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pH-Controlled association of PEG-containing terpolymers of *N*-isopropylacrylamide and 1-vinylimidazole

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Abstract

Temperature- and pH-controlled association of terpolymers of *N*-isopropylacrylamide (NIPA) with 1-vinylimidazole (VI) and polyethylene glycol (PEG) has been investigated by light scattering and atomic force microscopy (AFM) in situ. The polymers contained 0–15 mol% VI and 0–2 mol% PEG. The phase transition temperatures (T_p) have been in the range of 32–45 °C and exhibited significant dependence on the pH of solution in the pH range between 5 and 8. The T_p of the polymers increased with increasing VI content and with decreasing pH, confirming major effect of VI ionization status on T_p. The presence of PEG grafts in the polymer structure had augmenting effect on the magnitude of pH-responsiveness and on the pH-independent colloidal stability of the polymer particles formed above T_p. Incorporation of VI into the polymer structure had similar, but pH-dependent effect on colloidal stabilization of the polymer particles. The size of the particles formed after the phase transition is driven by the association of the collapsed NIPA segments in the globule conformation and it decreased with decreasing pH. The phase transition temperature of the polymers could be adjusted to increase from temperatures below, to temperatures above body temperature upon decreasing pH from 7 to 6, suggesting that such polymers could provide a material platform for a variety of biomedical applications. AFM analysis in situ showed a fully reversible formation of particles in the solutions of the polymers above their T_p.

Keywords: *N*-Isopropylacrylamide; Vinylimidazole; PEG

1. Introduction

Polymers capable of undergoing a conformational coil-to-globule phase transition upon application of a stimulus, such as change of temperature or pH, show a great promise in a variety of biomedical applications [1–4]. The reversible coil-to-globule transition has been studied most extensively in solutions of poly(*N*-isopropylacrylamide) (PNIPA). PNIPA exhibits a phase transition temperature (T_p) (lower critical solution temperature) around 32 °C in aqueous solutions. Below this temperature the PNIPA chains are soluble in water and exist in extended random coil conformation and when temperature exceeds the T_p the polymer conformation changes to the globular form [5]. Although this class of polymers can be engineered to exhibit responsive behavior to a variety of stimuli, temperature has been the most widely investigated one for soluble polymers exhibiting the lower critical solution temperature behavior. These polymers have emerged, among others, as very promising components of drug and gene delivery systems and as molecular switches of protein activity [6–10]. It is the rapid, highly non-linear and reversible nature of their phase transitions in response to small changes of temperature that makes these polymers promising for applications in the above areas. Temperature-responsive systems used in the biomedical applications described thus far use synthetic NIPA-based copolymers or genetically engineered elastin-like polypeptides as the temperature responsive component [11].

The phase transition of PNIPA can be controlled in several ways, including incorporation of hydrophobic or hydrophilic residues into the polymer structure [12,13], variation of ionic strength of solution [14,15], and presence of surfactants [16]. The temperature responsiveness of the

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NIPA copolymers can be also combined with the ability to respond to changes of pH [17,18]. This can be most easily achieved by incorporating weak electrolyte functionalities into the structure of the temperature responsive polymer. The pH-dependent ionization of the weak electrolyte residues modulates the overall hydrophilicity/hydrophobicity of the polymer and leads to changes of T_p. The phase transition, however, still originates from the conformational changes of the temperature-responsive component. Selection of a weak acid or a weak base component is governed by the intended use of the polymers. Incorporation of weak acid groups results in polymers exhibiting the coil-to-globule phase transition upon decreasing pH. Incorporation of weak basic groups results in polymers exhibiting the coil-to-globule phase transition upon increasing pH. So far, copolymers responsive to changes of pH were prepared with a number of comonomers including acrylic and methacrylic acids, glycine acrylamide, 2-vinylpyridine, or 1-vinylimidazole (VI) [13,19–23].

This paper evaluates pH-responsive properties of terpolymers of NIPA with VI and poly(ethylene glycol) (PEG). VI was chosen for the studies because of its attractive pK_a suitable for a variety of biomedical applications and PEG was chosen because of its high aqueous solubility. The synthetic methods for the preparation of PNIPA-*g*-PEG copolymers have been reported by several authors [24–26]. A high molecular weight copolymer was prepared by free radical polymerization of NIPA with PEG macromonomer. Alternatively, better-defined copolymers were prepared by copolymerization of NIPA and either *N*-acryloylsuccinimide or glycidyl methacrylate followed by grafting with amino-terminated PEG.

