU ml⁻¹ penicillin, 45 µg ml⁻¹ streptomycin. The RTG-2 medium was also supplemented with 25mM HEPES and 1% non-essential amino acids. Cultures were maintained in a refrigerated incubator at a temperature of 20°C under normoxic atmosphere.

2.3.5.1. Cytotoxicity assays. For cytotoxicity tests, wells were plated with 100 µl of the following cell suspension concentrations: 2 x 10⁵ cells per ml for 24 h exposure periods, 1.8 x 10⁵ cells per ml for the 48 h exposures, and 1.6 x 10⁵ cells per ml for the 72 and 96 h exposure periods (Davoren et al., 2005). Test particles were prepared in a reduced serum medium (5% FCS) to lessen the effects of the particles binding serum proteins. Fish cells were exposed to the nanoparticles in a range of concentrations (mg l⁻¹) based on the preliminary range finding tests with the cell line. Six replicate wells were used for each control and test concentration per microplate.

The test medium was removed; cell monolayers washed with phosphate buffered saline (PBS) and prepared for the cytotoxicity assays. The Alamar Blue (AB) and Neutral Red (NR) assays were subsequently conducted on the same set of plates, as previously described (Davoren and Fogarty, 2006).

2.4 Statistics

All experiments were conducted in at least triplicate (three independent experiments). Toxicity was expressed as mean percentage inhibition for the Microtox[®] (bioluminescence), *P. subcapitata* (growth) and *D. magna* (immobilisation) and percentage mortality was measured for the *T. platyurus* assay. Fluorescence (AB and NR assays) as fluorescent units (FUs) was quantified using a microplate reader (TECAN GENios, Grödig, Austria). Raw data from cell cytotoxicity assays were collated and analyzed using Microsoft Excel[®] (Microsoft Corporation, Redmond, WA). Cytotoxicity was expressed as mean percentage inhibition relative to the unexposed control \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. Toxicity data was fitted to a sigmoidal curve and a four parameter logistic model used to calculate EC/LC₅₀ values. This analysis was performed using Xlfit3[™] a curve fitting add-in for Microsoft[®] Excel (ID Business Solutions, UK).

3. Results

3.1 Nanoparticle characterisation

3.1.1 Particle size measurement

The average particle sizes of PNIPAM, and the various NIPAM/BAM copolymer nanoparticles were measured as a function of increasing temperature. PNIPAM and NIPAM/BAM copolymers are thermoresponsive in 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 (data not shown). In our experiments the particle size of NIPAM and NIPAM/BAM copolymer nanoparticles were found to increase with increasing temperature when they were prepared in other media, however, as presented in Figure 1 for the example of the Microtox[®] diluent, MD. For the 50:50 copolymer, the particle size is seen to begin to increase significantly at ~10°C, close to its LCST and the particle size becomes as high as microns. This dramatic increase of particle size is characteristic of aggregation. The 63:35 and 85:15 copolymer particles remain unaggregated until ~ 15°C and 20°C respectively and the 100% PNIPAM until 25°C. Table 1 lists the average particle size of the various co-polymer particles in the respective test media, at the temperatures at which the respective tests were conducted. With the exception of the 50:50 copolymer, all particles are unaggregated

at the concentrations and temperatures employed for the eco- toxicological studies. In cell culture medium, both the 50:50 and 65:35 copolymer particles were seen to be highly aggregated at 20°C, and so were not tested.

3.1.2 Zeta potential measurement

The zeta potentials of PNIPAM and the different NIPAM/BAM copolymer particles was measured in the various test media; MQ water, MD, AM, and DM. The results are shown in Figure 2. For all media types, increasing the ratio of BAM in the copolymer nanoparticles was shown to cause a decrease in their zeta potential. No correlation between zeta potential and particle size was observed.

3.1.3 Surface Area measurement

The surface area of PNIPAM, NIPAM/BAM 85:15, NIPAM/BAM 65:35 and NIPAM/BAM 50:50 particles was found to be 5.77 ± 0.07 m²/g, 9.57 ± 0.06 m²/g, 14.1 ± 0.1 m²/g and 8.5 ± 0.1 m²/g respectively. With the exception of the 50:50 copolymer, the surface areas correlate reasonably with the particle size.

