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Cell–polymer interactions of fluorescent polystyrene latex particles coated with thermosensitive poly(*N*-isopropylacrylamide) and poly(*N*-vinylcaprolactam) or grafted with poly(ethylene oxide)-macromonomer

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Abstract

Cell–polymer interactions of thermosensitive poly(*N*-isopropylacrylamide) (PNIPAM) or poly(*N*-vinylcaprolactam) (PVCL) coated particles with RAW264.7 macrophages and intestinal Caco-2 cells were evaluated. Nanosized particles were prepared by modifying the surface of fluorescent polystyrene (FPS) particles with the thermosensitive polymer gels or with poly(ethylene oxide) (PEO)-macromonomer grafts. The particles were characterized by IR-spectroscopy for functional groups, light scattering for size distribution and zeta-potential for surface charge. Effects of temperature and polymer coating/grafting on the cellular interactions were evaluated by cell association/uptake and visualized by confocal scanning microscope. PEO and PNIPAM inhibited the polymer–cell contact by steric repulsion, evidenced by weak attachment of the particles. PVCL-coated FPS was adsorbed on the cells more strongly, especially at 37 °C, because of more hydrophobic nature at higher temperatures. The results suggest feasibility of the PNIPAM and PVCL for biotechnological/pharmaceutical applications, as the cell–particle interactions may be modified by size, surface charge, hydrophobicity, steric repulsion and temperature.

Keywords: Thermoresponsive polymers; Nanoparticles; PVCL; PNIPAM; PEO; Cell cultures; Steric repulsion

1. Introduction

Cellular uptake and biodistribution of nano- and microparticles depend on the incubation time and temperature, on the properties of the particles used, e.g., size, charge, concentration and hydrophilicity/hydrophobicity, as well as on the used cell line (Florence et al., 1995; Desai et al., 1997; Zauner et al., 2001; Kidane et al., 2002; Panyam and Labhasetwar, 2003; Win and Feng, 2005). Also external stimuli, like temperature and/or pH, can affect the cellular uptake of polymeric materials (Twaites et al., 2004), as well as cell membrane or extracellular matrix proteins that regulate the adhesion of these polymers (Drotleff et al., 2004). Foreign materials, like nanoparticles, trigger an elimination reaction, which involves macrophages that recognize xenobiotics and remove them from the body. This has resulted in attempts to enhance the pharmacological effects of drugs by particulate carrier systems that avoid the macrophages (stealth drug carriers) (Cruz et al., 1997). Recognition by macrophages can be prevented by modification of the surface of the particles, for example by coating (Lemarchand et al., 2006) or by grafting hydrophilic polymers, like poly(ethylene oxide), PEO, on the particle surface. Due to its biocompatibility, PEO is one of the few synthetic polymers approved by the US Food and Drug Administration Agency. In a recent review by Otsuka et al., the utility of PEO in biological and pharmaceutical applications was widely shown (Otsuka et al., 2003). PEO is known to increase

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the biocompatibility by possessing stealth character, which is based on the steric repulsion around the macromolecule: the PEO-shell is expected to shield the core from binding to the cell surface and to avoid opsonization by proteins (Mosqueira et al., 2001; Ameller et al., 2003).

Novel materials responding to external stimuli are widely studied in pharmaceutical and biotechnological fields. Thermally responsive polymers have a reversible temperature dependent solubility and they are swollen in water below the lower critical solution temperature (LCST), but collapse and aggregate, when the temperature is raised above the LCST. A well-known thermosensitive polymer is poly(*N*-isopropylacrylamide) (PNIPAM), which may have potential, for example, in drug delivery (Hsiue et al., 2003; Eeckman et al., 2004), in tissue regeneration when modified with peptides (Stile and Healy, 2001; Smith et al., 2005), and as a carrier to transport the plasmid DNA to cell nuclei with cationic polymers (Twaites et al., 2004). No significant acute or subacute toxic signs after oral administration of PNIPAM have been observed even after 28 days of administration in mice (Malonne et al., 2005). Growing interest towards another thermosensitive polymer, poly(*N*-vinylcaprolactam) (PVCL), is justified because of even lower cytotoxicity compared to PNIPAM (Vihola et al., 2005), and due to the assumed biocompatibility *in vivo* (Peng and Wu, 2000). Phase separation of PNIPAM and PVCL takes place at approximately 32 °C (Heskins and Guillet, 1968; Kirsh, 1998) and, thus, these polymers are considered to be suitable for biotechnological applications, as the collapsing temperature is close to the physiological and can be adjusted with different comonomers (Taylor and Cerankowski, 1975).