The main objective of the present study has been to evaluate the effect of 1-vinylimidazole and poly(ethylene glycol) on the pH-dependent phase transition and association behavior of the NIPA terpolymers.

2. Experimental section

2.1. Materials

N-Isopropylacrylamide, 1-vinylimidazole, poly(ethylene glycol) methyl ether methacrylate with average molecular weight 2080 (PEGMA, 50% solution in water), azobisisobutyronitrile (AIBN) were from Aldrich and used as received. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. All other reagents and solvents were purchased from Fisher scientific and used as received.

2.2. Polymer synthesis and characterization

In a general polymerization procedure used, NIPA (0.35–1.67 M), VI (0.02–0.23 M), PEGMA (6–94 mM), and AIBN (4–17 mM) were dissolved in acetone, transferred into a glass ampoule, and deoxygenated by a stream of nitrogen. The ampoules were sealed and the polymerization was carried out in a water bath at 60 ± 1°C for 24 h. The reaction mixture was then added drop-wise to a cold diethyl ether, the precipitated polymer isolated by filtration, reprecipitated from methanol solution, and dried overnight in vacuum before analysis. The composition of the copolymers was determined in D_2O solutions by ^{1}H NMR analysis using varian 400 MHz NMR spectrometer. Area of –N–CH proton of NIPA ($\sim$4 ppm), protons of VI cycle (–CH, $\sim$7.2 ppm), and methylene protons of PEG (3.6 ppm) were used to calculate the composition of the copolymers. The VI content was also confirmed by titration with 0.01 M NaOH. The number-average (M_n), weight-average (M_w) molecular weight and polydispersity (M_w/M_n) of the polymers were determined by gel permeation chromatography (GPC) using Shimadzu LC-10ADVP liquid chromatograph equipped with CTO-10ASVP Shimadzu column oven and waters styragel HR-4E 7.8×300 mm column. The system was equipped with a 7-angle BIMwA static light scattering detector and BIDNDC differential refractometer (both from Brookhaven Instruments, Inc.). The BIMwA detector was equipped with 30 mW vertically polarized solid state laser (660 nm) as a light source. THF was used as an eluent at a flow rate of 1 mL/min at 30 °C. GPC data were analyzed using PSS WinGPC Unity software from Polymer Standards Services. Dissociation constants of the VI-containing copolymers were estimated from the titration curves of their aqueous solutions with 0.01 M HCl.

2.3. Measurement of hydrodynamic sizes and phase transition temperatures

The determination of hydrodynamic diameters was performed using Zetaplus Particle Size analyzer (Brookhaven Instruments) equipped with 35 mW solid state laser (658 nm). Scattered light was detected at 90° angle and a temperature of 25 °C. Mean hydrodynamic diameters were calculated for size distribution by weight, assuming a lognormal distribution. Copolymers were dissolved in water, 50 mM sodium phosphate (pH 8, 7, 6, or 5), DMEM, or DMEM supplemented with 1% FBS. All solutions were filtered through 0.45 μm PVDF filter prior measurement. To determine the phase transition temperature, temperature dependence of scattering intensity at 90° from 1 mL of 10 mg/mL solution of a copolymer in a glass cuvette was measured. The temperature was increased by 1 °C increments in the range 25–50 °C and the reading was taken after 3 min equilibration at each temperature. T_p was then determined as the onset temperature of increase of the scattering intensity. For each sample, the T_p was determined on two separate occasions to confirm the reproducibility.

2.4. In situ atomic force microscopy (AFM)

NIPA terpolymer was dissolved in 50 mM sodium

phosphate buffer pH 5 at the concentration 1 mg/mL. The solution was then diluted to 0.05 mg/mL with deionized water at room temperature. AFM imaging was conducted on a freshly cleaved mica using Nanoscope III from Digital Instruments in situ in a liquid cell by immersing the tip assembly in deionized water. Temperature variation of the solution during AFM imaging was conducted by model 2216e Temperature Controller from Eurotherm. An AFM E-scanner with a maximum scan area of 16×16 μm^2 was used. The E-scanner has been calibrated separately when used with the temperature controller in order to take into account temperature-induced dimensional changes. AFM imaging was conducted in the liquid tapping mode, which exerts less mechanical disturbance than the more common liquid contact mode. Silicon nitride integral tips (NP type, Digital instruments) were used with factory-specified nominal tip radius of curvature between 20 and 40 nm.

