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Avidin bioconjugate with a thermoresponsive polymer for biological and pharmaceutical applications

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Abstract

A thermoresponsive polymer, *N*-isopropylacrylamide-*co*-acrylamide (Mn 6 kDa) with a lower critical solution temperature (LCST) of 37 °C, was activated and conjugated to avidin to yield a derivative with 200 kDa molecular weight. Gel permeation analysis demonstrated that the new bioconjugate possessed an apparent size corresponding to a 220 kDa globular protein. Photon correlation spectroscopy and turbidometric studies showed that the bioconjugate underwent temperature dependent phase transitions. The protein–co-polymer bioconjugate displayed the same onset phase transition temperature (LCST) as the original synthetic co-polymer. Nevertheless, the aggregation profile of the bioconjugate shifted at higher temperature as compared to the original polymer. This indicated that the aggregation behaviour coil-to-globule transition of the co-polymer was modified by anchoring to the protein surface. Circular dichroism analysis showed that the co-polymer conjugation did not alter the protein tertiary structure tertiary the aromatic amino acid environment. The bioconjugate maintained 85 ± 3% of native avidin affinity for biotin and biotin-Mab, and high affinity was maintained after three heating cycles. Pharmacokinetic studies demonstrated that the co-polymer bioconjugation increased the avidin residence time in the bloodstream. The distribution phase of avidin-co-polymer was longer than the native protein by a factor of 20. The co-polymer conjugation decreased by three-fold the distribution extent of avidin and reduced significantly its up-take to the liver.

Keywords: Polymer conjugation; Thermoresponsive polymers; Avidin; Protein delivery; Poly-*N*-isopropylacrylamide

1. Introduction

The conjugation of synthetic soluble polymers to proteins and peptides is now a well-established strategy to enhance the pharmacokinetic, immunological and physicochemical properties of these biomolecules. The most used polymer in bioconjugation chemistry is poly(ethylene glycol) (PEG) owing to its favorable physicochemical and biological properties, specifically high solubility, complete elimination from the body and lack of local or systemic toxicity (Harris and Chess, 2003; Caliceti and Veronese, 2003).

Due to its advantageous properties, PEG has been widely used to produce new bioconjugated therapeutics, some of which have been rapidly adopted in the pharmaceutical market. These include, for example, L-asparaginase-PEG (Oncaspar), adenosine deaminase-PEG (Adagen) interferon-PEG (Pegintron and Pegays), filgastrim-PEG (Neulasta) and growth hormone-PEG (Pegvisomant) and many others are under development (Vicent and Duncan, 2006). Nevertheless, new polymers are being actively investigated in order to obtain derivatives with enhanced biological functionalities or physicochemical and biopharmaceutical features (Caliceti et al., 1999; Gregoriadis et al., 2005; Heredia and Maynard, 2007; Narain, 2006). Responsive polymers are of note in this regard, as a consequence of their capacity to change their structures and solubility by external stimuli. Amongst these polymers, poly(*N*-isopropylacrylamide) (pNIPAm) shows especial promise as it exhibits a coil-to-globule phase transition at its lower critical solution temperature (LCST) of about 32 °C in water (Schild, 1992).

Polymerization of NIPAm with co-monomers allows tuning of the LCST across a broad temperature range, thus with a hydrophilic co-monomer the LCST can be raised to a higher

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temperature making it of interest for pharmaceutical applications. PNIPAm co-polymer conjugates have the potential of changing the solubility, stability and even the functional properties at temperatures close to those in the body. Therefore, a new class of polymer bioconjugates with specific on/off temperature switching properties might be generated. These derivatives can be advantageously exploited for innovative therapeutic applications as either external or pathophysiological thermal stimuli can induce structural changes and modify their biological behaviour. In particular, the higher temperatures of disease tissues such as tumors or inflamed tissues with respect to healthy tissues as well as external ultrasound-induced local hyperthermia (Chilkoti et al., 2002; Meyer et al., 2001), can be exploited to yield targeted switch on/switch off activity.

In order to evaluate thermoresponsive polymers as functional alternatives to PEG in protein conjugation, we have investigated the modification of avidin with poly(*N*-isopropylacrylamide-*co*-acrylamide) (pNIPAm-*co*-Am). As a consequence of its high affinity interaction with biotin, the avidin–biotin complex has become a central tool in several biochemical and medical applications (Wilchek et al., 2006; Chen et al., 2000). The large array of applications could be expanded still further if new avidin derivatives with responsive controllable physicochemical and biopharmaceutical properties could be prepared. For example, long lasting and low immunogenic PEGylated avidins have been investigated for use in drug delivery and targeting protocols (Caliceti et al., 2002; Salmaso et al., 2005).

Responsive polymer derivatives of avidin potentially offer the possibility to combine the prolonged circulation properties of PEGylated derivatives with the ability to switch the biological and biopharmaceutical properties through a temperature pulse. Potential biomedical applications of responsive avidins include reversible protein capture (Galaev and Mattiasson, 1999; Malmstadt et al., 2003) switchable drug targeting (Lackey et al., 2002) and activated clearance of drugs and diagnostic agents through controllable 'avidin chase' (Kobayashi et al., 2003).

