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Molecularly designed water soluble, intelligent, nanosize polymeric carriers

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Abstract

Intelligent polymers, also referred as "stimuli-responsive polymers" undergo strong property changes (in shape, surface characteristics, solubility, etc.) when only small changes in their environment (changes in temperature, pH, ionic strength light, electrical and magnetic field, etc.). They have been used in several novel applications, drug delivery systems, tissue engineering scaffolds, bioseparation, biomimetic actuators, etc. The most popular member of these type of polymers is poly(*N*-isopropylacrylamide) (poly(NIPA)) which exhibits temperature-sensitive character, in which the polymer chains change from water-soluble coils to water-insoluble globules in aqueous solution as temperature increases above the lower critical solution temperature (LCST) of the polymer. Copolymerization of NIPA with acrylic acid (AAc) allows the synthesis of both pH and temperature-responsive copolymers. This paper summarizes some of our related studies in which NIPA and its copolymers were synthesized and used as intelligent carriers in diverse applications.

Keywords: Intelligent polymers; Poly(NIPA) and copolymers; Conjugates with amino acids; Gene therapy; Nonviral vectors

1. Introduction

In recent years, intelligent polymers have been promoted as useful tools in biotechnological applications like enzyme immobilization, thermal affinity separation, controlled drug release, immunodiagnostics, gene therapy, etc.

Intelligent polymers, also the so-called "stimuli-responsive" polymers exhibit large reversible physical changes in response to external stimuli such as, temperature, pH, ionic strength, solvents, electrical or magnetic field, radiation, etc. These polymers may be in solid gel form, immobilized on solid surfaces or even as water-soluble chains. A stimuli, for instance increasing temperature over a certain critical value cause a response such as shrinkage of the solid gel, or collapse of the polymer chains on the solid surface, or polymer chains change from water-soluble coils to water-insoluble globules in aqueous media, which are most of the cases reversible.

The most popular member of these type polymers is poly(*N*-isopropyl acrylamide), poly(NIPA), which exhibits temperature-sensitive character, in which the polymer chains are in an extended form in aqueous media at room temperature, but when the temperature is raised to a critical value (about 29–30 °C), which is also known "lower critical solution temperature" (LCST), polymer chains form globular structures. A typical AFM micrograph taken

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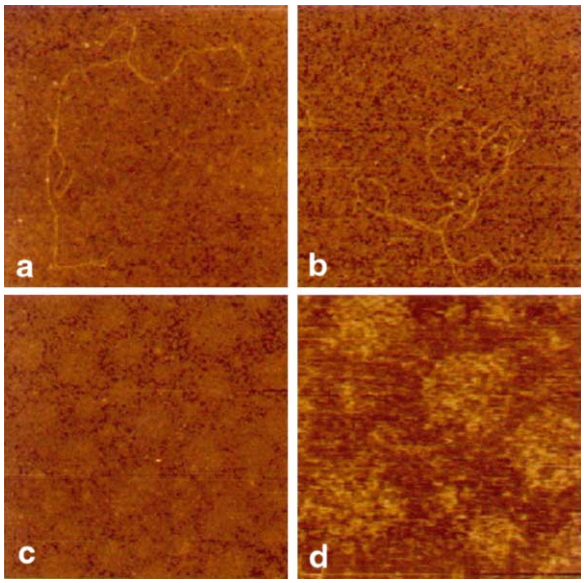

Fig. 1. AFM micrographs showing phase transition of a poly(NIPA) based polymer by increasing temperature: (a) extended form in solution at room temperature; (b) at an increased temperature but lower than LCST; (c) collapsed polymer chains which form globular structures over LCST; (d) magnified form of images at c.

by us is given in Fig. 1, which shows phase transition of poly(NIPA-co-AAc) with the increase in temperature (Zareie et al., 2000). Copolymers of NIPA with several comonomers including acrylic acid (AAc), 4-pentenoic acid, *N*-acryloxysuccinimide, allylamine, 2-(dimethylamino) ethyl methacrylate, 2-(dimethylamino) propyl methacrylamide, 2-(dimethyl) acrylamide, 3-acrylamidophenylboronic acid, maleic or citraconic anhydride, maleic or itaconic acids, etc., exhibiting both temperature and pH sensitivities have been used in diverse application (Kitano et al., 1991; Schild, 1992; Takeuchi et al., 1993; Chen and Hoffman, 1995; Hoffman, 1995; Ding et al., 1996; Chen and Hsu, 1997; Huglin et al., 1997; Hisamitsu et al., 1997; Kim and Park, 1999; Park, 1999; Tuncel, 1999; Kohori et al., 1999; Durand and Hourdet, 1999; Diez-Pena et al., 2002).

This paper summarizes some of our related studies in which NIPA and its copolymers were synthesized and used as intelligent carriers in diverse applications. Details of these studies can be found in our related publications (Bulmuş et al., 2001; Dinçer et al., 2002a,b; Zareie et al., 2002; David et al., 2002; Koçum et al., 2002; Bulmuş et al., 2003; Kesim et al., 2003; Köseli et al., 2003; Kalaycıoğlu et al., 2003).

2. Conjugates of poly(NIPA-co-AAc) copolymers with glycine, alanine and serine mono-, di-, tri-peptides

Peptides, glycopeptides, oligopeptides and/or their conjugates with carrier molecules have a great potential in therapeutical or protective medicine as drugs (or prodrugs) or as vaccines (Katre, 1993; Delgado et al., 1992; Nucci et al., 1991). Soluble polymers, especially polyethylene glycols have attracted attention as carriers of peptides (Herman et al., 1995; Gravert and Janda, 1997). The soluble character of these polymers allows one to carry out the desired manipulations on the conjugates in a homogenous solution state by preserving their macromolecular properties that meet the particular requirements for the application. Numerous PEG-peptide conjugates show improved physicochemical and pharmacological properties such as extended plasma half-life, reduced antigenicity, increased solubility, resistance to proteolysis, and higher biological activity (Suzawa et al., 2000; Katre, 1990; Conover et al., 1998).