3. Results and discussion

Copolymerization of NIPA with various comonomers is a reliable approach used to adjust the phase transition behavior to the temperature desired for intended application. Incorporation of hydrophilic residues typically leads to an increase of the phase transition temperature, while incorporation of hydrophobic residues results in its decrease. Incorporation of weak electrolyte residues provides additional dependence of the phase transition behavior on the pH of solution, especially in the pH interval around pK_a of the electrolyte groups. NIPA copolymers exhibiting pH-sensitivity in the slightly acidic pH range between pH 5 and 7 are of particular interest, because of their potential biomedical applications [27,28]. In the present study, NIPA terpolymers with VI and PEG macromonomer have been synthesized as materials potentially exhibiting such a pH-sensitive behavior in the slightly acidic pH. A library of the polymers containing 83–100 mol% of NIPA, 0–15 mol% of VI, and 0–2 mol% of PEG grafts was synthesized using a conventional free radical polymerization in acetone (Fig. 1). Vinylimidazole was selected as the component providing the pH-responsiveness because of the suitable pK_a value of its imidazole ring [13]. The PEG grafts were introduced into the polymer structure in order to prevent precipitation and permit evaluation of the association properties above the phase transition temperatures of the synthesized polymers. The macromonomer approach to the synthesis of NIPA graft copolymers has been successfully used before using PEG methacrylates [24]. On the other hand, Virtanen et al. reported a gel formation due to unwanted crosslinking during the copolymerization of NIPA and PEG methacrylates [26]. In this study, however, no gel formation was observed as documented by the molecular weights and polydispersity values of the prepared copolymers shown in Table 1. The main disadvantage of the selected synthetic approach is the likely compositional heterogeneity of the prepared copolymers due to significant differences in the reactivities of PEG macromonomer and NIPA and VI monomers [29].

Phase transition temperatures of NIPA copolymers are usually determined from temperature dependences of transmittance of their aqueous solutions. In this study, the phase transition temperatures were determined from the temperature dependence of scattering intensity measured at 90° scattering angle. The temperature was increased stepwise by 1 °C increments and scattering intensity and mean hydrodynamic radius were measured after 3 min equilibration at each temperature [30]. This approach permitted sensitive determination of T_p even in copolymer solutions of low concentration exhibiting low levels of molecular association. To evaluate the effect of VI on the pH-controlled phase transition behavior of the NIPA copolymers, phase transition temperatures were first determined for three P(NIPA-*co*-VI) copolymers at four different pH values (Fig. 2). The behavior was evaluated at pH range 5–8 because of its relevance for intracellular endo/lysosomal processing and extracellular acidification of solid tumors. It has been reported, however, that the phase transition of P(NIPA-*co*-VI) copolymers is influenced by pH levels as low as 4 [13]. As expected, the T_p of P(NIPA-*co*-VI) copolymers increased with increasing VI content and with decreasing pH. The incorporated charged VI units perturb hydrogen-bonding structure of hydrating water molecules. As a result, T_p of the NIPA-*co*-VI copolymer with ionized VI units is higher than that with non-charged VI units and, therefore, the phase transition temperature should change fastest in the pH range around pK_a of the copolymers. The pK_a of a model compound *N*-ethylimidazole is 7.0 and pK_a reported for similar P(NIPA-*co*-VI) copolymers in water was 5.2 [13], which is similar to that reported for P(VI) gels [31]. The pK_a of the P(NIPA-*co*-VI) copolymers used in this study was estimated to be around six, which is slightly higher than previously reported. This difference is likely to be the result of the phosphate buffers used in the present study compared to pure water used previously. The pK_a of polyelectrolytes is strongly dependent on the presence of salts. For example, pK_a of the P(VI) hydrogels increased by almost two units in the presence of 100 mM NaCl when compared with pK_a determined in pure water [31]. In addition, the ions present in solution of NIPA copolymers usually strengthen hydrophobic interactions, which stabilize the globular conformation of the copolymers and lower their T_p.

The increase of T_p of the P(NIPA-*co*-VI) compared with control PNIPA homopolymers at specific pH is dependent on both the total VI content and the VI degree of ionization. The degree of VI ionization is the dominant factor as documented by comparison of the T_p at pH 8 and 5 (Fig. 2). The magnitude of pH-responsiveness of the P(NIPA-*co*-VI) can be estimated from the difference of T_p determined at pH 8 and 5. The pH-responsiveness increases almost linearly