However, while covalent attachment of pNIPAm co-polymers to proteins has been shown to alter in a controllable fashion the biochemical and physicochemical properties of the resultant conjugates (Hoffman and Stayton, 2004; Shimoboji et al., 2003; Ding et al., 1996, 1998; Lee and Park, 1998; Yamanaka et al., 2003; Yoshizako et al., 2002; Shimoboji et al., 2002a,b; Pennadam et al., 2004), there have been no prior reports of how the pharmacokinetic properties of a protein vary when conjugated to pNIPAm-based co-polymers. In this paper we show that a new avidin derivative with pNIPAm-*co*-Am exhibits improved properties for in vivo and in vitro applications as a result of polymer modification of the biological, biopharmaceutical and physicochemical properties of this protein.

2. Materials and methods

2.1. Materials

Standard reagents and chemicals for poly-(*N*-isopropylacrylamide-*co*-acrylamide) (pNIPAm-*co*-Am) synthesis were purchased from Fisher Scientific (Loughborough, UK) and Aldrich (Sternheim, Germany) and used as received. Acrylamide (Am) and *N*-isopropylacrylamide (NIPAm) were purchased from Aldrich: the *N*-isopropylacrylamide was recrystallized from hexane before use. Purification of solvents for preparative chemistry was performed by standard methods.

Egg white avidin and biotin conjugated EPB2 (LL2)-L2 monoclonal antibodies were obtained from BIO-SPA (Milan, Italy). *N*-hydroxysuccinimidyl ester activated 5 kDa monomethoxy-poly(ethylene glycol) (PEG-NHS) was purchased from Nektar (Huntsville, AL, USA). Biotin and 4′-hydroxybenzene-2-carboxylic acid (HABA) came from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H] succinimidyl propionate was obtained from Amersham International (Amersham, UK). Soluene 350, Instagel and Ionic Fluor for radioactivity determinations were obtained from Canberra Packard (Groningen, The Netherlands). The Superose 6 gel filtration column and the FPLC system were supplied by Pharmacia Biotech (Uppsala, Sweden). The ultra filtration system came from Amicon Inc. (Beverly, MA, USA). All other reagents of analytical grade, were from Fluka (Buchs, Switzerland).

The fed ad libitum male Balb/c mice weighting 22 ± 2 g used for the in vivo studies were from the Department of Pharmaceutical Sciences, University of Padua. The body temperature was measured with a prismatic thermometer between 11.00 a.m. and 4.00 p.m. The animal experiments were performed in accordance with Italian law (DL n. 116/92) and European guidelines (EEC n. 86/609).

2.2. Co-polymers preparation and activation

Co-polymers of *N*-isopropylacrylamide and acrylamide were prepared via free-radical polymerization in 2-propanol using 3-mercaptopropanoic acid as a chain transfer agent to generate a carboxyl terminus according to our previously published protocols (Cunliffe et al., 2003). Owing to the well-documented problems obtaining reliable molecular weights by gel permeation chromatography (GPC) for pNIPAm-based polymers the molecular weights of the carboxyl-terminated polymers were determined by titration of dissolved polymer (∼100 mg) in deionised water (50 ml) with freshly prepared 10 mM sodium hydroxide solution. The end point was either determined potentiometrically or by using phenolphthalein solution as indicator. GPC using THF as the eluent was also used as a secondary technique for establishing the molecular weight profile.

2.3. Avidin–pNIPAm-co-Am and avidin–PEG preparation

Avidin from pure egg white in 0.2 M borate buffer, pH 8.0 (5 mg ml^{-1}) was reacted with *N*-hydroxysuccinimidyl ester activated 6 kDa pNIPAm-*co*-Am (pNIPAm-*co*-Am-NHS) or 5 kDa PEG-NHS using 1:2 protein amino group/polymer molar ratio. The solutions were maintained under stirring for 2 h at room temperature and then extensively ultra-filtered using a 50 kDa cut off membrane. The concentrated solutions were purified by gel filtration FPLC with a Superose 6 column eluted with 0.05 M ammonium acetate, 0.5 M NaCl, pH 4, to eliminate the un-reacted polymers. The eluted fractions were

analyzed via UV absorption at 282 nm to detect protein and by using iodine to detect the polymer components (Sims and Snape, 1980). The fractions corresponding to avidin–pNIPAm-*co*-Am or avidin–PEG were collected and concentrated by ultra-filtration using a 10 kDa cut off membrane. The avidin concentration in the final product was quantified by UV absorption measurements at 282 nm. The mean number of polymer chains bound to the protein was determined by NH_2 analysis according to the TNBS test (Habeeb, 1966).

2.4. Cloud point determination

Polymer or polymer–protein conjugate solutions (1 mg ml^{-1} in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4) were first cooled to 5 °C and then the temperature was increased to 60 or 70 °C at 0.5 °C min^{-1} in a thermostated UV spectrometer. At scheduled temperatures, the samples were maintained for 10 min and absorption at 500 nm was determined. The lower critical solution temperature was calculated as the mean value between the highest temperature at which no turbidity was observed and the lowest temperature at which the minimal optical absorbance variation was detected.