Recently we have attempted to prepare water-soluble peptide (or oligopeptide)-polymer conjugates which would allow to design prodrugs formulations with novel stimuli-responsive characteristics, which may increase the stability and effectiveness, and allow targeted delivery (especially into the cells) of the bioactive peptides or oligopeptides.

2.1. Experimental

A series of poly(NIPA-co-AAc) copolymers were synthesized in DMF by a free radical copolymerization by using 2,2′-azobisisobutyronitrile (AIBN) as the initiator. The mono-, di- and tri-peptide conjugates were synthesized by using carboxylic-end groups-protected peptides (namely glycine (Gly), alanine (Ala) and serine (Ser)) by a three step procedure, described in detail elsewhere (Bulmuş et al., 2001). Conjugation reactions were conducted in anhydrous DMF, using 1-cyclohexile-3-(2-morpholinoethyl) carbodiimide metho-ρ-toluene sulphonates as activating agent, at 0 °C, for 15 h under N_2 atmosphere. The

amount of amino acid conjugated during each conjugation reaction was determined by the ninhydrin test (Moore, 1968).

Chemical structures of the copolymers and conjugates were analyzed by FTIR and ^{1}H NMR. The average molecular weight of the copolymers were determined by using an Ubbelohde viscometer in methanol. LCST measurements, for both copolymers and conjugates were performed in a UV-Vis spectrophotometer at pH 4.0 and 7.4. For STM imaging, 10 μl solutions of the amino acids, copolymers and conjugates dried on freshly cleaved highly oriented pyrolitic graphite (HOPG) at room temperature (25 °C, 40% relative humidity). STM images were then taken at room temperature at atmospheric pressure, using a 1–1.5 V sample bias and a tuneling current of a 10–20 pA.

2.2. Summary of results

A series of poly(NIPA-co-AAc) copolymers with different molecular weights and AAc contents were synthesized by changing the polymerization recipe and characterized.

A copolymer with 3.1 mmol of carboylic acid per gram of the copolymer and with an average molecular weight of 1400 g/mol was used to synthesize polymer-peptide conjugates. The FTIR and ^{1}H NMR spectra revealed the formation of conjugates. Conjugation yields obtained at each step are given in Table 1. The conjugation yields for Gly and Ala were over 90%, but for Ser it was about 31% in the first step. The yields decreased as the amino acid sequence increased.

Table 1
Conjugation yields

	Conjugation yields (mmol amino acid/g copolymer) (amino acid conjugated/amino acid in feed × 100)		
	Gly	Ala	Ser
Step I	2.94 (95%)	2.89 (93%)	0.97 (31%)
Step II	2.26 (99%)	1.60 (68%)	0.20 (22%)
Step III	1.15 (64%)	0.77 (57%)	0.08 (21%)

Fig. 2 shows that poly(NIPA) homopolymer (LCST: 29.9 °C) was only sensitive to temperature, while incorporation of AAc resulted a copolymer (LCST: 37.7 °C) sensitive to both pH or temperature. Conjugation of oligopeptides to the NIPA-co-AAc copolymer chains caused slight decreases in the LCST values in the first step, however, significant increases were observed after 2nd and 3rd conjugation steps, especially at pH 7.4 (Table 2).

Representative STM images of alanine, the copolymer, and a conjugate carrying one Ala and one Gly are

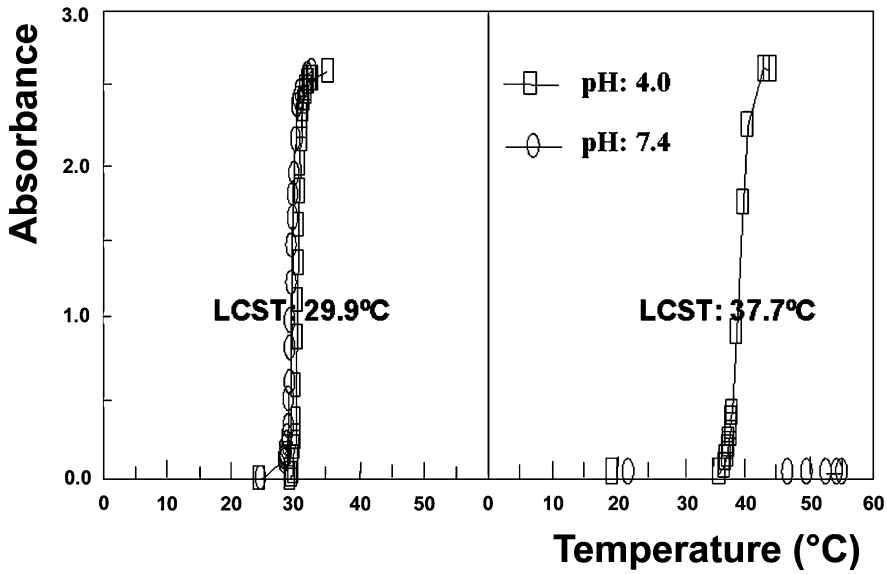

Fig. 2. Temperature–absorbance curves of poly(NIPA) homopolymer and poly(NIPA-co-AAc) copolymer.