Core-shell polymer particles/micelles consisting of temperature responsive polymers with an inner hydrophobic core and a hydrophilic shell have been used to control drug release with temperature changes (Chung et al., 2000; Lo et al., 2005; Soga et al., 2005). The core has been loaded with hydrophobic drugs while the hydrophilic shell responds to the temperature and stabilizes the structure. As a result of the collapse of the shell, the structural deformation of the core controls the release of the drug. Furthermore, the small size and hydrophilic surface have enabled the polymeric micelles to avoid the recognition by macrophages. This has allowed the accumulation for example in tumors, where the temperature is usually higher than the normal body temperature (Meyer et al., 2001). In the present study, a new type of core (fluorescently labeled polystyrene as a model polymeric core)—shell (crosslinked temperature responsive PNIPAM or PVCL) particles were prepared and their physical chemical properties and cellular interactions characterized. Core particles grafted with an amphiphilic PEO-macromonomer, $MAC_{11}EO_{42}$, were also prepared in order to study the effect of the PEO chains on the recognition and interactions with the cells (expected stealth action). The effects of temperature and polymer coating or grafting on the cellular attachment and interactions were evaluated by cell association/uptake and visualized by confocal scanning microscope in RAW 264.7 macrophages and in intestinal Caco-2 cells.

2. Experimental

2.1. Materials

Styrene (99% Merck, Germany) was distilled under reduced pressure prior to use. *N*-Isopropylacrylamide (NIPAM; 99% Acros Organics, USA) was recrystallised from *n*-hexane and dried in vacuum. *N*-Vinylcaprolactam (VCL; 98% Aldrich Chemicals Corporation, Germany) was recrystallized from benzene. Fluorescence label for the core, fluorescein dimethacrylate (FDMA; 99% Polysciences, USA), sodium dodecylsulfate (SDS; 99% Merck, Germany), potassium peroxydisulfate (KPS; 99% Merck, Germany), *N*,*N*-methylenebisacrylamide (BA; 99% Sigma–Aldrich, Germany), and 2,2′-azo-bis[2-methyl-*N*-(2-hydroxyethyl) propionamide] (VA-086; Wako Chemicals, Japan) were used as received. Water used as a solvent was purified with an Elgastat UHQ-PS purification system (United Kingdom). Filter papers (Whatman, 2V, United Kingdom) were of pore size 8 μm. Cellulose membrane tubings (MWCO 12000–140,00 g/mol, CelluSep T4, USA) were used in particle purification by dialysis. The synthesis and characterization of the amphiphilic PEO-macromonomer, $MAC_{11}EO_{42}$, has been previously reported (Laukkanen et al., 2000). The chemical structures of the PNIPAM and PVCL polymers and the PEO-macromonomer are shown in Fig. 1 and the summary of the syntheses and the abbreviations for the particles are shown in Table 1.

2.2. Polymerisations

All polymerisations were carried out in a sealed round-bottom flask equipped with a magnetic stirrer and an oil bath to control the reaction temperature. The fluorescent core particles (fluorescent polystyrene, FPS, as a model polymeric core), were prepared by radical copolymerisation of styrene and fluorescein dimethacrylate in aqueous emulsion. Covalent linking of the fluorescent label to the core PS particles was used in order to ensure easy and reliable detection of cell–particle association. Surfactant, SDS, was dissolved in water in the reaction

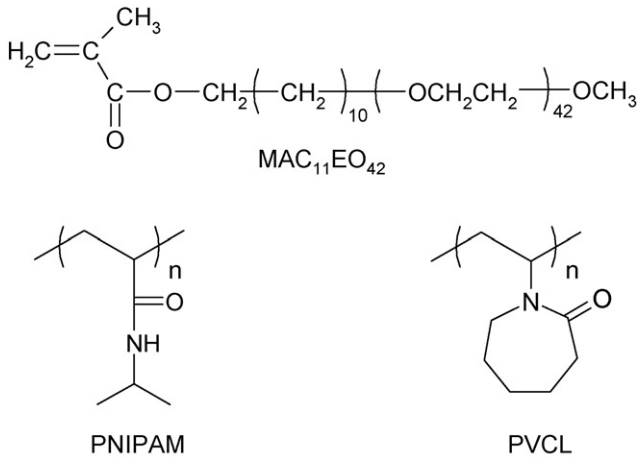

Fig. 1. Chemical structures of the thermosensitive polymers and PEO-macromonomer ($MAC_{11}EO_{42}$).

Table 1
Syntheses of the polymeric particles

Particle sample	Water/ seed (ml)	Styrene (mmol)	FDMA (mmol)	KPS (mmol)	SDS (mmol)	NIPAM (mmol)	VCL (mmol)	BA (mmol)	VA-086 (mmol)	$MAC_{11}EO_{42}$ (mmol)
FPS	10	7.7	0.043	0.074	0.347					
FPS-PNIPAM	10 (FPS)			0.022		1.325		0.065		
FPS-PEO	10	6.2	0.043	0.148						0.136
FPS-PVCL	9 (FPS)						0.97	0.06	0.04	

flask, after which the flask was sealed with a septum and placed into a preheated oil bath at 70 °C. The solution was purged with nitrogen and stirred at 400 rpm. The monomers were injected to the reaction flask with a syringe, after which the nitrogen purging was continued for 5 min. The nitrogen inlet and outlet was removed and polymerisation was initiated by injecting KPS dissolved in 2 ml of water to the reaction mixture.