2.5. Photon correlation spectroscopy

Equimolar avidin, avidin–pNIPAm-*co*-Am and avidin–PEG solutions (0.5–5 mg ml^{-1}) in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4, were filtered with 0.45 μm cut off filters. The solutions were thermostated at 21 and 40 °C and then analyzed using a single angle photon correlation spectrometer Nicomp 370 (Particle Sizing Systems, St. Barbara, CA) equipped with a 632.5 nm laser.

2.6. Circular dichroism analysis

Circular dichroism spectra were recorded in the far UV (193–250 nm) and near UV (250–330 nm) using 0.38 μM solutions of wild type avidin and avidin–pNIPAm-*co*-Am in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4. The analyses were performed out using a J-810 Jasco spectrodichrograph (Tokyo, Japan).

2.7. Biological activity evaluations

The avidin/biotin and avidin/biotinylated antibody (EPB2-biotin) binding was assessed according to the spectroscopic method based on HABA displacement (Caliceti et al., 2002). Briefly, 1 ml of protein solution (native avidin or protein equivalent avidin–pNIPAm-*co*-Am, 0.4 mg ml^{-1}) in 0.1 M phosphate buffer, pH 7.0, containing 15 mM HABA was added to fixed amounts of biotin or EPB2-biotin solutions to yield a final biotin or biotin equivalent concentration of 6.0 mM. The absorption changes of the protein solutions at 500 nm induced by biotin or EPB2-biotin displacement of HABA were measured and elaborated to calculate the biotin-binding sites and the biotin or EPB2-biotin affinity. The biotin-binding sites of avidin–pNIPAm-*co*-Am were expressed as percentages relative to the native protein binding sites (100%). The affinity of wild type avidin and avidin–pNIPAm-*co*-Am for biotin and EPB2-biotin was estimated from the slopes of the avidin–pNIPAm-*co*-Am/HABA displacement curves and expressed as a percentage of the slope obtained with wild type avidin (100%).

2.8. Pharmacokinetic studies

The pharmacokinetic study was carried out by intravenous injection of tritium labelled wild type avidin, avidin–pNIPAm-*co*-Am and avidin–PEG to Balb/c mice. The protein radio-labelling was carried out according to the method reported elsewhere (Caliceti et al., 1999).

Three groups of 12 mice each were treated by injection into the tail vein of 100 μl solution of 1 mg ml^{-1} [^{3}H]-avidin or equimolar quantities of [^{3}H]-avidin–pNIPAm-*co*-Am or [^{3}H]-avidin–PEG in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2. At scheduled times the animals were bled and four animals per group were sacrificed at 1, 3 and 110 h. The radioactivity in the blood and in the main organs (kidneys, spleen and liver) was evaluated according to the protocols reported in the literature for the determination of the protein content (Caliceti et al., 1999). The data were elaborated to calculate the main pharmacokinetic parameters as well as the native avidin, avidin–pNIPAm-*co*-Am or avidin–PEG concentration in the tissues.

2.9. Statistical analyses

Experimental assays for biotin affinity and residence times were conducted at least in triplicate and errors quoted as standard deviations from the mean values.

3. Results

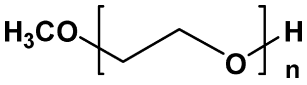
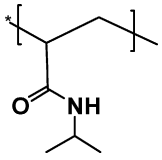
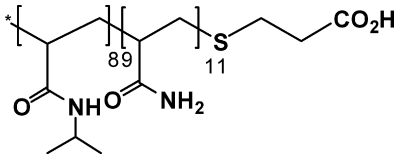
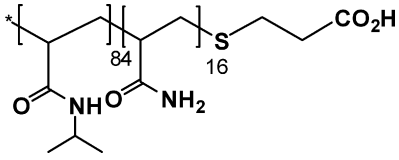
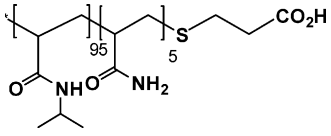
3.1. Polymer synthesis and characterization

Poly-*N*-isopropylacrylamide (pNIPAm) and co-polymers derived from *N*-isopropylacrylamide (NIPAm) and acrylamide (Am) polymerization (pNIPAm-*co*-Am) were prepared via conventional radical polymerization and their structures were confirmed by IR and NMR spectroscopies. Relative integral ratios of backbone protons at 1.7–2.4 ppm against side-chain isopropyl protons (1.23 and 3.98 ppm) enabled co-monomer ratios to be determined.

Cloud points of the co-polymers were determined as turbidity onset temperatures of polymer solutions (LCST), which were polymer concentration independent values. The LCST values were found to be above that of pNIPAm homopolymer and to depend on the polymer composition, as expected, with the properties shown in Table 1.

The co-polymer P1 was selected for further study because it displayed a LCST close to the body temperature of the male mice under the experimental conditions, which was in the range of 38.0–38.7 °C, in fair agreement with the values reported in literature (van Zutphen et al., 2001).