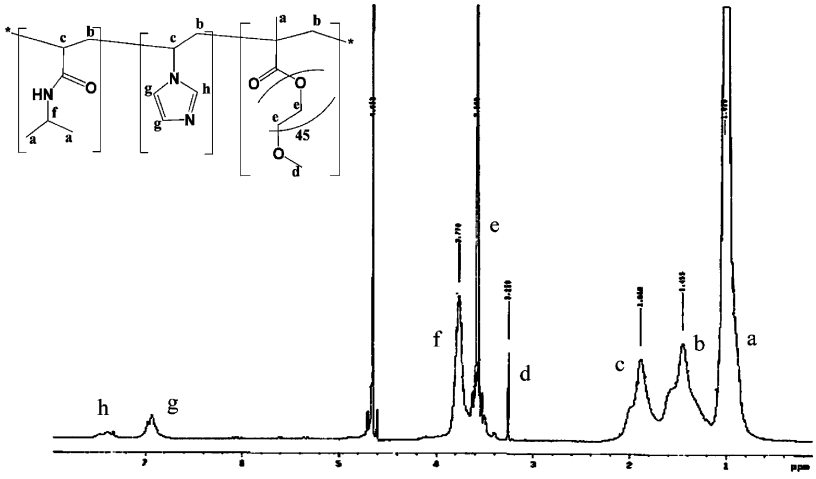

Fig. 1. Structure and typical ^{1}H NMR spectrum of P(NIPA-*co*-VI-*co*-PEGMA) in D_2O (spectrum of NVP9 terpolymer shown).

with increasing VI content as documented by the average increase of T_p per 1 mol% of VI (ΔT_p) upon decreasing the pH from 8 to 5. The ΔT_p was 0.38 °C for the copolymer with 8 mol% of VI units and 0.33 °C for the copolymers with 12 and 18 mol% of VI units. As expected, the determined ΔT_p values in 50 mM phosphate buffer are lower than those reported previously for similar copolymers in pure water [13].

Block copolymers of NIPA with hydrophilic polymers and graft copolymers containing NIPA grafts often exhibit T_p similar to that of NIPA homopolymers because the cooperative domains in NIPA blocks and grafts that undergo phase transition are not significantly perturbed by the other component [32–35]. On the other hand, T_p of graft copolymers with NIPA backbone grafted with hydrophilic polymers can be increased at high grafting densities, similar to NIPA copolymers with hydrophilic monomers. Fig. 3 shows the effect of PEG content on the T_p of PNIPA-*g*-PEG copolymers. The phase transition temperature increases only slightly with increasing PEG content up to 2 mol%. Increasing the PEG content further, however, resulted in the T_p increase of almost 5 °C and a very broad phase transition. The broad transition is probably caused by the compositional heterogeneity of the copolymers caused by large differences in reactivity ratios of the used monomers [29]. The phase transition temperatures in Fig. 3 were determined in 50 mM phosphate buffer and were lower by 1–2 °C than T_p measured in pure water. Based on these results, subsequent studies with VI and PEG-containing NIPA copolymers were limited to those with PEG content $\leq$2 mol%.

The pH-dependent phase transition behavior of NIPA terpolymers containing both VI and PEG units was evaluated in two series of polymers containing either 1 or 2 mol% of PEG (Fig. 4). Similar to the P(NIPA-*co*-VI) copolymers, the T_p of the PEG-containing terpolymers increased with increasing VI content and with decreasing pH. The vertical position of the curve for each terpolymer was governed by both the content of VI and PEG. Increasing the content of VI and PEG increased the T_p at all pH values.

Table 1
Synthesis and characterization of P(NIPA-*co*-VI-*co*-PEGMA) terpolymers

Sample	Composition (mol %)						Yield (wt%)	M_w (g/mol)	M_w/M_n
	In feed			In polymer					
	NIPA	VI	PEG	NIPA	VI	PEG			
NVP1	100	0	0	100	0	0	82	2.93×10^5	1.85
NVP2	95	5	0	92	8	0	90	1.29×10^5	2.56
NVP3	90	10	0	88	12	0	90	4.83×10^5	2.40
NVP4	85	15	0	82	18	0	51	2.25×10^5	2.75
NVP5	99	0	1	99	0	1	50	7.25×10^5	2.59
NVP6	98	0	2	98	0	2	90	1.20×10^5	1.97
NVP7	95	0	5	97	0	3	83	4.22×10^5	4.06
NVP8	94	5	1	93	6	1	95	1.59×10^5	1.43
NVP9	89	10	1	87	12	1	96	2.06×10^5	2.61
NVP10	84	15	1	84	15	1	66	2.05×10^5	1.72
NVP11	93	5	2	92	6	2	94	2.46×10^5	1.77
NVP12	88	10	2	82	16	2	87	9.84×10^5	6.60
NVP13	83	15	2	83	15	2	75	8.99×10^5	1.80