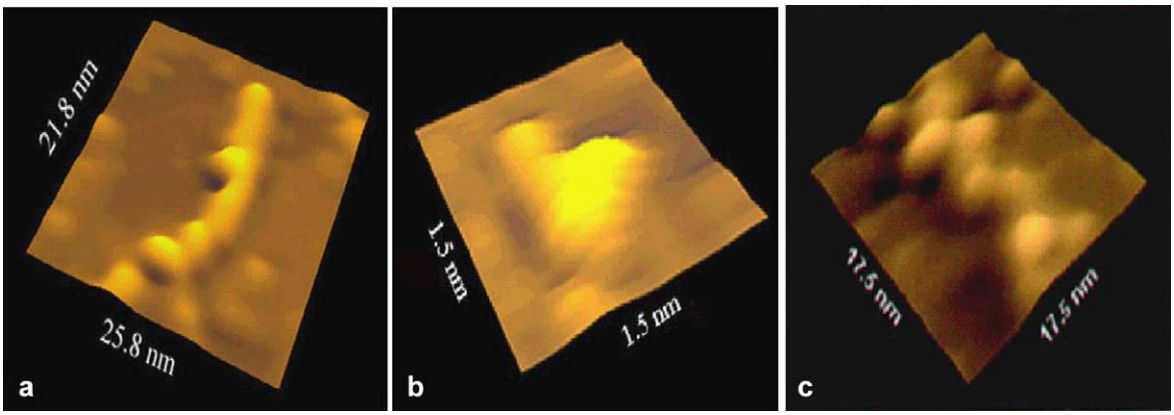

Fig. 3. Representative STM images of poly(NIPA-co-AAc) copolymer (a); alanine (b); and a conjugate carrying one Ala and one Gly (c).

Table 2
LCST values

	LCST (°C) at pH: 4.0/pH: 7.4		
	Gly	Ala	Ser
Step I	34.6/>60	35.1/41.6	36.0/43.9
Step II	38.9/>60	43.3/46.2	42.1/>60
Step III	42.6/>60	45.8/>60	50.8/>60

given in Fig. 3a–c, respectively. Fig. 3c exhibits the attached spherical amino acid molecules onto almost the rodlike copolymer backbone.

3. A potential gene delivery vector: *N*-isopropylacrylamide-polyethylenimine block copolymers

Gene therapy is used to correct or to modulate several diseases, in which genes are combined with a delivery system (a vector), and they are introduced to the patient to reach the DNA to be transfected. Both viral and nonviral vectors are used in clinical trials today (viral vectors are still the most widely investigated because of their high transfection efficiency). However, they do also associate important drawbacks, such as in safety, immunogenicity, mutagenesis, and also the limitation on the amount of genomic information that they can carry. Several lipid-, peptide, and polymer-based systems are currently under investigation for an effective and safe gene delivery. Water-soluble cationic polymers is one important class of nonviral vectors (Barron et al., 1999; Ruponen et al., 1999; Mahato et al., 1999; Plank et al., 1999; Finsinger et al., 2000; Han et al., 2000; De Smedt and Demeester, 2000; Boussif et al., 1995; Godbey et al., 1999; von Harpe et al., 2000).

Recently, we have also attempted to prepare water-soluble stimuli-responsive nonviral vectors. Here, we report synthesis and characterization of carboxyl-ended-poly(NIPA) homopolymers and their copolymers with polyethylenimine (PEI) as a stimuli-responsive polycationic DNA carriers for gene delivery.

3.1. Experimental

A series of carboxylic acid-ended poly(NIPA) were synthesized by a free radical polymerization of NIPA in ethanol by using different amounts the chain transfer agent mercapto acetic acid (MAA) and the initiator, AIBN, in the recipe. The polymerizations were conducted at 50 °C at a shaking rate of 400 cpm under nitrogen-atmosphere for 24 h. The carboxyl-ended poly(NIPA) obtained was then copolymerized with PEI different molecular weights (2000 and 25000 branched, 25,000 linear and 25,000 branched) by the reaction of the carboxylic acid groups activated with a water-soluble carbodiimide (EDAC), with the amine groups of PEI. These positively charged copolymers were then interacted with both genomic and plasmid DNA at the physiological conditions.

In the preliminary transfection studies, a specific plasmid pEGFP-N3 (expressing green fluorescent protein) was used for tracing of plasmid in transfection.

HeLa (human cervix epitheloid carcinoma) cell line was cultured in flaks at 37 °C in a humidified 5% CO_2 atmosphere. The medium was Dulbacco's modified Eagle's medium containing also 10% fetal calf serum. Copolymer solution was gently mixed with the solutions containing plasmid DNA in 0.15 M NaCl at room temperature for 20 min for conjugation. Then, this mixture was added to the six-well plates containing about 2×10^5 cells in serum free medium. Gene expression was followed about 72 h.

Chemical structures of homo and copolymers were analyzed by FTIR and ^{1}H NMR. The average molecular weight of the copolymers were measured with an Ubbelohde viscometer in methanol. LCST measurements were performed in a UV-Vis spectrophotometer at pH 4.0 and 7.4. STM images both polymers and DNA conjugates were taken in air at atmospheric pressure with tip-sample bias voltages of up to 800 mV and tunneling currents ~1 nA.

3.2. Summary of results

The FTIR and ^{1}H NMR spectra revealed the formation of block copolymers of poly(NIPA) and PEI. Satisfactorily high copolymer yields, in the range of 78–95% were achieved in the homopolymerization of NIPA. However, in the case of block copolymerization of poly(NIPA) and PEI, the copolymer yields were relatively lower, and were in the range of 42–56%. The maximum molecular weight of the carboxylic acid-ended poly(NIPA) homopolymer achieved was 40×10^3. There was no significant effect of the initial AIBN concentration on the molecular weight, while higher molecular weights were achieved when the initial concentration of the chain transfer agent, MAA, decreased.