Table 1
Structure and main physicochemical properties of pNIPAm and pNIPAm co-polymers

Polymer	Structure	LCST	Molar mass (kDa)
PEG		–	5
P0		32.0	6
P1		37.7	6
P2		39.4	3
P3		34.2	11

3.2. Protein modification and structural characterization

Activation of pNIPAm-*co*-Am (P1) took place via esterification of the chain terminal carboxyl to form an ω-hydroxy NHS ester intermediate, and which in turn was reacted with primary amino groups on avidin to form the conjugate (Fig. 1).

For comparison, avidin was modified with NHS-activated 5 kDa PEG to yield a derivative with similar polymer composition compared to avidin–pNIPAm-*co*-Am.

Analysis of protein primary amine after reaction of avidin with the NHS-activated pNIPAm-*co*-Am and PEG indicated that both conjugates (avidin–pNIPAm-*co*-Am and avidin–PEG) contained a mean of 25 polymer-derivatised amino groups from the 40 potentially available amino groups.

The molecular mass of avidin–pNIPAm-*co*-Am determined by gel filtration analysis was found to correspond to that of a 220 kDa globular protein, in reasonable agreement with the theoretical molar mass of 200 kDa based on the molecular weight of the native protein (48 kDa) and the total polymer mass (150 kDa). By contrast, the PEGylated counterpart was eluted with the solvent front indicating that PEG-conjugation increased significantly the hydrodynamic volume of avidin, though

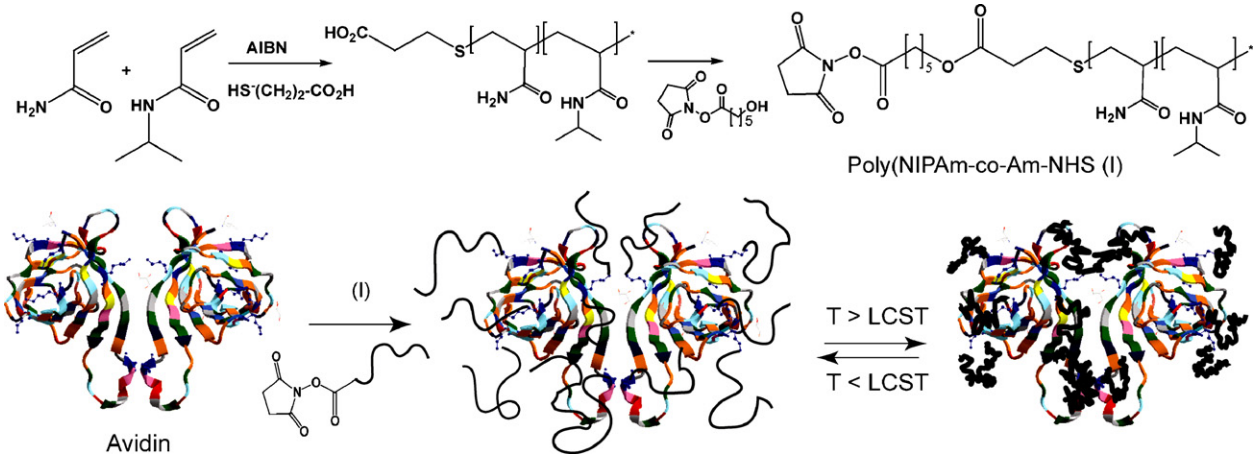


Fig. 1. Synthesis of thermoresponsive co-polymers and conjugation chemistries for attachment to avidin.

the theoretical molecular weight was calculated to be about 170 kDa.

Photon correlation spectroscopy (PCS) experiments performed at room temperature confirmed the gel permeation results. The pNIPAm-*co*-Am and PEG conjugate mean diameters were found to be about 80 and 320 Å, respectively, which were significantly higher as compared to the 25 Å mean diameter reported for native avidin (Livnah et al., 1993).

The different hydrodynamic size between the pNIPAm-*co*-Am and the PEG derivatives is ascribable to the different hydration behaviour of the two polymers. PEG is known to be strongly hydrated as each monomer unit can coordinate three water molecules (Caliceti and Veronese, 2003), while amphiphilic PEG co-polymers are well-known for forming micelles in solution (Kabanov et al., 2002). By contrast, the small hydrodynamic size of the pNIPAm-*co*-Am derivative indicated that the polymer was poorly hydrated even at a temperature below its LCST value. This suggested that the pNIPAm-*co*-Am chains remained associated with the protein surface either in the extended conformation at temperature below the LCST or in the coiled collapsed form at temperature above the LCST.

The PCS results obtained with avidin–PEG at 40 °C were similar to those obtained at 21 °C indicating that this polymer did not convey significant thermosensitive properties to the conjugate. The light scattering data for avidin–pNIPAm-*co*-Am at 40 °C indicated that the system was instable and large particles with broad size distribution were detected.