The poly(ethylene oxide)-grafted particles, FPS-PEO, were prepared as follows: KPS and the macromonomer, $MAC_{11}EO_{42}$, were dissolved in water in the reaction flask. The flask was sealed with a septum and the solution was purged with nitrogen and stirred at room temperature for 30 min. Styrene and fluorescein dimethacrylate were injected to the reaction flask and the flask was placed into the preheated oil bath at 70 °C. The macromonomer consists of a reactive methacrylate group, a hydrophobic alkyl chain (11 methylene units) and a hydrophilic PEO segment (42 ethylene oxide units). After grafting, the hydrophobic end is located at the hydrophobic polystyrene core, leaving the hydrophilic PEO-part of the macromonomer at the surface of the particle.

In the synthesis of FPS-PNIPAM, NIPAM-monomer and the crosslinking monomer (BA) were separately dissolved in the aqueous seed particle dispersion, FPS, and both solutions were transferred to a reaction flask. The flask was sealed with a septum and polymerisation was initiated and carried out by following the procedure (N_2, KPS) described for the synthesis of fluorescent seed particles.

In the synthesis of FPS-PVCL, VCL-monomer, crosslinking agent (BA), initiator (VA-086) and the seed FPS-latex dispersion were added to a 50 ml round bottom flask. The mixture was purged with nitrogen for 30 min at 20 °C to remove dissolved oxygen. At the same time the mixture was stirred to solubilise all the components, after which the flask was placed into a preheated oil bath at 80 °C.

All the polymerisation reactions were allowed to proceed at 70–80 °C for 3 h with stirring (600–700 rpm). The reactions were stopped by cooling to ambient temperature. After that, all the products were filtered to remove possible precipitates and purified by dialysis for 7 days (in darkness) against distilled water that was refreshed daily. The purified particle dispersions were stored as such in the refrigerator.

3. Characterization of the particles

3.1. DLS

The size distributions of the particles were measured before and after the coating reaction (Table 2). The size distributions of the particles were obtained with dynamic light scattering (DLS) by using the instrument of Bookhaven Instruments (BI-200SM goniometer, BI-9000AT digital correlator, USA) equipped with a laser at a wavelength of 633 nm. Scattering was collected at 90° scattering angle and the obtained time correlation functions were analysed with CONTIN Laplace-inversion program. The polymer concentration in the samples was 0.01 mg/ml obtained by diluting the particle dispersion with deionised water. Temperature of the sample unit (20 °C) was controlled with Lauda RC6 CP-thermostate (Germany).

3.2. Zeta-potential

Surface charges of the polymeric nanoparticles (Table 2) were measured by zeta-potential analyzer (Malvern Zetasizer 3000HSA, Malvern Instruments Ltd., UK). The particle dispersions were diluted to 1/10 with the buffer used at cellular experiments (see below). With the same equipment and the same samples it was also possible to perform the measurements of particle size distributions at ambient temperature and compare the results with the DLS measurements.

3.3. IR-spectroscopy

The incorporation of PNIPAM, PVCL or PEO-macromonomer on the particles was detected by IR spectroscopy. FTIR-spectra were measured from freeze-dried particles with the instrument of Perkin-Elmer Instruments (Spectrum One FT-IR-spectrometer, Spectrum One FT-IR-software, Universal ATR Sampling Accessory).

3.4. Cell cultures

Murine RAW 264.7 macrophages were cultured in 75 cm^2 culture flasks (Corning, USA) using Dulbecco's modified Eagle medium (DMEM, Euroclone, UK) supplemented with 10% fetal

Table 2
Characteristics of the fluorescence labeled polymer particles

Sample	R_h (nm, mean)[a]	ζ (mV)[b]
FPS	22	-40 ± 0.5
FPS-PEO	23	3 ± 1.4
FPS-PNIPAM	58	3 ± 0.6
FPS-PVCL	24	-1 ± 0.9

[a] Hydrodynamic radius, measured by DLS at 20 °C in pure water ($n \geq 3$).
[b] Zeta-potential, measured by Zetasizer at 25 °C in HBSS-Hepes (mean ± S.D., $n = 3$).

bovine serum (Biowhittaker, Belgium), 1% L-glutamine and antibiotics penicillin (100 IU/ml) and streptomycin (100 μg/ml), all from Euroclone, UK. Caco-2 cells were cultured in 75 cm^2 culture flasks (Corning, USA) using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml), all from Euroclone, UK. Both cells were maintained at 37 °C and 5% CO_2. The growth medium was changed every other day until the time of use. The macrophages were harvested using ethylenediamine tetra-aceticacid (EDTA, Sigma–Aldrich, Germany)-phosphate buffered saline (PBS without Ca^{2+} and Mg^{2+}, Euroclone, UK) and Caco-2 cells (p 36) were harvested using trypsin (0.25%, Euroclone, UK)-EDTA-PBS solution. After harvesting, the cells were diluted to a density of 5×10^5 cells/ml and seeded into sterile 96 well plates (surface area 32 mm^2, Corning, USA) 100 μl/well. The cells were incubated at 37 °C for 3 h before the uptake experiments in order to attach to the well.