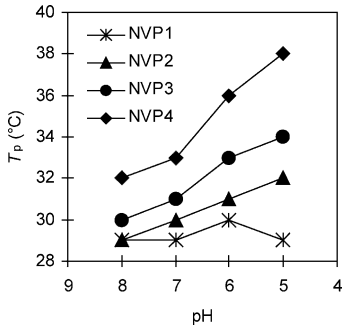

Fig. 2. Influence of vinylimidazole content on the pH-dependent phase transition temperature (T_p) of NIPA-*co*-VI copolymers. The analyses were performed using 10 mg/mL copolymer solutions in 50 mM phosphate buffers pH 5, 6, 7, and 8.

The increase of T_p of the NIPA terpolymers depends on the VI and PEG content as well as VI degree of ionization. The presence of PEG appeared to have a synergistic effect on the T_p of the polymers. The T_p of P(NIPA-*co*-VI) containing 12 mol% of VI units was 34 °C at pH 5. With the incorporation of 1 mol% of PEG into terpolymer with the same VI content, the T_p increased to 38 °C at pH 5 (Fig. 4(a)). Incorporation of 2 mol% of PEG into similar terpolymer resulted in T_p increasing to 42 °C at pH 5 (Fig. 4(b)). At the same time, the T_p of PNIPA-*g*-PEG copolymer with 1 and 2 mol% of PEG was similar to that of pure NIPA homopolymers. The results also show that the presence of PEG grafts in the terpolymer structure has augmenting effect on the magnitude of pH-responsiveness as determined by the T_p increase induced by decreasing pH of the solution from 8 to 5 (ΔT_p). While the ΔT_p for P(NIPA-*co*-VI) copolymers was 0.33–0.38 °C; the ΔT_p values were increased to 0.42–0.70 and 0.52–0.80 °C for copolymers containing 1 and 2 mol% of PEG, respectively. In both cases, polymers containing more VI units exhibited smaller ΔT_p values than those with lower VI contents.

With the exception of extremely diluted solutions, the coil-to-globule phase transition of NIPA homopolymers in aqueous solutions leads to a macroscopic precipitation. Incorporation of hydrophilic components, such as in case of

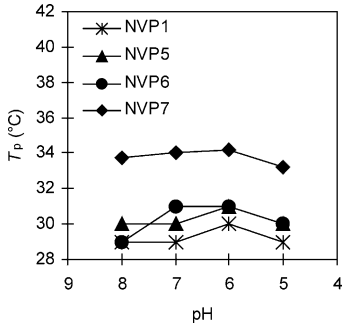

Fig. 3. Effect of increasing content of PEG grafts on the phase transition temperature (T_p) of PNIPA-*g*-PEG copolymers. The transition temperatures were determined using 10 mg/mL copolymer solutions in 50 mM phosphate buffers pH 5, 6, 7, and 8.

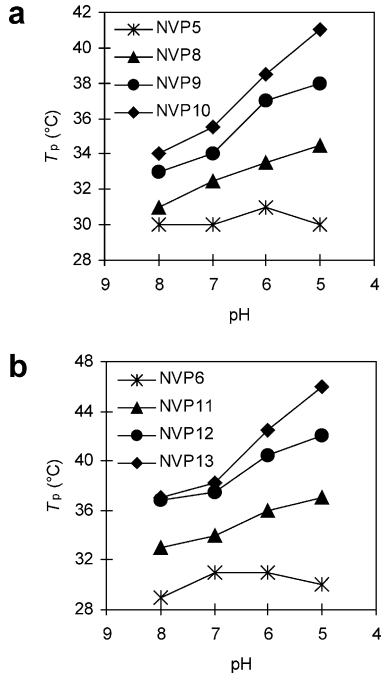

Fig. 4. Combined effect of VI and PEG on the pH-dependent phase transition temperature (T_p) of P(NIPA-*co*-VI-*co*-PEGMA) terpolymers. The transition temperatures were determined using 10 mg/mL polymer solutions in 50 mM phosphate buffers pH 5, 6, 7, and 8. The polymers contained 1 mol% of PEG grafts (a) or 2 mol% of PEG grafts (b).

block or graft copolymers with PEG, prevents the aggregation and leads to a formation of colloidal associates or micelles [30,34]. Similar stabilizing effect is achieved here by the incorporation of VI units. Although P(NIPA-*co*-VI) copolymers precipitate above T_p at pH 8 and 7, electrostatic repulsion between positive charges on ionized VI units prevents aggregation of the globules in the lower pH range. The presence of PEG grafts in the structure of the terpolymers provides them with pH-independent colloidal stabilization. This is documented in Fig. 5 which represents temperature-dependent changes of hydrodynamic radius in solution of the polymer NVP9 containing 12 mol% of VI and 1 mol% of PEG measured in 50 mM phosphate buffer of