Near UV circular dichroism (CD) analysis showed that the pNIPAm-*co*-Am conjugation did not modify the avidin tertiary conformation (Fig. 2). This result was attributable to the high structural stability of avidin, which is stable to denaturation conditions as well to proteolytic degradation (Caliceti et al., 2002; Green, 1975). Far UV circular dichroism spectra showed that the pNIPAm-*co*-Am bioconjugation did not induce significant modification in the aromatic aminoacid environment. These results were confirmed by the fluorescence analysis of native avidin and pNIPAm-*co*-Am modified avidin, which displayed similar fluorescence profiles (data not shown). Similar results were obtained with PEG derivatised avidin, indicating that the environmental exposure of the tryptophan and other aromatic residues involved in the biotin binding was not altered by the polymer conjugation, despite their different hydrophilic character (Livnah et al., 1993).

3.3. Thermal properties of pNIPAm-co-Am, native avidin and avidin–pNIPAm-co-Am

The conjugation of pNIPAm-*co*-Am conferred temperature sensitive properties to avidin, which, under the experimental conditions, per se does not undergo temperature induced aggregation or irreversible conformational irreversible alteration. The phase transition onset was observed at 37.7 °C for both co-polymer and co-polymer conjugate, indicating that the LCST of the co-polymer was not affected through bioconjugation. Nevertheless, at the same overall polymer concentration, the conjugate exhibited self-association profile, which was shifted slightly to higher temperatures as compared to the free co-polymer. This behaviour indicated that anchoring of pNIPAm-*co*-Am onto a soluble support, namely the hydrophilic protein, altered the overall co-polymer dehydration, chain–chain interactions and final aggregation. Therefore, though the co-polymer concentration in both experiments, free or conjugate co-polymer, was the same, the free pNIPAm-*co*-Am chains in isolation were able to interact with each other more readily. This led to an overall aggregation phase transition of pNIPAm-*co*-Am polymer chains in solution, whereas, when the co-polymer was anchored to the protein in the avidin–pNIPAm-*co*-Am conjugate the chains were not so freely available to interact and cause bulk aggregation.

3.4. Biological properties of native and pNIPAm-co-Am derivatised avidin

The pNIPAm-*co*-Am conjugation was found to be less detrimental to the biotin-binding properties of avidin as compared to PEG derivatization.

The extensive avidin modification with pNIPAm-*co*-Am yielded a reduction of about 23% of the available biotin-binding sites of the protein while the affinity for the small vitamin and the biotinylated Mab (Mab-biotin) was estimated to decrease by 15% (Table 2). PEG-conjugation was previously reported to

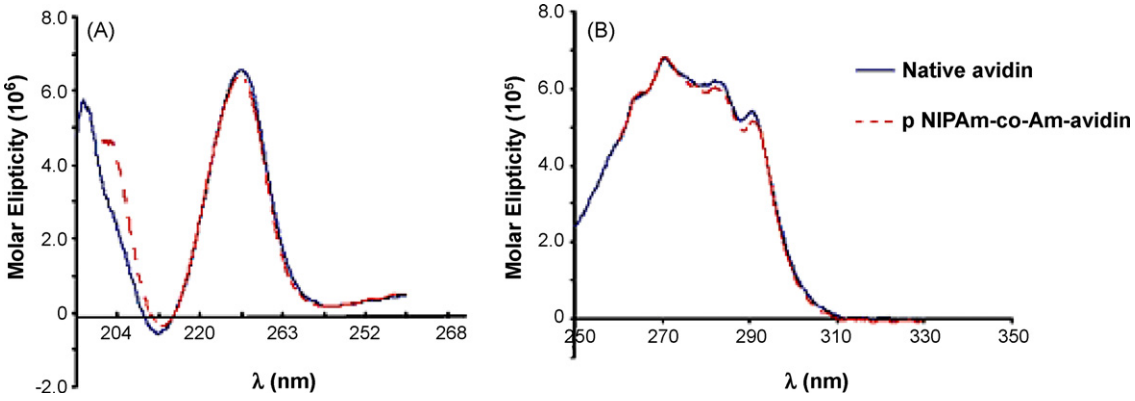


Fig. 2. Near-UV (A) and far-UV (B) circular dichroism spectra of avidin (blue line) and avidin–pNIPAm-*co*-Am (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Affinity assays for avidin and avidin–pNIPAm-*co*-Am with biotin and biotinylated antibody

	Residual affinity (%)	
	Wild type avidin	Avidin–pNIPAm-*co*-Am
Biotin	100	85 ± 3
Biotin (after heat cycles)	97 ± 3	91 ± 4
Mab-biotin	100	86 ± 2
Mab-biotin (after heat cycles)	94 ± 4	88 ± 3

decrease the biotin-binding sites by about 47% and the reduction in the biotin or biotinylated antibodies (Mab-biotin) affinity was about 17 and 64%, respectively (Salmaso et al., 2005).

These results indicated that the more hydrophilic PEG masked the biotin-binding sites to the vitamin recognition more efficiently as compared to pNIPAm-*co*-Am, thus reducing the number of possible biotin/avidin interactions.

