Reactivity of Temperature-Sensitive, Protein-Conjugating Polymers Prepared by a Photopolymerization Process

HASAN ULUDAG, MATTHEW WONG, JONATHAN MAN

Department of Biomedical Engineering, Faculty of Medicine and Oral Health Sciences, 10-102 Clinical Sciences Building, University of Alberta, Edmonton, Alberta T6G 2G3

Received 23 March 1999; accepted 29 April 1999

ABSTRACT: This study was carried out to characterize the reactivity of temperature-sensitive, protein-conjugating polymers prepared by a photopolymerization process. Polymers were based on *N*-isopropylacrylamide (NiPAM) and *N*-acryloxysuccinimide (NASI). A photoinitiator, 2,2-dimethoxy-2-phenyl-acetophenone, and monomers at desired ratios were polymerized in a glass flask using an UV source. Polymers were characterized for composition, molecular weight (MW), cloud point temperature (CPT), hydrolysis and aminolysis rates (using ethanolamine as a model compound), and protein conjugation. The monomer feed ratio was found to effectively control the composition of the synthesized polymers. The polymer MWs were between 10 and 20 kD, depending on the polymerization solvent. The CPT of NiPAM/NASI polymers did not depend on NASI content ($\leq$5.6%), nor did the hydrolysis and aminolysis rates. Compared to NASI monomer, the polymerized NASI exhibited a 6- and 120-fold slower rates of hydrolysis and aminolysis, respectively. Although hydrolysis and aminolysis rates were higher at higher pHs, the relative aminolysis : hydrolysis rate was highest at a pH of 7.4, which also gave the most effective protein conjugation. We conclude that characterizing the polymer reactivity is useful for predicting the optimal conditions for protein conjugation and may facilitate the design of polymers with improved protein conjugation kinetics. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 75: 583–592, 2000

Key words: protein conjugation; polymer reactivity; photopolymerization; hydrolysis; aminolysis

INTRODUCTION

Protein–polymer conjugates are being pursued as useful tools in pharmaceutical industry. Such conjugates are finding potential applications in enzyme immobilization,[1,2] in novel immunoassays[3] and in combinatorial synthesis. The protein component of the conjugate binds to a target ligand in a population of unspecific molecules. The polymer component, on the other hand, exhibits a particular physiochemical characteristic that can be conveniently manipulated to separate the ligand-bound conjugate from the rest of the molecules. Temperature, pH, ionic strength and solubility/insolubility in a particular media have been successfully used to induce such a separation.[4] Although protein–polymer conjugates can be obtained by chemically crosslinking a polymer and a protein or by polymerizing a "protein-monomer,"[5] both methods can cause nonspecific modification of the protein and lead to alterations in the protein's biological activity. An alternative approach is the preparation of "pre-activated" polymers, which contain protein-reactive monomer units. The active groups utilized to date include amino-reactive epoxide,[2] maleic anhydride[6,7] and succin-

Correspondence to: H. Uludag (hasan.uludag@ualberta.ca).

Journal of Applied Polymer Science, Vol. 75, 583–592 (2000)
 CCC 0021-8995/00/040583-10

imide esters,[8,9] and thiol-reactive vinylsulfone[10] and maleimide.[11] Depending on the properties of the protein to be conjugated (availability of specific functional groups, sensitivity to reaction conditions, etc.), the choice of the reactive groups will be significant. Polymers containing succinimide esters are particularly appealing because they are readily reactive with amine groups (the most common side group in proteins) under mild conditions. Despite successful conjugation of proteins to polymers containing succinimide esters,[8,9] no detailed study of the polymer reactivity has been reported. Understanding the reaction of such polymers with amine compounds is important, not only to optimize the conditions for protein conjugation, but also for rational design of polymers with improved specificity and reactivity.