There were no significant differences in the LCST values obtained at two different pH, which indicated that the carboxylic acid-end groups were not enough to add a pH-sensitivity to the temperature-sensitive poly(NIPA) chains. Copolymerization of poly(NIPA) chains with more hydrophilic PEI chains caused significant increases in the LCST (upto 43.1 °C) which was more profound at pH 4.0 due to protonation of amine groups of PEI. LCST values of the copolymer-DNA conjugates were even higher, but conjugates were still temperature and pH sensitive.

Typical STM images of poly(NIPA) homopolymer (M_w: 1958) and its copolymer, poly(NIPA)-co-PEI (M_w: about 4000) are shown in Fig. 4a and b, respectively. The image of poly(NIPA) reveals the rodlike homopolymer chains with average size of 5 nm. A typical STM image of poly(NIPA)-PEI block copolymer is given in Fig. 4b with the same scan area (30 nm × 30 nm). A globular structure at one end, which is most probably representing the PEI block of the copolymer, is combined with the rodlike poly(NIPA) chain.

Fig. 4c gives a typical STM image of the copolymer-plasmid DNA conjugates. The globular structure is formed at physiological pH and temperature, as expected, the size of the globular structures were around 100–200 nm which was smaller than the size of plasmid DNA (around 400 nm) which shows the squeezing effect of the copolymer. Some aggregations were also observed in incorrect initial ratios of copolymer and plasmid.

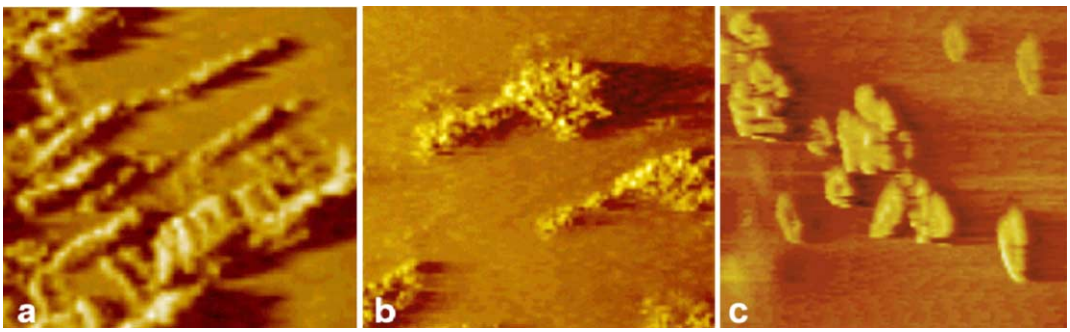

Fig. 4. Representative STM images of poly(NIPA) homopolymer (a); its copolymer poly(NIPA)-co-PEI (b); plasmid DNA-poly(NIPA)-PEI block copolymer (c).

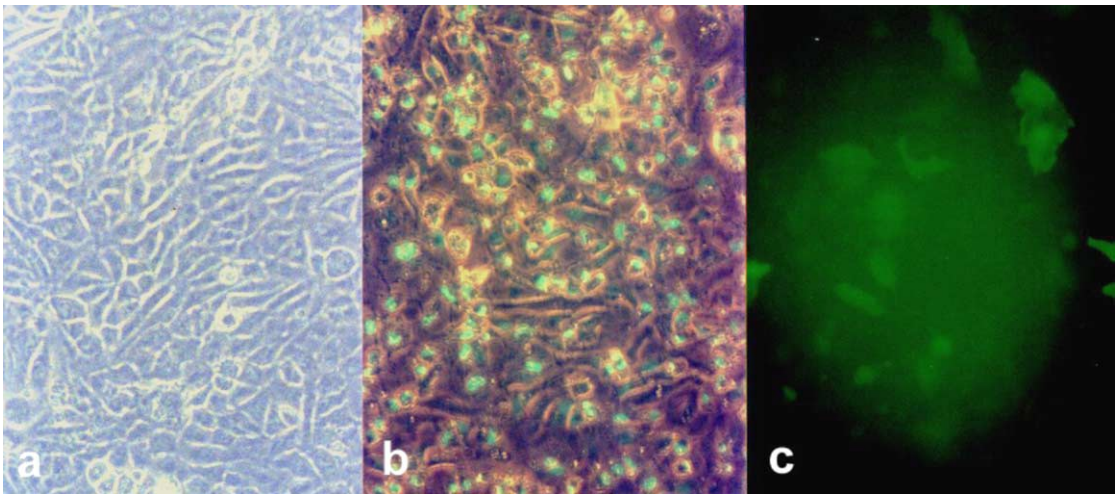

Fig. 5. Representative optical and fluorescence microscopy photographs: (a) HeLa cell line in petri dishes; (b) the same cell line transfected with plasmid DNA-copolymer conjugates; (c) GFP expressed in the transfected HeLa cells.

The optical and fluorescence micrographs taken in the cell culture studies are given in Fig. 5a–c. As seen here, the preliminary transfection studies showed that efficiency of the conjugates was high, up to even 70% of the cells were transfected. However, the percentage of cells expressing GFP was rather low around 30%. Optimization of structure of the carrier molecules is underinvestigation to increase the expression percentage.

4. Poly(NIPA-co-DMAPM) copolymers for albumin determination

Albumin is the most abundant blood plasma protein (55–60% of total proteins, 3.5–5.0 g/kg body weight). Albumin has a strong negative charge. Albumin takes an important role in many body functions, such as; contributes up to 80% of the normal osmotic pressure; carries several substances in blood; contributes pH balance; has an antioxidantfunction, passivates blood-material interactions, etc. Therefore, it is important to measure albumin concentration in blood plasma by a fast, cheap and effective method.