PEG and pNIPAm-*co*-Am grafts were found to have similar effect on biotin recognition, while significant differences were obtained concerning the macromolecularized biotin (Mab-biotin). The reduction in affinity of biotinylated antibodies obtained with both PEG and pNIPAm-*co*-Am conjugates may be related to the large interacting agent size (Mab-biotin). The PEG chains on the protein surface interfered with the formation of stable Mab-biotin/avidin complex probably because of the hindrance of the hydrated polymer, which obstructed the interaction with a macromolecular biotinylated derivative. On the contrary, pNIPAm-*co*-Am had only a limited effect on the Mab-biotin affinity indicating that this polymer did not physically impair the approach of macromolecules to the avidin surface.

Importantly, avidin–pNIPAm-*co*-Am aggregation and redissolution over three heating cycles did not result in protein structural alteration or further biotin affinity decrease. In the case of the PEG-avidin conjugates both biotin and Mab-biotin affinity were affected by the thermal cycles despite the high protein stability to physical stress. These results demonstrate that the structural changes of the polymer did not perturb the conformation of avidin, which was reported to be very stable under these denaturing conditions.

3.5. Pharmacokinetic behaviour of native and polymer modified avidins

The conjugation of pNIPAm-*co*-Am changed significantly the pharmacokinetic properties of avidin (Fig. 3).

As a consequence of the size enlargement due to polymer conjugation, the pNIPAm-*co*-Am derivative displayed higher systemic bioavailability. Area under the curve (AUC) was 8213 μg ml^{-1} min^{-1} for avidin–pNIPAm-*co*-Am and 4035 μg ml^{-1} min^{-1} for native protein. Native avidin was found to disappear from circulation very rapidly making the pharmacokinetic analysis very difficult. Soon after administration, 75% of the injected native avidin disappeared from the circulation with a distribution half life ($t_{1/2}\alpha$) of 1.4 min. Small protein concentrations were detected in the blood for about 60 min from injection. For the injected avidin–pNIPAm-*co*-Am, 80–90% of the conjugate was detected in the immediate few minutes after

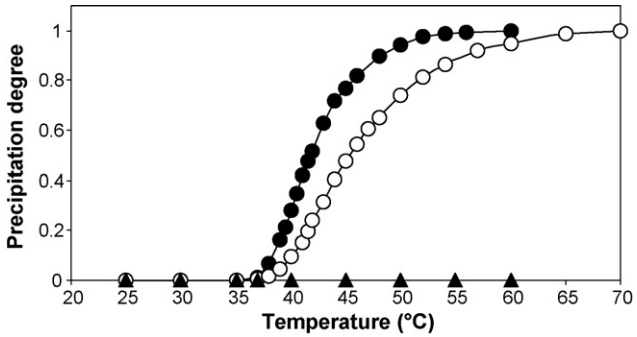


Fig. 3. Temperature induced aggregation profiles of pNIPAm-*co*-Am (●), avidin–pNIPAm-*co*-Am (◯) and native avidin (▲).

injection and its plasma concentration was detectable few for up to 4 h (Fig. 4). The distribution and elimination half times ($t_{1/2}\alpha$ and $t_{1/2}\beta$) of avidin–*p*-NIPAm-*co*-Am were 28 and 634 min, respectively.

The longer distribution phase of the bioconjugate demonstrated that the polymer attached on the biomolecule surface slowed the diffusion of the protein into the peripheral compartment. The distribution volumes (Vss) of the bioconjugate and native protein (7.8 and 22.5 ml, respectively) indicated also that the former distributed to the peripheral compartments at a lower extent than the latter. As compared to the PEG derivative, avidin–pNIPAm-*co*-Am displayed a shorter residence in the circulation with $t_{1/2}\alpha$ and $t_{1/2}\beta$ about 8 times lower than that of avidin–PEG and with about 35 times lower AUC. This was most likely due to the higher hydrodynamic volume of the PEG derivatives, which slowed the protein diffusion to the peripheral compartments and prolonged the distribution phase and the glomerular ultrafiltration.

By contrast to the PEG-avidin conjugate, which was mainly confined into the central compartment, the avidin–pNIPAm-*co*-Am partially distributed in the liver, though to a lower extent than the native protein, while no significant distribution was observed in kidneys and spleen. After 1 h from injection about 18 and 8% of the injected native avidin and avidin–pNIPAm-*co*-Am dose were, respectively, found in the liver. In both cases, the avidin content in this tissue rapidly decreased as less than 3 and 1% of native avidin and avidin–pNIPAm-*co*-Am was determined after 3 h from injection. The reduced accumulation in the

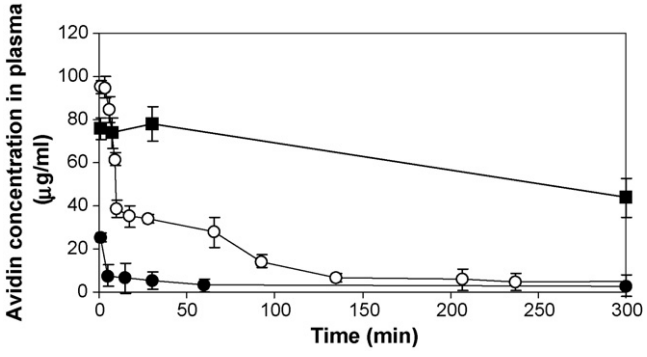


Fig. 4. Pharmacokinetic profiles of native avidin (●), avidin–pNIPAm-*co*-Am (◯), avidin–PEG (■).

liver of avidin–pNIPAm-*co*-Am may be ascribed to the masking of the strong charge and recognition sites to the liver cell surface receptors, which promoted the protein interaction with the reticuloendothelial cells and increased cell up-take.