3.5. Polymer association with the cell membranes and cellular uptake

The polymer samples were diluted to a concentration of 20% (v/v) of original polymer dispersion with Hank's balanced buffer solution (HBSS). The fluorescent core particles were observed to aggregate with divalent cations, so the uptake experiments were performed with HBSS solution without calcium and magnesium (HBSS w/o $CaCl_2$ & $MgCl_2$, Gibco Invitrogen corp., USA). HBSS-solution was buffered at 7.4 with Hepes (10 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid], Sigma–Aldrich, Germany). RAW 264.7 macrophages were exposed to the polymer dispersions for 3 h either at 4 °C, 23 °C or 37 °C and Caco-2 cells at 37 °C for comparison. Based on previous experiments (Desai et al., 1997; Win and Feng, 2005), 3 h incubation was considered to reach the plateau in cellular binding/uptake. After incubation, the polymer dispersions were removed and each well was washed five times with 100 μl of fresh HBSS-Hepes and the cells were lysed with 20% sodium dodecyl sulphate (Sigma–Aldrich, Germany) in dimethylformamide (Riedel de Haen, Germany):H_2O (1:1). The polymer dispersions, washing solutions and lysing media were collected individually to a new black 96-well plate (Nalge Nunc International, USA). Black wells were used to attenuate the background noise from the fluorescence measurements. The amount of fluorescence was measured by fluorescence plate-reader (Wallac VICTOR® 1420 multilabel counter, Finland) from polymer dispersions, from all the washing media and from the lysing media at exitation and emission wavelengths of 485 and 535 nm, respectively. Cellular autofluorescence and the signals from the solvents were subtracted. As the fluorescence was shown to be diminished by the lysis-media, separate standard curves for each of the polymers were determined both in HBSS-buffer and lysis-media. The results were calculated as μg of polymer particles per well surface area (μg/mm^2) taking into account each initial amount of the polymer sample. These procedures allowed comparisons between the polymers at different temperatures and between the two different cell lines. The recoveries of polymers in each experiment were also calculated. Anionic fluorescence labeled dextran (FD, molecular weight 10,000 g/mol, Molecular Probes, USA), was used as a control for the uptake by the endocytotic pathway (Lencer et al., 1990). FD was dissolved in HBSS-Hepes at a concentration of 50 μg/ml and the experiments were carried out by the procedure above.

3.6. Fluorescence and confocal scanning microscopy

RAW 264.7 cells were cultured at a density of 5×10^5 cells/ml on glass coverslips at the bottom of 12-well plates. After attaching to the coverslips, the cells were incubated with the polymer samples for 3 h at 37 °C. After incubation, the cells on the coverslips were washed quickly with PBS and incubated for 20 min at room temperature with 3 ml of fixative (PBS containing 1% paraformaldehyde, 100 mM L-lysine and 10 mM sodium metaperiodate, all from Sigma–Aldrich, Germany). After incubation, the cells were rinsed three times with PBS for 5 min. The glass coverslips were then inverted (cell side down) onto a drop of Vectashield (Vector Laboratories Inc., CA, USA). The excess of Vectashield was removed and the edges of the coverslips were sealed.

The samples were monitored first by fluorescence microscopy (Nikon Eclipse TE 300, Nikon Corp., Japan) and after that the images were captured by using confocal scanning microscopy (Leica TCS SP2 AOBS, Germany) with oil 63× objective (HCX PL APO oil 63×/NA 1.4-0.6). Depth scanning with confocal microscopy enabled the localization of the fluorescence in the cell structures. The brightness of the confocal images was adjusted to the whole image (Adobe Photoshop 6.0, Adobe Systems, USA). Labels were placed on the images that were reduced to the desired size, to yield 300 dpi.

3.7. Statistical analysis

The interactions with the polymers and cells presented in Figs. 3 and 4 are expressed as average values ± S.E.M. The variations of the different tests in Fig. 3a and b were analyzed by two-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons by using SYSTAT® for Windows-software (Version 5.1, Systat Inc., USA). The results presented in Fig. 4 were analyzed by using Student's two-tailed *t*-test. The differences were regarded statistically significant when $p < 0.05$.

4. Results

4.1. DLS

Hydrodynamic radii (mean) from the intensity weighted size distributions in aqueous particle dispersions at 20 °C are shown in Table 2. The values are calculated from at least three repeated measurements. The particle size was not affected when the synthesis was performed using SDS as a surfactant (FPS, radius = 22 nm) or $MAC_{11}EO_{42}$-macromonomer (FPS-PEO, radius = 23 nm). FPS was used as a seed particle in the synthesis of FPS-PNIPAM and FPS-PVCL (Table 1). From

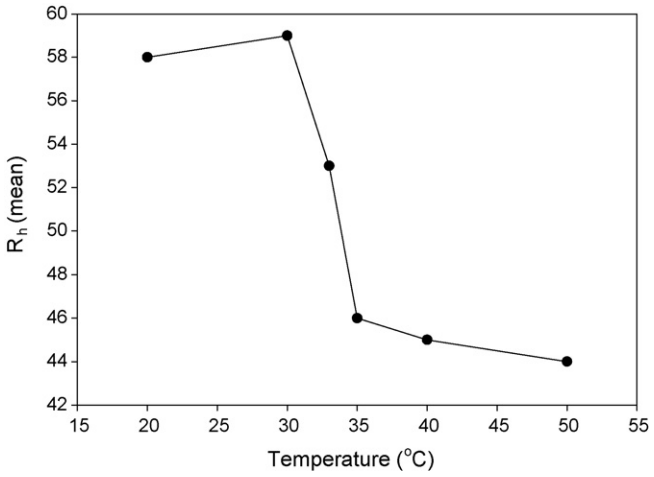

Fig. 2. Mean hydrodynamic radius (R_h mean, $n = 5$) of FPS-PNIPAM as a function of temperature in pure water.