In this study, a relatively new copolymer, i.e., a copolymer of NIPA and a cationic-monomer, *N*-[3-(dimethylamino)propyl] methacrylamide (DMAPM) was synthesized. The response behavior of this copolymer to the presence of albumin was investigated for possible use as a reagent in the quantitative determination of albumin.

4.1. Experimental

A series of NIPA-co-DMAPM copolymers were synthesized by solution copolymerization of NIPA and DMAPM by varying the DMAPM/NIPA feed ratio between 0/100 and 18.8/81.2 mol/mol in 1,4-dioxane using AIBN as catalyst.

Chemical structures of copolymers were analyzed by FTIR and ^{1}H NMR. The isolation yields of copolymers were determined by gravimetric analysis. The average molecular weight of the copolymers were measured by using an Ubbelohde viscometer in THF. LCST measurements were performed in a UV-Vis spectrophotometer.

The phase transition temperatures of aqueous solutions containing Bovine Serum Albumin (BSA) and NIPA-co-DMAPM copolymer (i.e., at a concentration of 1% (w/v)) were determined in a UV-Vis spectrophotometer by measuring absorbances of the solutions at a wavelength of 540 nm as a function of temperature. BSA concentration was varied between 0 and 4000 μg/ml and these measurements were performed at pH 5.0, 7.0 and 11.0, using NIPA-co-DMAPM random copolymers produced with different DMAPM/NIPA feed ratios.

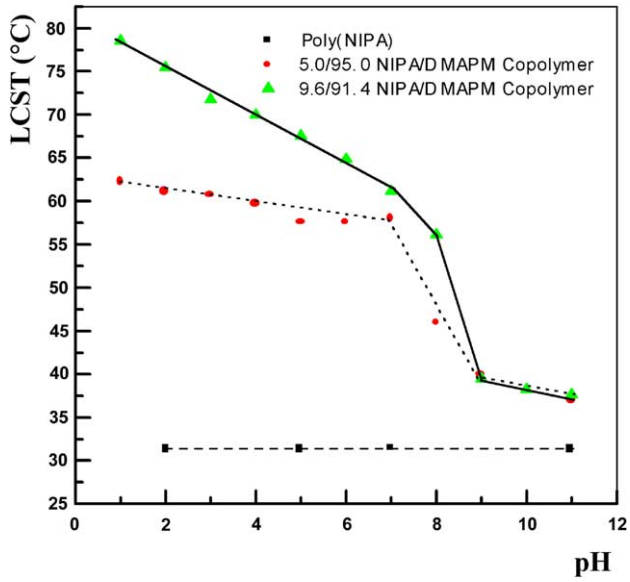

Fig. 6. Changes of LCST values NIPA-co-DMAPM copolymers with the DMAPM feed concentration at three different pH.

4.2. Summary of results

FTIR and ^{1}H NMR spectra described the copolymer structure. Satisfactorily high copolymer yields (90–98%) were achieved in all cases. The copolymer yield slightly increased with increasing the DMAPM feed concentration. Low DMAPM feed concentrations provided copolymers with a viscosity average

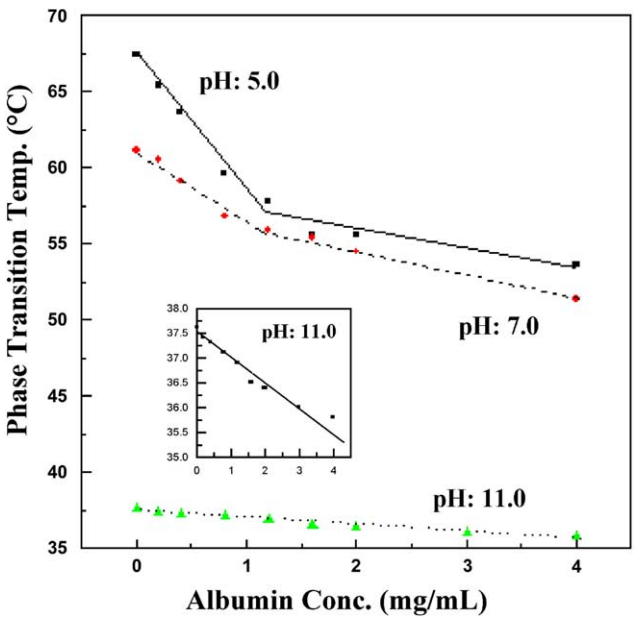

Fig. 7. Variation of phase transition temperatures (PTT) of the NIPA-co-DMAPM copolymers with the albumin concentration at three different pH.

molecular weight of approximately 3×10^5. However, the copolymers with lower molecular weights were obtained with the DMAPM feed concentrations higher than 5% mol.

As seen in Fig. 6, the LCST of NIPA-co-DMAPM copolymers increased with increasing DMAPM feed concentration at all pH. LCST drastically decreased with increasing pH for NIPA-co-DMAPM copolymers while no significant change was observed in the LCST of poly(NIPA) homopolymer.

The copolymers prepared with different DMAPM/NIPA feed ratios exhibited thermally reversible phase-transition in the absence of BSA, in the pH region between 5.0 and 11.0. On the other hand, the phase-transition induced by the temperature change was reversible at pH 11.0, in the presence of BSA. However, thermally irreversible transitions were observed in the presence of BSA, at pH 5.0 and 7.0. Fig. 7 shows the variation of phase transition temperature (PTT) with the albumin concentration at pH 5.0, 7.0 and 11.0. As seen here, PTT linearly decreased with increasing albumin concentration in the range of 0–2000 μg/ml at both pH 5.0 and 7.0. The magnitude of change in the PTT is also important since more accurate determination of albumin concentration is possible when the PTT change occurs in a wider range. At pH 11.0, PPT decreased linearly in the albumin concentration range of 0–4000 μg/ml. The results indicated that NIPA-co-DMAPM copolymer could be utilized as an appropriate reagent for the determination of albumin concentration only by measuring the temperature at which the transparent albumin-copolymer solution became turbid.