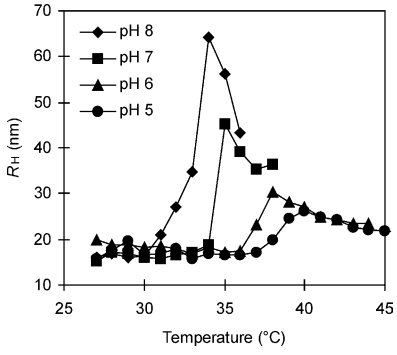

Fig. 5. Temperature and pH-dependence of hydrodynamic radii in the solution of NVP9 terpolymer. The hydrodynamic radius (R_H) was measured in 10 mg/mL polymer solutions in 50 mM phosphate buffer of indicated pH.

various pH values. With decreasing pH, the R_H vs temperature curves shift toward higher temperatures and the slopes of the transitions become smaller. The size of the associates formed after the phase transition is driven by the association of the collapsed NIPA segments in the globule conformation and it decreases with decreasing pH. The decrease of the size and the curve slopes with decreasing pH is at least partly caused by the contributing effect of ionized VI residues on the colloidal stability of the formed associates. The globules formed at pH 8, when essentially all of the VI units are present in non-ionized form, are relatively hydrophobic and thus, higher degree of association and larger particles are formed. The hydrodynamic radius of the associates formed upon the phase transition decreased slightly as the temperature was increased further above T_p. This size decrease was not accompanied by corresponding decrease in scattering intensity, suggesting further association of more densely packed particles. The particle size distributions of the associates formed above T_p were typically characterized by polydispersity values 0.20–0.25.

The effect of polymer concentration on pH-dependant association properties of the NVP9 terpolymer containing 12 mol% of VI and 1 mol% of PEG was studied next (Fig. 6). The measurements of R_H were conducted at three different concentrations (0.5, 2.5, and 10 mg/mL) in 50 mM phosphate buffer solutions of indicated pH. The temperature was adjusted to 5 °C above T_p of the copolymer at a given pH and the solution was left to stabilize for 30 min before measurement of R_H. The size of the formed particles is dependent on the hydrophobic/hydrophilic balance within the copolymer. Since, the hydrodynamic sizes were determined above T_p, it is reasonable to assume that primarily the ionization degree of the VI units will determine the balance. The hydrodynamic sizes of the particles formed by the association of the terpolymer molecules decrease, in general, with decreasing pH. Decreasing the polymer concentration resulted in further decrease of size of the formed associates. For example, the radius of the particles decreased from 34 nm, observed at the highest concentration studied, to 25 nm at 0.5 mg/mL in solution of pH 7.0. The concentration dependence of the hydrodynamic sizes was less pronounced at more acidic pH, at which the ionization of VI prevents a significant association of the terpolymer molecules even at the higher concentrations. At pH 5, the R_H of the particles decreased only slightly, from 25 to 22 nm, upon decreasing the polymer concentration. The stabilization of the particles at higher pH is primarily determined by the PEG grafts present in the terpolymer structure, while the ionization of VI units contributes mainly at lower pH.

The above results established that the phase transition behavior of the studied copolymers is controlled by temperature and pH. A possible application of polymers exhibiting the phase transition controlled by the pH in the slightly acidic pH range is in the design of drug delivery devices capable of exploiting acidic pH gradients existing in cells and solid tumors [27,36]. To be applicable for such applications, a suitable polymer should undergo the globule-to-coil phase transition at a constant temperature corresponding to the normal body temperature of 37 °C after small decrease of pH. The results shown above document that pH-responsive behavior of the NIPA terpolymers with VI and PEG can be engineered so that T_p changes from below to above body temperature upon decreasing pH from 8 to 5. Fig. 7 demonstrates pH-dependent dissociation of the NVP9 terpolymer containing 12 mol% of VI and 1 mol% of PEG at constant temperature of 37 °C. Starting in a solution of phosphate buffer pH 8.0, the T_p of the studied polymer is below 37 °C and, therefore, association into particles with hydrodynamic radius of 37.5 nm takes place. Decreasing the pH to 7.0 at which the terpolymer T_p is still below 37 °C leads only to a negligible decrease of size of the particles. However, decreasing the pH further to 6.0 increases the T_p of the copolymer above 37 °C and this leads to a dissociation of the formed particles as documented by the abrupt decrease of the observed hydrodynamic radius and intensity of scattered light. Further decrease of pH has little effect on the properties of the polymer solution. Fig. 7, therefore, demonstrates globule-to-coil phase transition of