In this study, we report the relative rates of hydrolysis and aminolysis of succinimide-containing polymers prepared by a convenient photopolymerization process. Hydrolysis of succinimide esters under aqueous conditions is a side reaction that competes with the conjugation to amine compounds. The polymer backbone was based on temperature-sensitive *N*-isopropylacrylamide (NiPAM), so that reversible changes in the polymer solubility can be induced without the need to introduce exogenous molecules to the polymer system. The results provided in this study will guide efforts to design protein-reactive polymers with improved conjugation characteristics.

MATERIALS AND METHODS

Materials

N-acryloxysuccinimide (NASI) initially was obtained from Sigma (St. Louis, MO) but later was synthesized as described below. All buffer salts, bovine α-lactalbumin, bovine serum albumin, and a nonspecific goat IgG were from Sigma. Benzene, ethyl ether, chloroform, ethylacetate, dimethylformamide (DMF), dioxane, and tetrahydrofuran (THF) were from Caledon Laboratories (Georgetown, ON, Canada). Hexanes (85% *n*-hexane) was from J. T. Baker (Phillipsburg, NJ). Laboratory-grade ethanolamine was from Fisher (Fair Lawn, NJ). NiPAM, Na, benzophenone, triethylamine, acryloyl chloride, *N*-hydroxysuccinimide (NHS), 2,2-dimethoxy-2-phenyl-acetophenone (polymerization initiator) and 2,6-di-tert-butyl-4-methylphenol (polymerization inhibitor) were from Aldrich (Milwaukee, WI). Fetal Bovine Serum (FBS) was from GIBCO BRL (Grand Island, NY). The NMR solvent deuterated chloroform ($CDCl_3$) was from Cambridge Isotope Laboratories (Andover, MA). The electrophoresis reagents and apparatus were from Bio-Rad (Hercules, CA). The molecular weight (MW) standards were polystyrene of 3.7 kDa (M_w/M_n = 1.09), 13.7 kD (M_w/M_n = 1.01), 44.0 kDa (M_w/M_n = 1.07) and 212.4 kDa (M_w/M_n = 1.11), all obtained from Aldrich.

NASI Preparation

NHS (57 g) and triethylamine (55 g) were dissolved in 750 mL chloroform at 0°C.[12] To this solution, 50 g acryloyl chloride was added dropwise over a 10-min period under stirring. The reaction mixture was allowed to stir for another 20 min. The reaction was stopped by washing the chloroform with ice-cold water (400 mL) and saturated brine (400 mL). After drying with $MgSO_4$, the mixture was filtered through a Buchner funnel, and 25 mg of 2,6-di-*tert*-butyl-4-methylphenol was added to the chloroform solution. The solution was concentrated to 150 mL by a rotary evaporator and the product was filtered. Ethylacetate and *n*-hexane (100 and 10 mL, respectively) were added sequentially to the chloroform solution under stirring, and the precipitate formation was allowed to proceed at 40°C overnight. The precipitate was filtered and washed with ice-cold 100 mL of *n*-hexane/ethyl acetate (4 : 1), 100 mL of *n*-hexane/ethyl acetate (9 : 1) and finally twice with 100 mL *n*-hexane. The crystals were dried at ambient temperature under vacuum. The final product was $\sim$ 30 g (65% yield), and a single spot was obtained on thin-layer chromatography, indicating the relative purity of the product.

Polymerization

The polymers were synthesized by solution photopolymerization. Two types of solvents were used: THF and benzene : THF (95 : 5%). THF was refluxed for at least 12 h over Na/benzophenone, distilled, and stored under positive N_2 pressure. Benzene was reagent grade (99%) and used without purification. All glassware was washed with soap, distilled water, and acetone and baked under vacuum overnight. The glassware was assembled under nitrogen after cooling. The monomers at desired ratios were added to a round-bottom flask, followed by the addition of the solvents using glass syringes. 2,2-dimethoxy-2-phenyl-acetophenone (5% w/v) was then added, and the

solution was degassed by nitrogen purging. The flask was placed on a magnetic stirrer and illuminated with a long-wave UV source at 3–5 cm (100 W; UVP, Upland, CA). After 40 min, the reaction mixture was filtered through glasswool and precipitated in ethyl ether. The precipitate was washed twice with ethyl ether, and the white powder was dried under vacuum at room temperature.