3.2 Ecotoxicity

Testing of reference chemicals in tandem with the polymer nanoparticles was carried out to ensure the validity of each test method. Phenol was used as the reference chemical to validate the Microtox[®] acute test and the 5 min EC₅₀ was determined as 16.5 mg/L. This was in accordance with the Microtox[®] acute toxicity basic test procedures (Azur Environmental, 1998) which stipulates values between 13 and 26 mg/L. In the algal growth inhibition test with *P. subcapitata* a 72 h EC₅₀ of 1.3 mg/L

was determined for the reference toxicant potassium dichromate which showed good agreement with published results (Nyholm, 1990). The *T. platyurus* bioassay determined a 24h LC₅₀ value of 0.1 mg/L for the reference chemical potassium dichromate. This value was within the range reported in the test procedure Thamnotoxkit F (1995) protocol. In the *D. magna* test a 24h EC₅₀ value of 0.1 mg/L was determined with the reference chemical potassium dichromate which was consistent with that published in the standard test guideline (BS EN ISO 6341, 1996).

No mortality was observed in the negative controls for the *T. platyurus* or *Daphnia* bioassay at any of the endpoints investigated.

The dose dependent effects of the PNIPAM and NIPAM/BAM particles in the algal tests are shown in Figure 3. The effects of each of the materials on the invertebrate *Daphnia magna* and *Thamnocephalus platyurus* are presented in Figures 4 and 5 respectively. The cytotoxicity of P(NIPAM) and NIPAM/BAM 85:15 particles was tested in the concentration range 25-1000 mg l⁻¹ in the RTG-2 cell line at four time points up to 96 h. No significant toxicity was found at any of the test concentrations with either of the endpoints studied at any of the time points (results not shown).

3.2.1 Ecotoxicity test results with PNIPAM

The results of the ecotoxicity assessment of PNIPAM particles with the four test systems are presented in Table 2. Employing the calculated ecotoxicity value (EC₅₀/LC₅₀), in terms of species sensitivity the ranking order for PNIPAM particles is

as follows *Daphnia magna* > *Thamnocephalus platyurus* > *Vibrio fischeri* > *Pseudokirchneriella subcapitata*.

3.2.2 Ecotoxicity test results with NIPAM/BAM 85:15

The results of the ecotoxicity evaluation of NIPAM/BAM 85:15 with the test systems are presented in Table 3. Based on the calculated ecotoxicity value (EC_{50}/LC_{50}), in terms of species sensitivity, the ranking order for NIPAM/BAM 85:15 nanoparticles is as follows *Daphnia magna* > *Thamnocephalus platyurus* > *Vibrio fischeri* > *Pseudokirchneriella subcapitata*, as was the case for the parent polymer PNIPAM.

3.2.3 Ecotoxicity tests with NIPAM/BAM 65:35

The results of the ecotoxicity assessment of NIPAM/BAM 65:35 particles with the different test systems are presented in Table 4. Employing the calculated ecotoxicity value (EC_{50}/LC_{50}), in terms of species sensitivity the ranking order for NIPAM/BAM 65:35 particles is as follows *Vibrio fischeri* > *Daphnia magna* > *Thamnocephalus platyurus* > *Pseudokirchneriella subcapitata*.

3.2.4 Ecotoxicity tests with NIPAM/BAM 50:50

The results for NIPAM/BAM 50:50 particles from the different test systems are presented in Table 5. Employing the calculated ecotoxicity value (EC_{50}/LC_{50}) in

terms of species sensitivity the ranking order for NIPAM/BAM 50:50 nanoparticles to the test species is as follows *Vibrio fischeri* > *Daphnia magna* > *Thamnocephalus platyurus* > *Pseudokirchneriella subcapitata*.