4. Discussion

This study was aimed at evaluating the primary biological and pharmacokinetic behaviour of proteins modified with responsive polymers. We set out to establish whether pNIPAm-*co*-Am could be conjugated to avidin to an extent such that it would both confer responsive properties and increase residence time in vivo, but without adversely affecting the biochemical activity of the protein.

Avidin was selected as the protein model because this natural macromolecule possesses poor pharmacokinetic and immunological properties, which limit its exploitation in immuno-pretargeting in tumor diagnosis and therapy. While a degree of increased residence time in the body is required for therapeutic purposes, it is also the case that more prolonged persistence in the circulation is problematic as it increases the systemic basal signal in diagnosis as well as systemic toxicity. Thus, avidin conjugates that are controllable in terms of their pharmacokinetic properties are desirable. However, despite the many potential biomedical uses of 'smart' polymers (Heredia and Maynard, 2007) and the wealth of literature on PEG-protein conjugates, responsive polymer-avidin or polymer-streptavidin derivatives have thus far only been investigated for binding interactions with biotin (Malmstadt et al., 2003; Ding et al., 1999; Garret-Flaudy and Freitag, 2001). Binding with biotinylated macromolecules, namely Mab-biotin, or pharmacokinetic performance of avidin conjugates with thermoresponsive polymers have not been evaluated, even though there have been prior reports of the synthesis of these derivatives (Heredia et al., 2005; Bontempo et al., 2005). The principal reason may be the perception that acrylamide based polymers and especially pNIPAm are cytotoxic, even though co-polymers of 2-hydroxypropylmethacrylamide (HPMA) have been extensively investigated as polymer therapeutics (Satchi-Fainaro et al., 2006; Satchi et al., 2001; Kopecek et al., 2001). However, recent studies have suggested that the potential toxicity of pNIPAm polymers may not be a problem in reality. For example, Malonne et al. (2005) found that pNIPAm polymers did not cause acute cytotoxicity in mice when administered orally at up to 4000 mg kg^{-1}, nor was cumulative toxicity observed with pNIPAm and two related co-polymers. Vihola et al. (2005) showed that pNIPAm was well-tolerated by Caco-2 cells when the polymer was below LCST and for incubation times of up to 3 h above LCST. Some reduction in viability of Caco-2 cells occurred when assays were conducted above LCST for 12 h incubation times, suggesting that hydrophobic polymer aggregates may have induced membrane damage, but more hydrophilic thermosensitive co-polymers were not found to be toxic under the same conditions. It seemed likely to us at the start of this study, therefore, that for shorter-term biomedical applications, pNIPAm-based polymers or similar thermosensitive materials might be used without inducing significant toxic effects.

We accordingly designed pNIPAm-based co-polymers containing a more hydrophilic co-monomer (acrylamide) in order to reduce the potential cytotoxicity of pNIPAm and to increase the phase transition temperature above that of the homopolymer. Co-polymers P1–P3 prepared from NIPAm and acrylamide were found to exhibit phase transition temperatures (34–39 °C) spanning the physiological range. Of these polymers we selected P1, as its LCST of 37.7 °C was considered most suitable for in vivo application because the physicochemical properties of this material would most likely be unaltered the most after administration into the body while they could vary at under slight temperature changes. Environmental induced structural changes of the polymer could be exploited for tissue targeting as the temperature of few certain disease sites, namely inflammatory or cancer tissues, is higher than that of healthy tissues (Bull, 1984; Neta and Oppenheim, 2001). In this way, P1 should serve as the exemplar material within the class of thermosensitive polymers to establish the fundamental biopharmaceutical and biological properties of PNIPAm-based bioconjugates.

The similar degree of avidin modification obtained with PEG and pNIPAm-*co*-Am indicated that the different hydration of the two polymers did not affect their reactivity with the protein amino groups. Probably, the unmodified amino groups were located at inaccessible sites inside the protein structure and thus could not react with the single end-terminal reactive groups of the synthetic polymers.

The P1 co-polymer and the P1–avidin-conjugate showed the same LCST (37.7 °C), defined as the turbidity onset of polymer solutions, even at 1 mg ml^{-1} (the concentration used for turbidity measurements). However, the extent of aggregation for the P1–avidin-conjugate was only 50% at 45 °C, well above body temperature. This was because the LCST is a concentration-independent indicator of the phase transition which occurs at very low concentrations via intra-chain (i.e. intramolecular) interactions without precipitation (Fujishige et al., 1989; Yamamoto et al., 1989; Kubota et al., 1990; Wang et al., 1998), whereas at higher concentrations, inter-chain and inter-particle interactions become important allowing for aggregate precipitation. Thus, by preparing a avidin–pNIPAm-*co*-Am conjugate with the onset of LCST at 37.7 °C we could be reasonably sure that individual polymer–protein conjugates contained the P1 component in the globule conformation, but that bulk aggregation and rapid clearance via opsonisation would not take place. Furthermore, the retention of biotin binding through LCST heat-cycles confirmed that the protein retained its activity after conjugation and repeated thermal stress.