Table 2 it can be seen that the particle size increased during the coating polymerisation of PNIPAM (radius = 58 nm). After coating, the temperature dependent phase behaviour of PNIPAM was observed (Fig. 2). The hydrodynamic radius of FPS-PNIPAM was decreased with increasing temperature and the volume transition was found to be reversible.

After the coating reaction with PVCL, the hydrodynamic radius of the FPS-PVCL particles was only slightly increased (24 nm, Table 2). The size distribution remained narrow after the reaction and, thus, secondary nucleation during the polymerisation was probably not taking place. No remarkable change of the particle size upon heating was observed (data not shown).

4.2. Zeta-potential

Zeta-potentials of the particles in HBSS-Hepes are shown in Table 2 and expressed as mean values (±S.D., $n = 3$). The coating and grafting had a clear impact on the surface charge of the polymer particles. The FPS core particles were clearly anionic (−40 mV), whereas the grafted FPS-PEO and coated FPS-PNIPAM and FPS-PVCL particles were found to be virtually neutral (Table 2). These measurements support the assumption that the PEO-macromonomer, PNIPAM and PVCL were bound to the FPS-core during the manufacture. Particle size analyses with Zetasizer were consistent with the DLS results (data not shown).

4.3. IR-spectroscopy

The characteristic bands of ethylene oxide units of PEO (for example a strong band at 1104 cm^{-1}) were observed in the spectrum of FPS-PEO. FPS-PNIPAM showed the characteristic bands of both the core and the shell polymer (polystyrene at 755 and 697 cm^{-1}, aromatic ring and PNIPAM strong band at 1645 cm^{-1}, amide group). Also the PVCL coated FPS particles showed a characteristic band at wavelenght 1627 cm^{-1} attributed to the amide group in the lactam ring of PVCL. The spectra of FPS did not differ clearly from the spectra of pure polystyrene, so no conclusions about the presence of small amount of the fluorescent label in the core particles could be drawn based on the IR-measurements.

4.4. Polymer association with the cell membranes and cellular uptake

Cellular adhesion of the PVCL-coated FPS-particles with RAW 264.7 macrophages was more than twofold compared to that of the FPS core or the FPS-PNIPAM particles at 37 °C ($p < 0.05$, Fig. 3a). This indicates that the two thermosensitive polymers exhibit different behaviour and surface properties at 37 °C. As the temperature was decreased, the interactions between the cells and the particles diminished, but again the amount of highest cell association was found with the FPS-PVCL particles ($p < 0.05$, Fig. 3a), although anomalously high cell association of FPS could be seen at 23 °C. Attachment of the FPS-PEO particles to the RAW 264.7 cell membranes was essentially negligible (with the exception of 23 °C) compared to the FPS particles alone or the FPS particles coated by the thermosensitive polymers ($p < 0.05$, Fig. 3a). The statistical differences