4. Discussion

The particle size of the different materials was measured in different media. The expected particle size decrease as a function of temperature expected for these thermoresponsive polymers is observed only in the MQ environment. The particle size of NIPAM and NIPAM/BAM copolymer nanoparticles was found to increase with increase in temperature in AM, DM, and MD media, however, the average particle size increasing to several microns as the materials under go the phase transition. This behaviour is characteristic of aggregation., as a consequence of the screening effect of the salts in the various test media. It is proposed therefore that the particles interact with different components of the media and aggregate when the temperature is increased above the respective LCST.

The zeta potential of PNIPAM and the different NIPAM/BAM copolymer nanoparticles was shown to decrease (NIPAM < NIPAM/BAM 85:15 < NIPAM/BAM 65:35 < NIPAM/BAM 50:50) with increasing BAM ratio as presented in Figure 2. This increasingly negative value of the zeta potential of the co-polymer particle surface is consistent with the reduced hydrophilicity (as determined by contact angle measurements) as a result of the increased BAM content (Lynch et al., 2005; Allen et al., 2003). A similar trend was observed with all the media tested but was most significant when the nanoparticles were tested in MQ water. Such a decrease in the zeta potential of nanoparticles in growth media is commonly observed

(Hang et al., 2007) and is attributable to shielding of the potential due to adsorption of and/or interaction with components of the medium by the nanoparticles, supporting the conclusions of the particle size measurements.

In terms of response, Poly *N*-isopropylacrylamide particles were found to be the least toxic among all the NIPAM/BAM copolymer particles tested in this study. The most sensitive test species to PNIPAM nanoparticles was the *Daphnia magna* (48 hour EC₅₀ 413.6 mg l⁻¹) and the least sensitive test species was the *Pseudokirchneriella subcapitata* (72 hour EC₅₀ > 1000 mg l⁻¹).

The NIPAM/BAM 85:15 nanoparticles demonstrated a very similar toxicity profile to PNIPAM with *Daphnia magna* being the most sensitive test species (48 hour EC₅₀ is 449.6 mg l⁻¹) and *Pseudokirchneriella subcapitata* the least sensitive (72 hour EC₅₀ is > 1000 mg l⁻¹).

NIPAM/BAM 65:35 nanoparticles were shown to be more toxic than PNIPAM and, and this increase in toxicity was attributed to the increase in BAM ratio in the copolymer nanoparticles. There was a change in the test species sensitivity to this particle in comparison to PNIPAM and NIPAM/BAM 85:15 nanoparticles with the bacterium *Vibrio fischeri* (5 minutes EC₅₀ 40.5 mg l⁻¹) showing greater sensitivity than the *Daphnia magna* bioassay in this case. The least sensitive test species are *Pseudokirchneriella subcapitata* (72 hour EC₅₀ 727.1 mg l⁻¹).

NIPAM/BAM 50:50 nanoparticles were found to be the most toxic to the test battery compared to the other three copolymer nanoparticles. The species sensitivity to these particles was found to be in the same order as that of the NIPAM/BAM 65:35 nanoparticles with *Vibrio fischeri* (5 minutes EC₅₀ 25.7 mg l⁻¹) as the most sensitive

and *Pseudokirchneriella subcapitata* (72 hour EC₅₀ 706.7 mg l⁻¹) as the least sensitive test.