On the basis of the thermal behaviour of the conjugate, it is likely that at the amount used in the injections (100 μg of avidin equivalents) the conjugates were at low concentrations soon after injection (about 50 μg ml^{-1} of protein and 150 μg ml^{-1} of polymer), and thus not present as aggregates even though, due to the close difference between polymer LCST and body temperature, individual pNIPAm chains could partially be in the globule conformation due to above-LCST intra-chain association. The enhanced residence time of the avidin–pNIPAm-*co*-Am-avidin conjugate was thus most probably a consequence of the structural alteration of the protein. Though pNIPAm-*co*-Am P1

induced less dramatic morphological protein changes than PEG, the former co-polymer was also able to effect noticeably the glomerular ultrafiltration and liver up-take. The increased residence time was perhaps predictable, since polymer conjugation endowed the avidin derivative with a size (200 kDa) above the ultrafiltration limit usually considered to correspond to the albumin size (65 kDa). Furthermore, even in chain-collapsed form the residual hydration shell of the pNIPAm would have generated an extra hydrodynamic shielding of the overall conjugate which contributed to reducing the glomerular ultrafiltration. Gel permeation chromatography showed, in fact, that pNIPAm-*co*-Am had an apparent size corresponding to a 220 kDa protein. Polymer conjugation was also found to affect significantly the liver up-take which is the main elimination pathway of avidin and was responsible of the prompt disappearance of about 75% of the injected dose from the circulation (Chen et al., 2000). After pNIPAm-*co*-Am conjugation, only 20% of the administered protein was rapidly eliminated from the bloodstream as a consequence of the reduced liver uptake; in this case it was probably be due to the charge, and glycosilic binding site masking. The disappearance of about 20% of the administered conjugate was probably due to the heterogeneous modification of the protein surface, which yielded incomplete protein covering. As a consequence, of reduced ultrafiltration and liver up-take, avidin–pNIPAm-*co*-Am was found to maintain high systemic levels for a time period of 3 h. This level of increased residence time in the bloodstream would in fact be suitable for in vivo applications in immuno-pretargeting.

The low accumulation of avidin–pNIPAm-*co*-Am in the liver and spleen indicated that the co-polymer attachment did not promote the non-specific accumulation of the conjugate into permeable tissues via passive diffusion. This result was significant as it suggested that P1–avidin might be exploited for active immuno-pretargeting as avidin–pNIPAm could diffuse in the organs where it would be trapped only after recognition with binding moieties, such as biotinylated antibodies that are already used in the immuno-pretargeting protocols. These assays used only a model pNIPAm co-polymer with an LCST above body temperature when conjugated, however, additional variation in polymer structure should allow the preparation of bioconjugates that partition controllably between the bloodstream and peripheral tissues dependent on local temperature or pH. Local temperature control is already used clinically by the application of ultrasound (Lewin, 2004; Lavon and Kost, 2004), whereas pH-variance of LCST has been demonstrated in a number of pNIPAm co-polymers (Sawant et al., 2006; Lee et al., 2005). This would allow further selectivity in targeting by tuning the polymer response and hence the bioconjugate pharmacokinetic properties to tissue- or disease-specific factors. We are currently investigating the synthesis and pharmacokinetic properties of new polymer–protein conjugates that can be switched by these mechanisms in vivo.

Importantly, despite the extensive polymer conjugation, avidin–pNIPAm-*co*-Am maintained a high affinity towards macromolecularized biotin, which is the essential substrate of avidin either in vivo or in vitro protocols. Though at pathophysiological temperatures (above the LCST) the biological activity of the bioconjugate could not be evaluated, it should be observed that in vivo its concentration in the tissues is expected to be very low, thus it should not undergo aggregation and precipitation. Therefore, aggregation phenomena which might impair the avidin biological activity should be avoided, while the molecular recognition properties of the avidin–pNIPAm-*co*-Am conjugate, which are pre-requisites for pharmaceutical and medical applications, are maintained, making this derivative potentially effective for in vivo application.

5. Conclusions

Conjugates of avidin with pNIPAm-*co*-Am and PEG were prepared and showed enhanced residence times compared with native avidin. The phase transition of the pNIPAm-*co*-Am-avidin conjugate was demonstrated in vitro and avidin activity in terms of biotin binding was retained after three cycles above and below polymer LCST. The results indicate that thermoresponsive polymers can be successfully coupled to biopolymers for subsequent exploitation as new protein conjugates with unusual and potentially valuable physicochemical and biological properties. According to the polymer properties, the conjugates might be targeted specifically for various applications such as in vitro bioassays as well as for in vivo protocols.

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