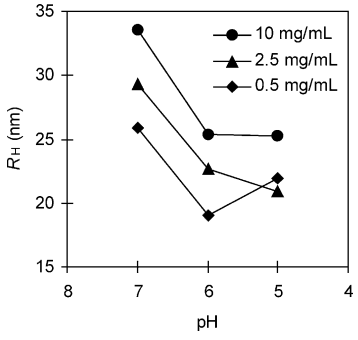

Fig. 6. Concentration and pH-dependence of the hydrodynamic radius (R_H) of associates of NVP9 terpolymer. The hydrodynamic radii were measured in 50 mM phosphate buffer of required pH at temperatures 5 °C above T_p of the polymer at the respective pH.

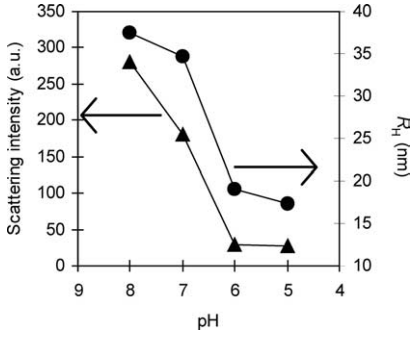

Fig. 7. pH-Controlled association behavior of NVP9 terpolymer at 37 °C. Hydrodynamic radius and intensity of scattered light (90°) from solution of the polymer (10 mg/mL) were measured in 50 mM sodium phosphate buffer (pH = 5, 6, 7, and 8) after 15 min equilibration at 37 °C.

the selected terpolymer induced by decreasing pH at constant temperature of 37 °C.

The association behavior of the polymers was investigated also by AFM analysis in situ (Fig. 8). The NVP9 terpolymer was imaged in 2.5 mM phosphate buffer pH 5.0 at the concentration 0.05 mg/mL. Similar to the T_p determination, the solution temperature was increased stepwise from room temperature to 50 °C and then the solution temperature was gradually reduced back to room temperature. The heating and cooling cycle was repeated several times for the same sample. The results confirmed that the morphological changes observed in the terpolymer solution are fully reversible with temperature. Fig. 8 shows typical AFM images below and above T_p during one heating/cooling cycle. All three images have a scan size of 2×2 μm^2 with a z-range of 10 nm. At room temperature, the image reflects the packing structure of the polymer coils, which are flexible enough to form a smooth and continuous film (Fig. 8(a)). Above T_p, the images reflect the association structures formed from the terpolymer globules (Fig. 8(b)). The polymer associates appear as spherical particles of fairly uniform size distribution. The height of particles ranges from 1 to 4 nm and width ranges from 20 to 40 nm. However, the width is overestimated due to tip convolution. When temperature was reduced to below 42 °C, number of particles has been significantly reduced to yield the same flat film below T_p (Fig. 8(c)). Identical features were reproducibly observed in repeating cycles. The T_p observed in the AFM analysis was somewhat higher than T_p determined from the light scattering analysis. Lower terpolymer concentration and lower buffer concentration used in the AFM studies, together with the adsorption of the polymer to the substrate, are most likely the main reasons behind the difference.

Phase transition behavior of NIPA polymers is often studied in pure water or under conditions, such as the 50 mM phosphate buffers used in this study, which may not be fully relevant for biomedical applications. Analysis of the phase transition behavior under conditions more relevant for the intended use of the polymers is necessary in order to translate any promising results into real settings. The properties of the NIPA terpolymer containing 12 mol% VI and 1 mol% of PEG (NVP9) were, therefore, examined in a common cell culture medium (DMEM) (Fig. 9). To test the behavior of the terpolymer further, the phase transition was also studied in DMEM containing 1% of fetal bovine serum (FBS). DMEM is a solution with pH ~ 7.1–7.4 and containing mixture of essential amino acids, glucose, vitamins, and inorganic salts. The FBS, on the other hand, is a complex mixture of proteins. The components of the DMEM and FBS may affect the structure of hydrating water and, therefore, in turn also the phase transition of the NIPA polymers. In addition, possible interactions of serum proteins with VI residues of the terpolymers may also affect the T_p. Fig. 8 shows changes in the mean hydrodynamic radius of the terpolymer (Fig. 9(a)) and control PNIPA homopolymer (Fig. 9(b)) in the temperature interval 25–40 °C. Both figures reveal that the presence of serum had no significant effect on the phase transition temperature of the NVP9 terpolymer or PNIPA homopolymer. The phase transition behavior of both evaluated polymers in DMEM also corresponds to the behavior observed in 50 mM phosphate buffer of corresponding pH. The effect of PEG on colloidal stability of the formed particles is clearly observed for the terpolymer with the formation of particles with $R_H \sim 45$ nm above T_p. The fluctuation of sizes at temperatures below T_p in the solution of the terpolymer in DMEM in the presence of serum compared to that in DMEM alone is caused by the protein molecules present in the serum.