Polymer Composition

The proton NMR of polymer solutions (10 mg/mL in $CDCl_3$) was determined by a Bruker AM-300 (Billerica, MA) or Varian Unity 300 (Palo Alto, CA). The distinctive chemical shifts of monomers (N$\underline{H}$ at 4.3–3.8 ppm for NiPAM units, C$\underline{H}_2$—C$\underline{H}_2$ at 3.0–2.7 ppm for NASI units) were integrated, normalized by the number of monomer hydrogens, and used quantitatively to calculate a percent monomer composition.

Molecular Weight

The MW of synthesized polymers was estimated on a 7.8 × 300 mm Styragel™ HMW 6E gel permeation column [(GPC) Waters Inc.], using monodisperse polystyrene standards. The column was attached to a high-pressure liquid chromatography (HPLC) system consisting of Rheodyne Manual Injector (Cotati, CA), Waters 600 Pump (Rochester, MN), and Waters 996 Photodiode Array Detector. The polymer standards and the synthesized polymers were dissolved in dioxane (typically at 0.5 mg/mL) and 20 μL of polymer solution was injected to the column. The column was eluted with 0.2 mL/min dioxane, and the elution profiles were detected at 214 nm. A semi-log calibration curve, $\log M_w = 13.535 - 0.179 \times$ (elution time) ($R^2 = 0.990$), was used to estimate the MW of the polymers.

Cloud Point Temperature

The polymer cloud point temperature (CPT) was determined in distilled, deionized water, 0.1*M* phosphate buffer (pH 7.4), and fetal bovine serum. The initial experiments indicated no effect of polymer concentration on CPT (between 1 and 10 mg/mL NiPAM/NASI and 1–4 mg/mL for NiPAM/MMA), so that the results shown were from 1 mg/mL solutions. All polymers were allowed to dissolve in desired solvents at 4°C for 1–2 h and ~ 1 mL solution was added to 5-mm-diameter glass NMR tubes. The tubes were placed in a water or silicone oil bath at 4°C, and the temperature was slowly (~ 5°C/h) increased. The temperature at which visual phase inversion (i.e., turbidity) was obtained was taken as the CPT.[9]

Polymer Succinimide Content

A spectrophotometric procedure[13] was adopted for the determination of succinimide ester amount in polymers, based on NH_4OH aminolysis of NASI. A linear standard curve was obtained between 0 and 50 μg/mL (0 and 296 μM) when NASI was dissolved in aqueous 0.1 *N* NH_4OH. The polymers were dissolved at 100 mg/mL in dry DMF and diluted to 0.5 mg/mL with aqueous 0.1 *N* NH_4OH. The absorbance of polymer solutions was determined at 260 nm. The absorbance of a NiPAM homopolymer was subtracted from the polymers to account for the polymer "background."