5. Poly(NIPA-co-MAH) copolymers for affinity separation of human immunoglobulin-G

Antibodies (i.e., immunoglobulins, IgGs), especially monoclonal antibodies, play a dual role in biomedical technology. They offer exciting potential as diagnostic and therapeutic substances and also serve as bioaffinity ligands for purifying other high-value proteins of pharmaceutical importance such as cytokines and blood-clotting factors.. Several affinity separation techniques are used for purification of antibodies. Pseudospecific ligands can be used to purify a wide range of biomolecules, thereby offering more structural flexibility as compared with biospecific ligands for bioaffinity separation (Bueno et al., 1996). They have low binding constants and consequently, belong to the family of weak affinity ligands. Recently, it has been found that amino acids as pseudospecific ligands may hold certain advantages for industrial bioaffinity separations, as they are not likely to cause an immune response in the case of leakage into the product. These ligands are also much more stable than protein ligands because they do not require a specific tertiary structure for maintaining biological activity (Huang and Carbonell, 1999). They offer additional advantages over biological ligands in terms of economy, ease of immobilization and high adsorption capacity.

Histidine has been used as a ligand in the affinity chromatography of proteins (Alvarez et al., 1997; Legallais et al., 1997; El-Kak and Vijayalakshmi, 1992), Done et al., 1998). Histidine interacts through its carboxyl, amino and imidazole groups with several proteins at around their isoelectric points and has shown particular efficacy in separation of IgG subclasses from human plasma and in the purification of monoclonal antibodies from cell culture or ascites fluids. For example, calf chymosine, myxaline and acid protease from *Aspergillus niger*, as well as catechol-2,3-dioxygenease, have been purified on histidine-immobilized supports. Histidine-linked matrices were used for the purification of IgG from human plasma.

In the present study, NIPA was copolymerized with a histidine carrying monomer to obtain a temperature sensitive copolymer for bioaffinity separation of human immunglobulin-G (HIgG) from aqueous media.

Histidine has been used as a ligand in the affinity chromatography of proteins (Vijayalakshmi, 1989; Haupt and Vijayalakshmi, 1993; Alvarez et al., 1997; Legallais et al., 1997; El-Kak and Vijayalakshmi, 1992). Histidine interacts through its carboxyl, amino and imidazole groups with several proteins at around their isoelectric points and has shown particular efficacy in separation of IgG subclasses from human plasma and in the purification of monoclonal antibodies from cell culture or ascites fluids. For example, calf chymosine, myxaline and acid protease from *Aspergillus niger*, as well as catechol-2,3-dioxygenease, have been purified on histidine-immobilized supports.

Histidine-linked matrices were used for the purification of IgG from human plasma.

5.1. Experimental

2-Methacryloamidohistidine (MAH) was synthesized, by the reaction of L-histidine and methacrylchloride in CH_2Cl_2 solution containing triethylamine and hydroquinone under nitrogen atmosphere at room temperature for 2 h.

A series of NIPA and MAH copolymers were synthesized by copolymerization in methanol by varying both the "NIPA/MAH" feed ratio and the initiator concentration at 70 °C for 24 h at a shaking rate of 120 cpm under a nitrogen atmosphere.

Chemical structures of copolymers were analyzed by FTIR and ^{1}H NMR. The MAH content of the copolymer was obtained by back-titration. The conversions were determined by gravimetric analysis. The average molecular weight of the copolymers were obtained by using an Ubbelohde viscometer. LCST measurements were performed in a UV-Vis spectrophotometer.

NIPA-co-MAH random copolymers produced with different NIPA/MAH feed ratios were interacted with HIgG solutions with different concentrations (0–8000 μg/ml) at pH 7.4. The poly(NIPA-co-MAH) concentration was kept constant at 1% (w/v). HIgG concentrations were determined with an UV-Vis spectrophotometer by measuring absorbance of the solutions at a wavelength of 280 nm.

For STM imaging, 10 μl of aqueous solutions of the copolymer and the copolymer interacted with HIgG were dried on freshly cleaved HOPG. Then, STM images were taken at room temperature at atmospheric pressure, using a 1–1.5 V sample bias and a tuneling current of a 10–20 pA.

5.2. Summary of results

FTIR and ^{1}H NMR spectra proved the formation of copolymers. MAH contents of the copolymers obtained by back-titration showed that MAH incorporated to the polymer chains during copolymerization are very similar that are used in the initial recipe in most of the polymerizations. Satisfactorily high polymer yields (81–91%) were achieved in all cases. The viscosity-average molecular weight of poly(NIPA) homopolymer was 83×10^3. Molecular weights of the copolymers synthesized by using the same amount of initiator but with different NIPA/MAH ratios were about 62×10^3, and did not change with the MAH content. However, there were noticeable increases in the molecular weights upto 78×10^3 when the initiator concentration was decreased which is expected.