4. Conclusions

Terpolymers of NIPA with VI and PEG described in this study exhibit temperature and pH-controlled phase transition and association behavior. While the VI units provide the polymers with pH-sensitivity in a slightly acidic pH range, the presence of PEG grafts permits the control of polymer association above their phase transition temperature. The phase transition temperature of the synthesized polymers increases with increasing VI content and with decreasing pH. Light scattering and in situ AFM analysis revealed that the polymers associate into well-defined

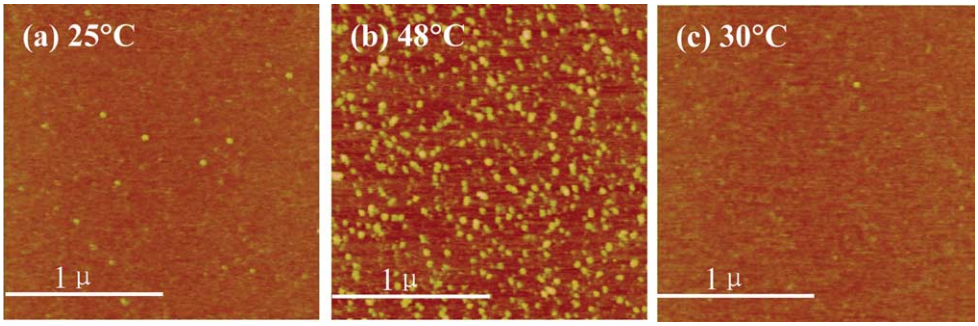

Fig. 8. In situ AFM images of NVP9 terpolymer on mica. The polymer was imaged at concentration 0.05 mg/mL in 2.5 mM phosphate buffer pH 5 at temperature below T_p (a), above T_p (b), and below T_p after heating cycle (c). The images have a scan size of 2×2 μm^2 with a z-range of 10 nm.

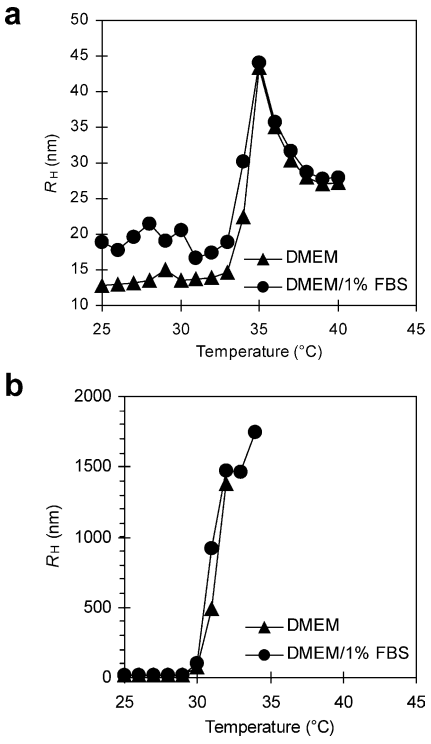

Fig. 9. Influence of fetal bovine serum (FBS) on the phase transition of NVP9 terpolymer (a) and PNIPA homopolymer (b). Temperature dependence of hydrodynamic radii was measured in 10 mg/mL polymer solution in Dulbecco's modified Eagle medium in the presence or absence of 1% FBS.

reversible particles at temperatures above the phase transition. The size of the particles is determined by the VI and PEG content, as well as pH of the solution. The phase transition temperature of the terpolymers can be adjusted to increase from temperatures below, to temperatures above body temperature upon decreasing pH from 7 to 6. Such globule-to-coil transition of the NIPA terpolymers induced by decreasing pH from 7 to 6 at 37 °C was demonstrated by the dissociation of particles formed via hydrophobically-driven association of the polymers. Overall, these studies suggested that NIPA-VI polymers could be suitable candidates for the design of biomedical devices capable of responding to small decrease of pH.

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