Hydrolysis and Aminolysis Rates

The NHS release from NASI monomer and NASI-containing polymers was determined in 0.1*M* phosphate buffer (pH 7.4), 0.1*M* HEPES buffer (pH 8.1), and 0.1*M* carbonate buffers (pH 9.4 and 10.8). The monomer/polymer stock solutions were prepared in dry DMF on the day of the assay and used immediately. For monomer hydrolysis, the stock solution was diluted with the buffer to a desired concentration (1000–100 μM), and the increase in absorption (Abs.) at 260 nm was monitored for 30 min. For monomer aminolysis, the stock solutions were diluted with the appropriate buffer containing different concentrations of ethanolamine, and Abs. at 260 nm was monitored. For hydrolysis and aminolysis of polymers, the release of NHS was determined as a function of ethanolamine concentration ([ethanolamine]). To calculate hydrolysis (k_{hyd}) and aminolysis (k_{am}) rate constants, Abs. versus time curves were first fitted with a first-order equation, $A(t) = A_0 + (A_\infty - A_0) \cdot (1 - e^{-kt})$. The apparent reaction rate, k_{obs} is given by k[14] and is a cumulative sum of aminolysis and hydrolysis rates. k_{obs} versus [ethanolamine] was then plotted, and the slope and the intercept was used for k_{am} and k_{hyd}, respectively.[14] This analysis assumes that aminolysis rate is equal to $k_{am} \cdot$ [ethanolamine], as observed with small succinimide esters.[15] Where indicated, a relative rate of aminolysis (k_{rel}) was computed by dividing the aminolysis rate with the hydrolysis rate: $k_{rel} = (k_{am} \cdot [\text{ethanolamine}])/k_{hyd}$.

Table I Synthesized Polymers and Their Characteristics

Polymer	No.	Monomer Feed Ratio	Solvent	Composition by Spectroscopy	Composition by NMR	*MW* (kD)	CPT (°C)		
							Water	Buffer	Serum
NiPAM	1	100	THF	—	100	26.6	31	29	28
NiPAM/NASI	2	95 : 5	THF	95.7 : 4.3	94.4 : 5.6	10.4	29	29	31
NiPAM/NASI	3	97 : 3	THF	97.2 : 2.8	96.2 : 3.8	7.1	29	29	31
NiPAM/NASI	4	99 : 1	THF	98.2 : 1.8	97.8 : 2.2	9.1	30	29	30
NiPAM/NASI	5	95 : 5	Benzene : THF	96.2 : 3.8	94.5 : 5.5	17.9	29	28	28
NiPAM/NASI	6	97 : 3	Benzene : THF	97.8 : 2.2	96.1 : 3.9	20.5	30	28	29
NiPAM/NASI	7	99 : 1	Benzene : THF	98.9 : 1.1	97.8 : 2.2	19.0	31	29	29
NiPAM/NASI*	8	95 : 5	THF	95.0 : 5.0	93.0 : 7.0	—	24	23	31

*: NASI monomer synthesized in-house and used for protein conjugation.

Protein Conjugation

The conjugation studies were carried out using NiPAM/NASI polymer #8 (#8, Table I) and the proteins α-lactalbumin, albumin, and IgG. The proteins were chosen because of (i) the presence of numerous lysines (11, 59, and 90 per α-lactalbumin, albumin, and IgG, respectively), (ii) their size range (14.2, 66, and 150 kD for α-lactalbumin, albumin, and IgG, respectively), and (iii) relatively similar isolectric point (pI: 4.5, 4.7, and 6.6 for α-lactalbumin, albumin, and IgG, respectively). The polymer was dissolved in a buffer at a desired concentration and mixed with a protein solution in the same buffer to give a final protein concentration of 0.5 mg/mL. After incubating for a specific period of time, an aliquot of reaction solution was diluted with electrophoresis buffer [62.5 mM Tris–HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 0.05% (w/v) bromphenol blue] and directly loaded onto SDS-PAGE gels. The acrylamide/*N′N*-bis-methylene-acrylamide concentration in the separation gel was 15%, 7.5%, and 4% for α-lactalbumin, albumin, and IgG, respectively. The stacking concentration was 4%. The proteins were detected by Coumassie R-250 staining. A qualitative scoring system was used to assess conjugation efficiency: (1) −, a lane (containing protein and polymer) in which protein migration was identical to native protein; (2) ±, a lane in which the majority of the protein migrated at the control protein band and a small fraction of higher MW species was present; (3) +, a lane in which the majority of the protein was in the higher MW region, but with a small fraction at the native MW band; and (4) ++, a lane in which the protein migrated at a higher MW with no native protein band.