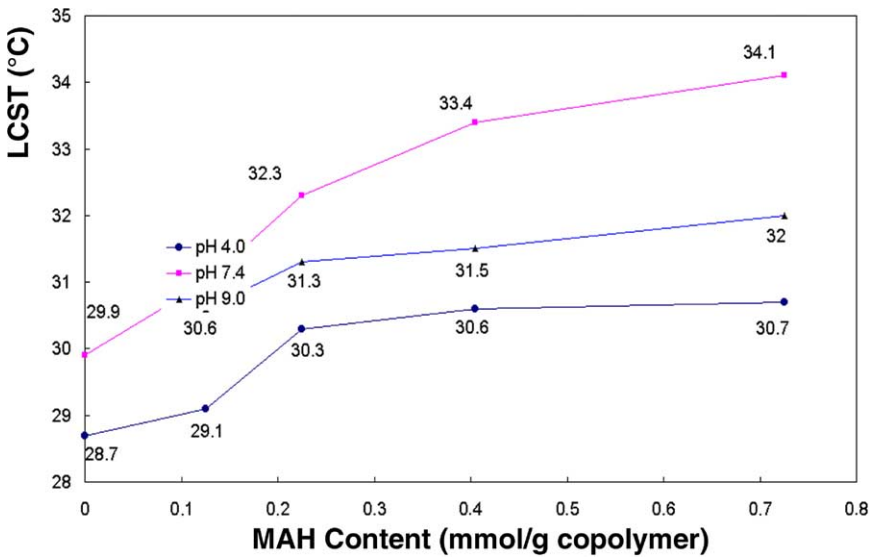

Fig. 8. Changes of LSCT values with the MAH content of the NIPA-co-MAH copolymer chains at different pH.

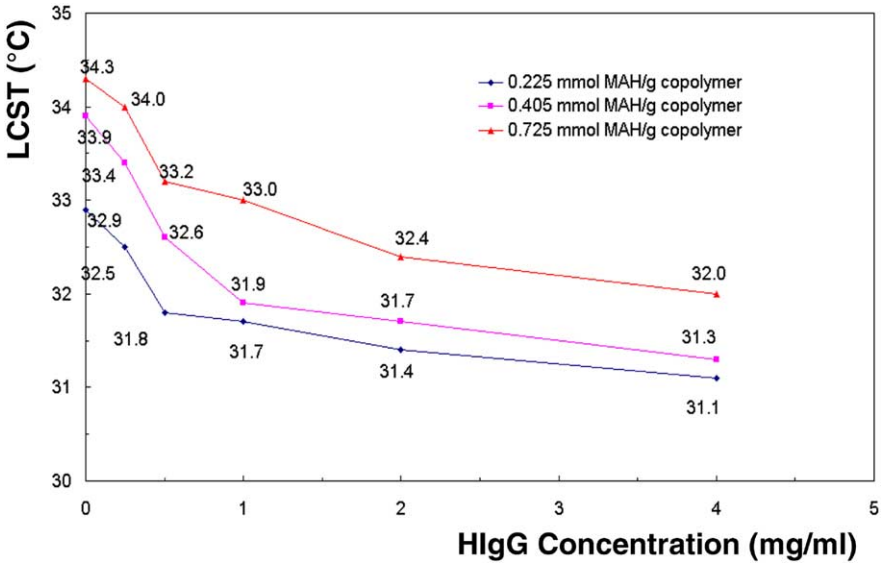

Fig. 9. Changes of LSCT values with HIgG concentration for three different NIPA-co-MAH copolymers with different MAH contents.

Stimuli-responsive behavior of both the poly(NIPA) homopolymer and its copolymers with MAH was studied at three different pH values of 4.0, 7.4 and 9.0. Fig. 8 shows the LCST values increases with the MAH content of the copolymer chain, for all three pH. It was also observed that there were decreases in the LCST values, not very significant but noticeable, when the molecular weight of the copolymers is increased.

Poly(NIPA-co-MAH) copolymers having different MAH contents were interacted with HIgG solutions at pH 7.4. Fig. 9 shows the changes of LCST values with HIgG concentration for three different copolymers with different MAH contents. Note that most probably the change in hydrophobicity–hydrophilicity balance of the copolymer chains due to he increase in hydrophobicity of conjugation with HIgG molecules caused the decrease in LCST. The decrease in LCST with HIgG concentration is not linear overall. However, if one considers two HIgG concentration regions (lower than 0.5 mg/ml and higher than that), the LCST-HIgG concentration relations could be accepted as linear. The results indicated that NIPA-co-MAH copolymer could be utilized as a new reagent for the determination of HIgG concentration in the aqueous medium.

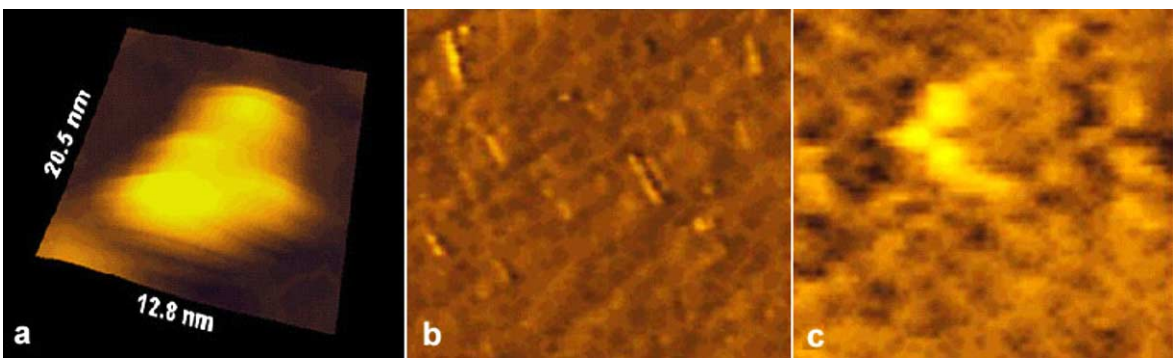

Fig. 10. Representative STM images of the “Y” shaped HIgG molecule (a); NIPA-co-MAH copolymer chains (b); HIgG-copolymer conjugate (c).