The sensitivity of the test species varied as the physicochemical characteristics of the PNIPAM and NIPAM/BAM copolymer particles changed. The most sensitive bioassay for PNIPAM and NIPAM/BAM 85:15 particles was found to be the immobilisation of *Daphnia magna* (48 hour EC₅₀) followed by mortality of *Thamnocephalus platyurus* (24 hour LC₅₀). The most sensitive test for NIPAM/BAM 65:35 and NIPAM/BAM 50:50 particles was the Microtox[®] assay (*Vibrio fischeri*, 5 minutes EC₅₀) followed by the immobilisation of *Daphnia magna* (48 hour EC₅₀) and mortality of *Thamnocephalus platyurus* (24 hour LC₅₀). The least sensitive bioassay was the *Pseudokirchneriella subcapitata* (72 hour LC₅₀) for the four test particles. The cytotoxicity of PNIPAM and NIPAM/BAM 85:15 was studied in RTG-2 cells and from this study it was observed that there was no significant difference in terms of cell viability between control and cells exposed to the polymer particles. Neither the PNIPAM nor NIPAM/BAM 85:15 particles were found to be toxic to the fish cells at the concentrations and timepoints tested in this study. Cytotoxicity in RTG-2 was assessed at the recommended growth temperature of 20°C. NIPAM/BAM 65:35 and NIPAM/BAM 50:50 were demonstrated to form large aggregates at this temperature, due to the low LCST, which led to both materials floating in the cell culture media. For a cytotoxicity assessment the particles should be fully dispersed and capable of interaction with the cells so in this case it was not considered practical to test these particles with the cell line.

The toxicity of the polymer nanoparticles gradually increased in all of the test species (except RTG-2 cells) as a function of increasing BAM ratio. This relationship with increasing ratio of BAM is shown for the EC₅₀ of the 24 and 48 h acute

immobilisation test with *Daphnia magna* in Figure 6. An increase in the ratio of the monomer BAM was also shown to cause a concomitant decrease in the LC₅₀ value (i.e. more toxic) following 24 h exposure of the invertebrate *Thamnocephalus platyurus* (Figure 7). The monomer ratio has, however, also been shown to be correlated to the measured zeta potential and therefore the measured ecotoxic response can be correlated with the zeta potential as shown in Figure 8.

No correlation with particle size was observed for the test results and thus the increased toxic response must be associated with the decreased hydrophilicity and therefore zeta potential of the copolymer particles with increasing BAM content. For a given particle composition, the zeta potentials are significantly reduced in all growth media, indicating a further decrease of hydrophilicity due to charge screening by the ionic salts of the media. It is worth noting that, for the 50:50 and 65:35 NIPAM/BAM copolymer nanoparticles, the degree of screening is highest for the Microtox diluent (Figure 2), and that this assay shows the highest sensitivity for these particles. For the PNIPAM and 85:15 NIPAM/BAM particles, the most screened zeta potential is in the *Daphnia* medium, and again the *Daphnia* assay was seen to be the most sensitive assay for these particles. This observation may point towards a better understanding of the mechanisms of the toxic response. In, for example, studies of the toxic response of mammalian cells to carbon nanotubes, it has been seen that medium depletion by interaction with the molecular components of the medium results in a significant indirect toxic response (Casey et al., 2008). The interaction with the medium components implied here could similarly contribute to the toxic response observed. The change to the effective composition of the media used here may be quantitatively represented by the reduction of the zeta potential of the respective particles on going from water to the media. Figure 9 shows a correlation of the observed toxic response in the *Daphnia magna* system after 24

h with the change in the zeta potential of the polymer nanoparticles in the *Daphnia* medium. Thus the interaction of the particles with the medium can be correlated with the toxic response suggesting a secondary toxic mechanism, similar to that observed in mammalian cells exposed to single walled carbon nanotube samples. Although the media for the test species employed here is very different in nature to that used for mammalian cytotoxicity, in that it is made up of salts to modify the aqueous environment rather than essential nutrients, the interaction with the medium components implied here could result in changes in the ionic strength of the media and similarly contribute to the toxic response observed. This correlation of response to reduction of zeta potential does not simply correlate to all endpoints for a given particle composition however, and the correlation with the remnant particle zeta potential demonstrated in Figures 8 implies an intrinsic primary toxic response which is dependent on nanoparticle composition. Further studies of particle uptake by the different species are therefore merited to further understand the mechanisms underlying the toxic response. Additionally, parallel studies on the test species in media systematically depleted of individual salt components may shed additional light on the toxicity mechanism.