RESULTS

Synthesis and Characterization of NASI Monomer

The preparation of NASI, the final yield and proton NMR (in $CDCL_3$: 6.0–7.0 ppm for $C\underline{H}_2{=}C\underline{H}$ and 2.85 ppm for $C\underline{H}_2{-}C\underline{H}_2$) were consistent with a previous report.[12] Aminolysis and hydrolysis rates for the NASI monomer are shown in Figure 1. Between 100 and 1000 μM NASI, a concentration-independent but pH-dependent hydrolysis rate was observed. At 10 mM ethanolamine (10–100-fold molar excess to NASI), the

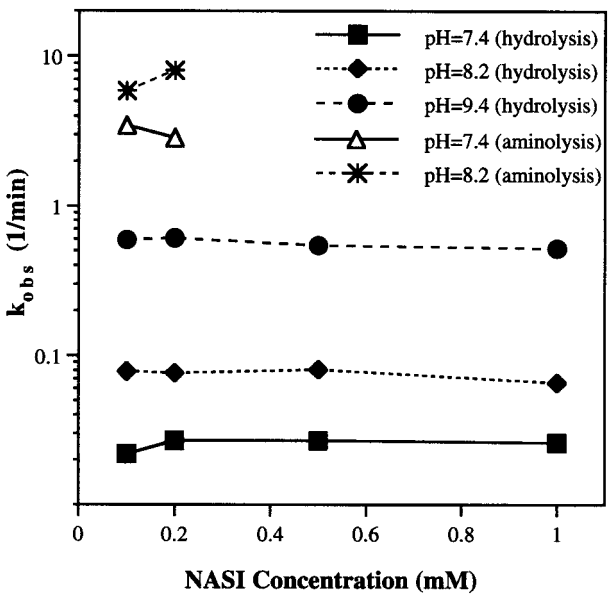

Figure 1 Hydrolysis and aminolysis rates (at 10 mM ethanolamine) for NASI monomer. Note that the hydrolysis and aminolysis kinetics were independent of the monomer concentration in the range tested.

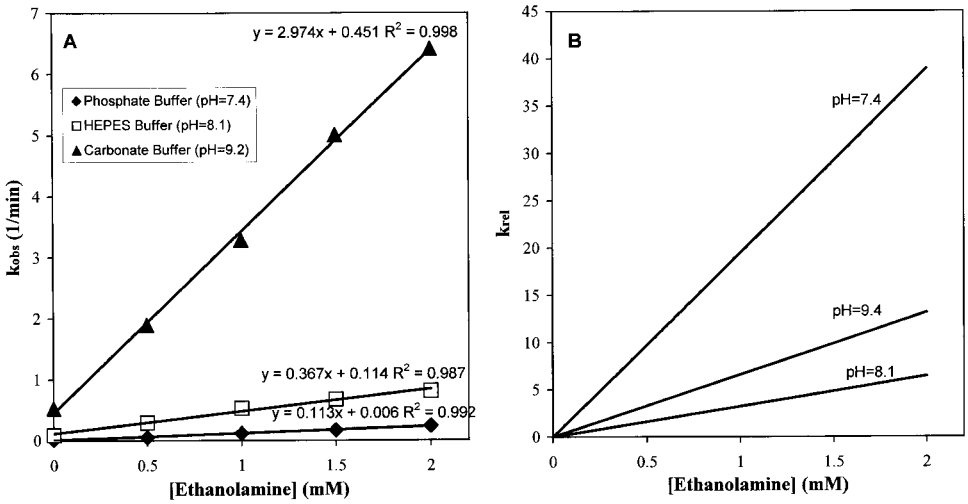

Figure 2 NASI aminolysis as a function of [ethanolamine]. (A) k_{obs} versus [ethanolamine]. (B) Relative reactivity (k_{rel}) versus [ethanolamine]. k_{rel} was obtaining by dividing the aminolysis rate at a particular [ethanolamine] by the hydrolysis rate of the polymer.