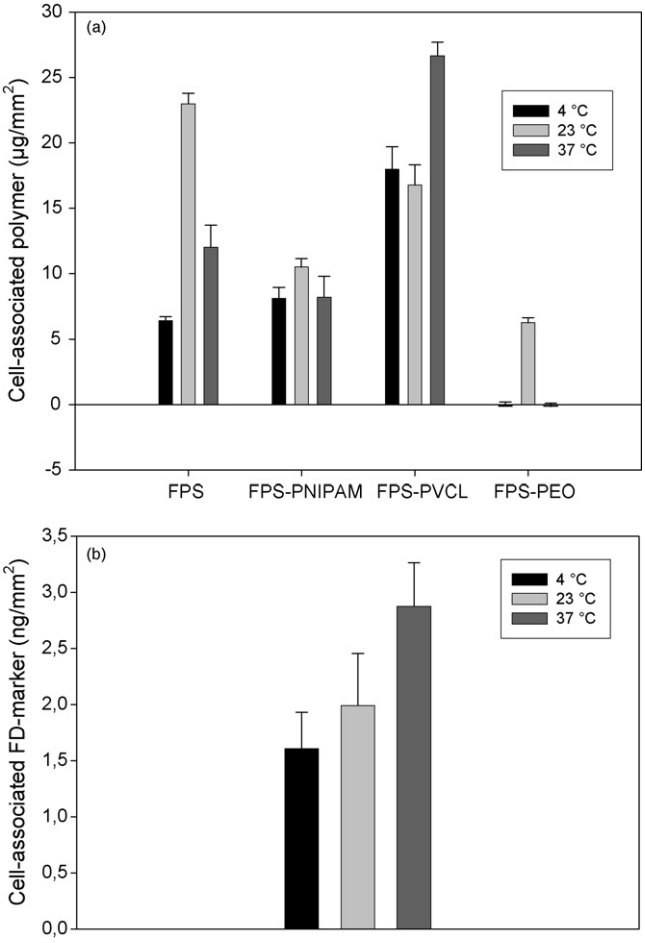

Fig. 3. (a) The effect of temperature on the amount of fluorescently labeled polymer particles associated with the cells (µg/mm^2) after 3 h of incubation with RAW 264.7 macrophages ($n = 10–18 \pm$ S.E.M.). (b) The effect of temperature on the amount of fluorescent dextran marker associated with the cells (ng/mm^2) after 3 h of incubation with RAW 264.7 macrophages ($n = 12–18 \pm$ S.E.M.).

between the temperatures within each polymer were significant with the exception of FPS-PNIPAM and the temperatures 4 and 23 °C in the case of FPS-PVCL. The amount of cell-associated fluorescence with the endocytotic marker, FD, was low (relative to that observed with the FPS, FPS-PVCL and FPS-PNIPAM particles) (Fig. 3b). Again, the cellular interactions were shown to be temperature dependent; as the temperature was decreased from 37 to 4 °C, the cellular association of FD was decreased ($p < 0.05$).

In the cases of the thermosensitive FPS-PVCL and FPS-PNIPAM, the total amount of fluorescence associated to the RAW 264.7 macrophages was significantly higher than in Caco-2 cells (37 °C, 3 h incubation; Fig. 4). In contrast, no clear difference was seen between the two cell lines in the cases of FPS (relatively high cell association) and FPS-PEO (negligible cell association). While the FPS-PVCL particles exhibited high adsorption also in the Caco-2 cells, cellular association of the FPS-PNIPAM particles was at similarly negligible level as with the FPS-PEO particles. Association of FD with the Caco-2 cells was similar to FD association with the RAW 264.7 macrophages (data not shown).

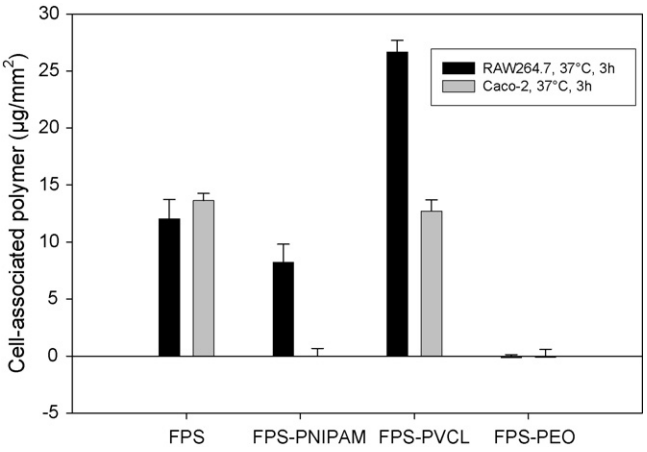

Fig. 4. Cellular interactions reported as μg/mm^2 of polymer particles associated with the cells after 3 h of incubation in RAW 264.7 macrophages or Caco-2 cells at 37 °C ($n = 10–18 \pm$ S.E.M. for RAW 264.7 cells and $n = 9–12 \pm$ S.E.M. for Caco-2 cells).

4.5. Visualization of polymer-cell associations

Fig. 5 represents confocal microscope images from the RAW 264.7 cells after 3 h of incubation at 37 °C either with FPS-PVCL (5a), FPS-PNIPAM (5b), FPS-PEO (5c) or with the endocytotic marker, FD (5d). The strong fluorescence in Fig. 5a could be observed at the surfaces of cell membranes and a weaker distribution through the cell membranes, suggesting that the FPS-PVCL was associated mainly with the cell membrane and had not been internalized into the cytoplasm at least within 3 h. In the case of FPS-PNIPAM, some fluorescent particles were seen evenly distributed inside individual cells (Fig. 5b), although most of the label was seen attached to the plasma membranes of