Conclusions

PNIPAM and NIPAM/BAM nanoparticles are well known thermoresponsive particles and to the best of our knowledge there is no ecotoxicity data of NIPAM/BAM nanoparticles available to date. The most sensitive ecotoxicological assay for PNIPAM and NIPAM/BAM 85:15 nanoparticles was the immobilisation of *Daphnia magna* (48 hour EC₅₀) and for NIPAM/BAM 65:35 and NIPAM/BAM 50:50 nanoparticles was the Microtox[®] assay (*Vibrio fischeri*, 5 minutes EC₅₀). The least sensitive bioassay was *Pseudokirchneriella subcapitata* (72 h EC₅₀) for

the four nanomaterials tested. An important conclusion from the study therefore is that the sensitivity of each assay is dependent on the physico-chemical characteristics of the particle, emphasising the importance of a multi-trophic approach. As the ratio of BAM increases in the copolymer nanoparticle the toxicity was increased in all the test species, despite the fact that the particles with the highest ratio of BAM were highly aggregated. The toxicity trend for different nanoparticles was NIPAM < NIPAM/BAM 85:15 < NIPAM/BAM 65:35 < NIPAM/BAM 50:50, which suggests that there is a significant effect due to particle hydrophobicity and the surface free energy (Lynch et al., 2005). This is confirmed by the correlation of the toxic response with the observed zeta potential of the particles in the medium. The correlation of the toxic response in *Daphnia magna* with the reduction in zeta potential points towards a contribution of secondary effects due to modification of the medium. No dependence of the toxic response on the particle size was observed however. Nevertheless the study gives a clear dependence of the toxic response on the particle composition pointing towards structure-activity relationships.

The observed effects and interpretation of their consequences for nanoparticle fate and behaviour in the environment will be probed in further detail via the utilisation of bulk-fluorescently labelled particles of identical composition, allowing visualisation of the interaction between the particles and test species.

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Figure 1. Particle size measurement of different nanoparticles in Microtox diluent (MD) as a function of increasing temperature. (—▲— PNIPAM; —△— P(NIPAM/BAM) 85:15; —◆— P(NIPAM/BAM) 65:35; and —■— P(NIPAM/BAM) 50:50 nanoparticles. Data is presented as the mean \pm SD (N=6).

Figure 2. Zeta potential of PNIPAM and P(NIPAM/BAM) copolymer particles in different media. (■) MQ; (□) AM; (□) MD; (■) DM. Data is presented as the mean \pm SD (N=6).

Figure 3. Effect of NIPAM and NIPAM/BAM nanoparticles on algal growth inhibition. (□) NIPAM; (□) NIPAM/BAM 85:15; (■) NIPAM/BAM 65:35; (■) NIPAM/BAM 50:50. Data is presented as the mean \pm SD (N=3). *Denotes significant difference from control ($P \leq 0.05$).

Figure 4. Effect of PNIPAM (a); P(NIPAM/BAM) 85:15 (b); P(NIPAM/BAM) 65:35 (c); and P(NIPAM/BAM) 50:50 (d) particles on immobilisation of *Daphnia magna* after (□) 24 hour and (■) 48 hours. Data is presented as the mean \pm SD (N=3). *Denotes significant difference from control ($P \leq 0.05$).

Figure 5. Effect of PNIPAM (□) and P(NIPAM/BAM) 85:15 (■) (a); P(NIPAM/BAM) 65:35 (b); and P(NIPAM/BAM) 50:50 (c) particles on lethality of

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Figure 6. Relationship between 24 hour (□) and 48 hour (■) EC₅₀ for immobilisation of *Daphnia magna* following exposure to PNIPAM and different NIPAM/BAM copolymer particles.

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Figure 8. Effect of zeta potential of different copolymer nanoparticles on immobilisation of *Daphnia magna* based on the (□) 24 hour; (■) and 48 hour EC₅₀ results.

Figure 9. Correlation between change in zeta potential and EC₅₀ in immobilisation of *Daphnia magna* with different NIPAM and NIPAM/BAM copolymer.

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Table 5. Ecotoxicity data for *N*-isopropylacrylamide-co-*N*-*tert*-butylacrylamide (NIPAM/BAM 50:50) copolymer nanoparticles on selected test species and endpoints

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