NHS release rate was approximately two orders of magnitude higher than the hydrolysis rate. Aminolysis at NASI concentration $> 200\ \mu M$ and pH > 8.1 could not be accurately determined because of the exceptionally rapid kinetics. To determine k_{hyd} and k_{am}, NASI (100 μM) was reacted with ≤ 2 mM ethanolamine, and k_{obs} was found to be a linear function of [ethanolamine] (Fig. 2A). k_{hyd} ranged from 0.006 to 0.451 min^{-1} and k_{am} from 0.113 to 2.974 min^{-1} mM^{-1}. The relative aminolysis rate, k_{rel} was the highest at pH 7.4, followed by pH 9.4 and pH 8.1 (Fig. 2B).

Polymer Properties

The utilized polymerization process was able to provide us with functional polymers. We have chosen to use a photosensitive initiator to carry out the reaction at the ambient temperature, using relatively short reaction times ($\sim$ 40 min) compared to commonly used azobisisobutyronitrile (AIBN)-initiated reactions (24 h). Two types of polymerization solvents, THF and benzene/THF (95 : 5%), were used in this study. Pure benzene was not used because of the nonsolubility of NASI in benzene. Even with 95 : 5% benzene : THF, yellow particles were visible during the initial stages of polymerization, after which the particles slowly dissolved. Diethyl ether used to precipitate the polymers is expected to effectively remove any unreacted initiator, since 2,2-dimethoxy-2-phenyl-acetophenone was readily soluble in ether. The NASI content of NiPAM/NASI was determined by two independent methods (Table I). Both proton NMR and photometric NHS assay indicated a NASI content that was proportional to the monomer feed ratio. NMR-based quantification appeared to give a higher NASI content than the photometric assay. The nature of polymerization solvent (THF versus benzene/THF) did not affect the obtained compositions. The polymer MW was determined using monodisperse polystyrene standards. The NiPAM homopolymer and NiPAM/NASI copolymers eluted as a single peak. The NiPAM homopolymer had a MW of 26.6 kD. With THF as the solvent, NiPAM/NASI polymers were between 7.1 to 10.4 kDa, and with THF/benzene, the MW was between 17.9 and 20.5 kDa. The NASI feed ratio (between 1% and 5%) did not affect the final polymer MW.

As expected, NiPAM homopolymer underwent a phase transition as a function of temperature (Table I) with a CPT of 28–31°C. The CPT of NiPAM/NASI copolymers was not affected by the % NASI in the range obtained in this study. An exception was the polymer that was prepared by in-house synthesized NASI (#8, also the highest percentage of NASI). This polymer exhibited a lower CPT in water and phosphate buffer, but the CPT in serum was similar to the CPT of other polymers.