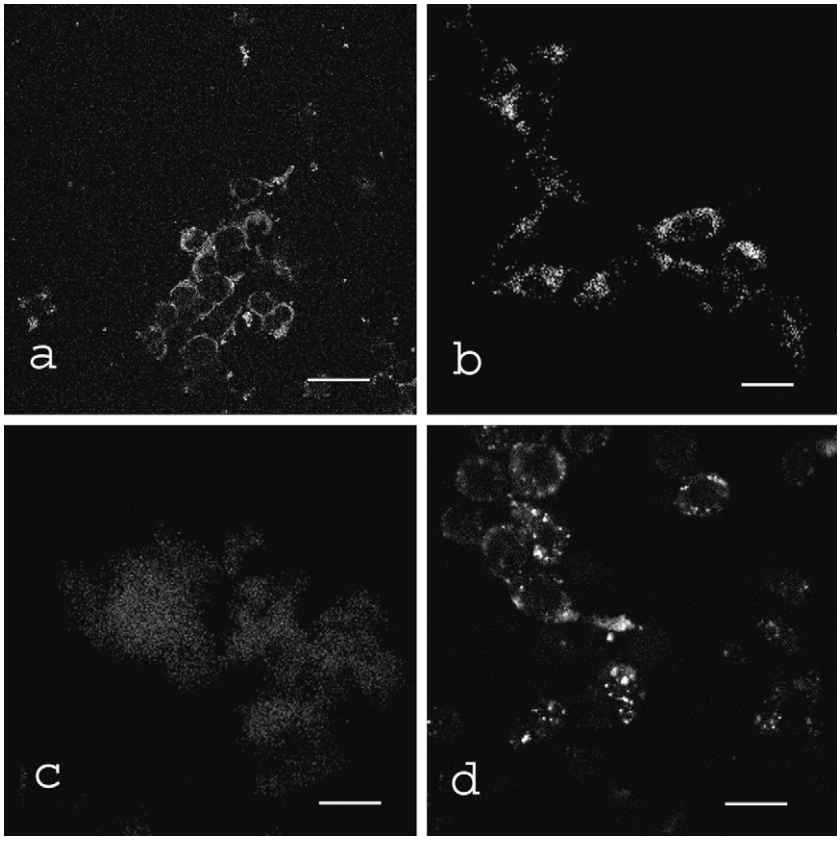

Fig. 5. Confocal scanning microscope images from the RAW 264.7 macrophages after incubation with the polymer samples. FPS-PVCL (a), FPS-PNIPAM (b), FPS-PEO (c) and fluorescently labeled dextran (FD) (d). Scale bar 20 μm.

the macrophages. In the case of FPS-PEO, hardly any fluorescence was detected as the polymer particles were not attached to the cells, evidencing the steric repulsion and rejection created by PEO on the FPS-particles (Fig. 5c). For the FPS core particles, the confocal scanning microscope images showed attachment to cell membranes of the macrophages, but no internalization (image not shown). It can be clearly seen from the Fig. 5d that the endocytotic marker, FD, was taken up by the macrophages, as small fluorescent vesicular structures could be observed inside the macrophages.

5. Discussion

5.1. Cell–particle interactions

To discuss the different cellular association of the thermosensitive FPS-PNIPAM and FPS-PVCL particles, the first consideration is directed towards the different size (radius of 58 nm versus 24 nm, respectively). During coating, the particle size of FPS-PVCL increased only a few nm (which is within the error margin of the DLS measurements), and no typical reduction of the size of these thermosensitive particles as a function of temperature were observed. The reason may be related to the more hydrophobic nature of VCL compared to NIPAM. Consequently, considerable amount of VCL is most probably distributed into the FPS latex before the initiation, and the formed polymeric coating layer may be partially interpenetrated within the FPS matrix.

PVCL is adsorbed to many proteins (Kirsh, 1998), which may explain the stronger interactions with FPS-PVCL and the cell membranes compared to the other polymers. Cell membranes, especially the plasma membranes of macrophages, are full of proteins, which can interact and form specific hydrogen bonds with the PVCL. The confocal scanning microscope images showed that the FPS-PVCL particles were attached mainly to the cell membrane and not internalized into the macrophages. Lipophilic segments and hydrophobic surface on the polymer particles contribute to hydrophobic interactions with lipophilic cell membranes, whereas hydrophilic segments are rejected (Maassen et al., 1993; Sahoo et al., 2002; Nori and Kopecek, 2005). In the case of FPS-PVCL, the increase in temperature induced dehydration and increased the hydrophobicity of PVCL, which could be observed as increased cellular associations of FPS-PVCL with increasing temperature.

In the case of FPS-PNIPAM, the reactivity ratios between the crosslinking agent, BA, and the monomer, NIPAM, are different (Wu et al., 1994). The reactivity of the crosslinker is higher than that of NIPAM and the rate of radical polymerisation is faster. Owing to this, the amount of crosslinker at the surface of the FPS-PNIPAM particle may be lower than in the vicinity of the core. As a consequence, linear, dangling chain segments with no crosslinker are most likely formed on the surface of the particles creating steric repulsion (Ballauff, 2003). The relatively weak attachment of FPS-PNIPAM to the cells can therefore be explained due to steric repulsion, which prevents the attachment onto the surface of the cells and, on the other hand, prevents the adsorption of proteins onto the surface of the polymer. However, when the temperature is increased and the particles collapse, the chains compress parallel to the particle surface, and it is assumed that the steric repulsion becomes less effective (Laukkanen et al., 2002).