Representative STM images given in Fig. 10 shows that the HIgG molecule is "Y" shaped with 15.4 nm × 9.5 nm dimensions, as also suggested in literature. The copolymer chains are like sticks and their dimensions are in the about (9.6–32.4) × 6.5 nm. The interaction of HIgG molecules with the copolymer chains were throug Fab region of HIgG, as also mentioned in the literature (El-Kak and Vijayalakshmi, 1992).

6. Thermal stabilization of penicillin G acylase with poly[(NIPA-co-MA)-G-PEO]/PEI

Pencillin G acylase (PGA) is a member of the *N*-terminal nucleophilic hydrolase superfamily of enzymes and as a periplasmic protein has been extensively studied genetically, biochemically and structurally (Done et al., 1998; Brannigan et al., 2000; Hewitt et al., 2000). One of major industrial importance properties of this enzyme is its catalyst activity in the hydrolysis reactions of many biomolecules: PGA is used for the production of artificial β-lactam antibiotics and as catalyst for the hydrolysis of amide bond in benzyl penicillin (Penicillin G) and many β-lactam nucleus such as penicillin V, cephalosporin G and V, etc. (Simons and Gibson, 1999). PGA activity increases with temperature, but it looses its activity rapidly. Therefore, stabilization of PGA which would allow it to work at elevated temperatures without loosing its activity is a critical issue to be solved. There are number of approaches have been proposed to increase the stability of PGA at high temperatures.

In present work, we aimed to synthesize (i) stimuli-responsive poly(NIPA-co-MA), (ii) their macrobranched derivatives using α-hydroxy-ω-methoxy-polyethylene oxides (PEO) with different molecular weight, (iii) PEI macrocomplexed derivatives and (iv) conjugates with pencillin G acylase and evaluation of kinetic parameters of PGA inactivation.

6.1. Experimental

Copolymerizations of NIPA with MA using various monomer feed ratios were carried out in 1,4-dioxane at 65 °C with AIBN radical initiator at a constant total concentration of monomers under the nitrogen atmosphere. Resulted copolymer with given composition was grafted with PEO (with molecular weight of 4000, 10,000 and 20,000) using copolymer/PEO (anhydride unit/end hydroxyl group) equimolar percentage ratios (50/50) in anhydrous THF at 40 °C during 30 min. The copolymer having carboxylic acid groups (–COOH) and also carrying the macrobranched PEO domains allowed to form complementary macrocomplexes with PEI (60,000) macromolecules in aqueous medium through proton transferring in the $–COO^{-}\cdot{}^{+}NH–$ different amine groups of PEI).

Chemical structures of copolymer and its macrobranched and macrocomplexed derivatives were analyzed by FTIR and ^{1}H NMR. Intrinsic viscosities of the copolymers with different structure and compositions were determined in THF at 25 °C using an Ubbelohde viscometer. LCSTs were obtained at pH 4.0, 5.0, 7.4 and 8.2 spectrophotometrically.

Conjugates of the copolymer and its macrobranched and macrocomplexed derivatives with the enzyme (PGA) were prepared by mixing of solutions containing these at 25 °C for 24 h. For the determination of the free and conjugated enzyme activities, a continuous colorimetric enzyme assay was used (Simons and Gibson, 1999). Inactivation rate constants and half life values for the both free and conjugated PGA at different temperature (45, 55 and 65 °C) were obtained according to the first order inactivation mechanism (Erarslan and Koçer, 1992).

6.2. Summary of results

FTIR and ^{1}H NMR spectra exhibited the formation of poly(NIPA-co-MA) copolymer and its derivatives. It was shown that the important properties of copolymer systems, such as viscosity, thermal and pH-sensitivity, LCST, and thermal behavior change in increasing molecular weights, copolymer compositions and length of macrobranched hydrophobic fragments. Copolymers containing about 17.7–19.1 mol% MA and with different molecular weights ($(\eta)_{in}$: 0.13–0.44 dl/g, in THF at 25 °C) were synthesized. LCST values of these copolymers were in the range of 31.1–45.0 °C. Higher LCST values were obtained with higher molecular weights and at higher pH values. The LCST values of the copolymer having more MA in their backbone were even higher.

Synthesized amphyphilic random and PEO grafted copolymers with reactive anhydride and carboxylic groups exhibit high complexing ability toward PEI

and allow to prepare new cation active self-organized macromolecular architecture which is used as enzyme carrier for PGA enzyme. Obtained some kinetic parameters of PGA inactivation indicated that complexed copolymer-enzyme conjugate essentially increases thermal stabilization of PGA (three times at 45 °C and two times at 65 °C). It was observed that copolymer-enzyme conjugate is more stable at high temperature than the free enzyme. Incorporation of PGA enzyme to the copolymer and its PEO macrobranched and PEI macrocomplexed derivatives provides protection against thermal inactivation of the enzyme at temperatures well above the LSCT of the studied copolymer systems.

The improvement of the thermal stabilization of the enzyme after modification by poly(NIPA-co-MA) with and without the addition of PEI may be because of the conjugation of polymer molecules with PGA enzyme. Observed an increase of LSCT value in the copolymer/PEI-PGA enzyme system (from 36.7 for the free copolymer/PE to 41.3 °C for PGA incorporated system) is indicated a formation of copolymer-enzyme complexed conjugate. The interactions between enzyme and copolymer increases the rigidity and resistance of the structure against thermal unfolding. It seems that the carboxyl groups of copolymer and different type of amine groups of PEI are play important role on the interaction of enzyme amino acid groups. So the conjugation of amine-carboxyl groups is essential. As a result, it can be assumed that synthesized new temperature-responsive copolymer systems have the potential for improving the thermal stabilization of enzymes.

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