In this study, temperature seemed to have no effect on the degree of repulsion of FPS-PNIPAM, as at higher temperatures no reduction in repulsion was observed. We assume that the PNIPAM chains at the outer surface of the crosslinked PNIPAM layer (on the FPS-core) do not entirely collapse, although most of the PNIPAM layer is collapsed at 37 °C as indicated by the DLS data. Recent results of polystyrene particles coated with crosslinked PNIPAM indicated that the phase transition was shifted towards higher temperatures when compared to PNIPAM microgels and that the temperature range of the phase transition was broader (Andersson et al., 2006); similar observations have been made by others studying different types of PNIPAM coated particles (Nuopponen et al., 2004; Shan et al., 2004; Xu et al., 2006). Moreover, in PNIPAM microgel particles a shell of slightly cross-linked polymer chains containing more ionic groups than the core (coronal polyelectrolyte layer) have been linked to higher collapse temperature (Daly and Saunders, 2000). Our results of zeta-potential measurements at 37 °C also support this assumption, as the FPS-PNIPAM was found slightly anionic when heated above the LCST (−7.0 mV at 37 °C versus +3.0 mV at 25 °C). Thus, the negative charge at the surface aids in maintaining the repulsion effective at 37 °C and the surface of the particles remains relatively hydrophilic.

Despite the negative charge repulsion uncoated/ungrafted FPS core particles displayed clear attachment to the studied cells. This was probably due to hydrophobic nature of the FPS core, which assisted cellular interactions. However, PEO-macromonomer grafted polystyrene particles had no affinity to the phagocytic or intestinal cells confirming the stealth characteristics of PEO chains. As discussed previously, the repulsion of both PEO and PNIPAM were considered to be sterical because of the dangling chains at the surface of the FPS-core. Recently, it has been shown that at elevated temperatures, the hydrophilic PEO sterically stabilizes also particles made from the thermosensitive polymers, PNIPAM (Virtanen et al., 2000) and PVCL (Laukkanen et al., 2002). It was also observed in our previous publication that PEO, when grafted to the PVCL, diminished the cellular toxicity of PVCL in MTT- and LDH-tests, presumably once again by steric repulsion (Vihola et al., 2005).

Based on the confocal images, endocytosis was not observed in any of the polymer samples at 37 °C, only plasma membrane binding or a small number of individual fluorescent particles inside the cytoplasm in the case of FPS-PNIPAM could be detected. Instead, the well-known endocytotic marker, FD, was clearly shown to be taken up inside the macrophages by endocytosis. Although the amount of internalized FD was relatively small, the cellular interactions of FD were shown to be temperature dependent as the cellular uptake was linearly increased by the temperature. It should be mentioned here that the uptake of FD (and possibly also of the polymeric nanoparticles) might have been weakened by the absence of the divalent cations in

the cell culture medium, as these are needed in the phagocytosis (Mason et al., 1973).

5.2. Cell lines

The cell type affects the total uptake and kinetics of the uptake (Zauner et al., 2001). Intestinal Caco-2 cells, being a colonic tumor cell line (the preferred model for intestinal drug absorbtion), have a layer of microvilli and high transepithelial electrical resistance, which act as barriers against foreign materials (Quaroni and Hochman, 1996). On the other hand, macrophages are rich in cell membrane proteins, secrete cytokines and have vacuoles in their cytoplasms (Nacife et al., 2000). The cell membrane proteins of macrophages enable opsonization, which further eases up the phagocytosis. Win and Feng (2005) found out that commercial polystyrene nanoparticles were attached to the apical membrane surface of Caco-2 cells (not inside the cells), whereas Foged et al. (2005) observed that polystyrene particles were associated with the cell membrane or were internalized inside the cell cytoplasm of dendritic cells.

Thus, not too surprisingly, the thermosensitive polymer particles in this study displayed lesser interactions with the Caco-2 cells than with the RAW 264.7 macrophages. This is most likely a result from the barrier properties of the Caco-2 cells. Differences in cellular association between the PVCL (high association) and PNIPAM (low association) in the Caco-2 cells implicates that also the intestinal drug delivery could possibly be affected by these materials. The increased interactions with the RAW 264.7 macrophages result from the surface characteristics of the macrophages (see above), which confirms that the macrophages recognize thermosensitive polymers differently than the Caco-2 cells and that the cell membranes of the macrophages are more responsive inducing the attachment of the thermosensitive polymers to the cell membrane. The FPS-particles (as such, grafted or coated) were not found inside cell cytoplasms of RAW 264.7 or Caco-2 cells (with the exception of FPS-PNIPAM inside some individual cells).

6. Conclusions

Despite the fact that temperature responsive phase transition can be detected both in PNIPAM and PVCL, the different structures and physical chemical characteristics of the polymers produced diverse cellular associations with FPS-PNIPAM and FPS-PVCL, respectively. PVCL enhanced the cellular attachment of FPS-particles as a function of temperature presumably by hydrophobic interactions with lipophilic cell membranes. PNIPAM was found to interact with the cells to a much lesser extent because of steric repulsion similar to PEO-macromonomer grafted polystyrene. RAW 264.7 macrophages exhibited a higher extent of interactions with the thermosensitive polymers than intestinal Caco-2 cells. The cell–particle interactions were considered to combine the effects of size, surface charge, hydrophobicity, steric repulsion and temperature. The thermosensitive nature and low cellular toxicity of PVCL and PNIPAM used as coating (or grafting) polymer may open up a range of biotechnological and pharmaceutical applications in the form of either stealth-carrier behaviour or enhanced cellular contact.

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