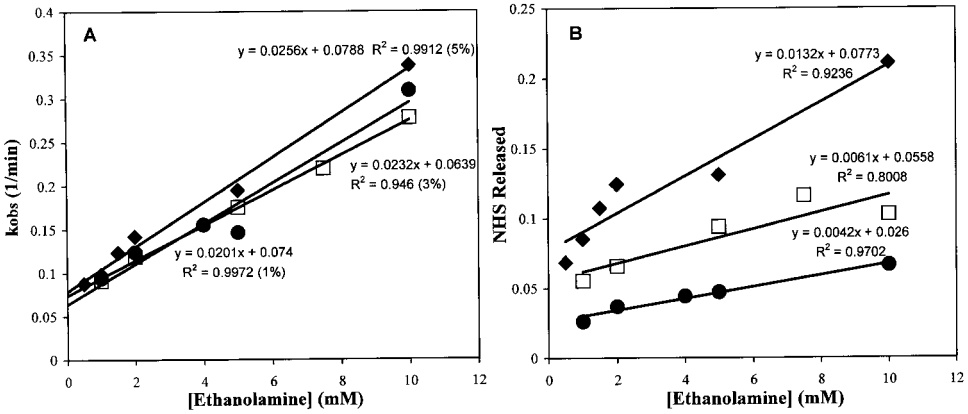

Figure 3 Aminolysis at pH 9.4 using NiPAM/NASI polymers with, 1%, 3%, and 5% NASI feed ratio (diamonds, circles and triangles, respectively). (A) k_{obs} versus [ethanolamine]. (B) Total NHS released versus [ethanolamine]. The total NHS released was calculated from the best-fit obtained for the k_{obs}.

Polymer Hydrolysis and Aminolysis

Initial studies to determine the polymer k_{hyd} without ethanolamine indicated a slow rate of NHS release that was erratic and could not be analyzed by first order equation (not shown). We have alternatively determined the aminolysis rate and obtained k_{hyd} by extrapolation to zero [ethanolamine]. There was no effect of polymer concentration on aminolysis rate (not shown) so that all polymer concentrations were kept at 100 μM NASI equivalent. At pH 9.4 (Fig. 3A), k_{am} was similar for 1–5% NASI (polymers 2, 3 and 4): 0.026, 0.020 and 0.023 min^{-1} mM^{-1}, respectively. k_{hyd} was also similar and ranged from 0.064 to 0.079 min^{-1}. The obtained k_{hyd} and k_{am} were approximately 6- and 120-fold less than the rates for the monomer. The total NHS released, $A_\infty - A_0$, was calculated from the first-order fit for k_{obs}, and, as expected, the amount of released NHS was proportional to the NASI mole content of the polymers (Fig. 3B). However, NHS release from an individual polymer was dependent on the [ethanolamine]. Two- to threefold more NHS was released at [ethanolamine] of 10 mM compared to the 1 mM concentration. As with NASI monomer, k_{hyd} and k_{am} was pH-dependent (Fig. 4A), k_{hyd} ranged from 0.003 to 1.199 min^{-1} between pH 7.4 and pH 10.8 and k_{am} from 0.002 to 0.841 min^{-1} mM^{-1}. The k_{rel} ranged from 0.2 to 5 between 1 mM and 10 mM ethanolamine (Fig. 4B), the highest being at pH 7.4.

Protein Conjugation

Incubating proteins with NiPAM homopolymer produced a protein migration identical to protein migration alone, indicating no association of base homopolymer with the proteins under SDS-PAGE conditions. The effect of polymer concentration on conjugation efficiency was then studied at a constant protein concentration of 0.5 mg/mL. For all proteins, consistent conjugation obtained for polymer concentration was $\geq$ 5 mg/mL, with no reaction occurring at $\leq$ 1 mg/mL (not shown). The effects of incubation time and pH on protein conjugation were summarized in Table II. For all proteins, successful conjugation was obtained between pH 7.4 and 9.2, but not at pH 10.8. For albumin, a prolonged reaction time (>8 h) was necessary to obtain a relatively efficient conjugation (i.e., to obtain a score of +), whereas α-lactalbumin and IgG readily formed conjugates as early as 1 h. The conjugation efficiency for all proteins appeared to be higher at the pH of 7.4 and 8.1 as compared to pH of 9.4. To determine the effect of temperature on conjugation efficiency, the conjugation reaction was carried out at 4°C, 22°C, and 37°C (Table III). The polymer solution gelled at the latter temperature but, upon incubating at room temperature, a solution suitable for SDS-PAGE analysis was obtained. As before, a better conjugation was obtained at a higher polymer concentration. Conjugation at 4°C appear to be less efficient compared to higher temperatures, and little difference was seen between 22°C and 37°C (except for IgG, which produced less conjugation at 37°C).

DISCUSSION AND CONCLUSIONS

The present study was carried out to characterize the reactivity of polymers containing succinimide

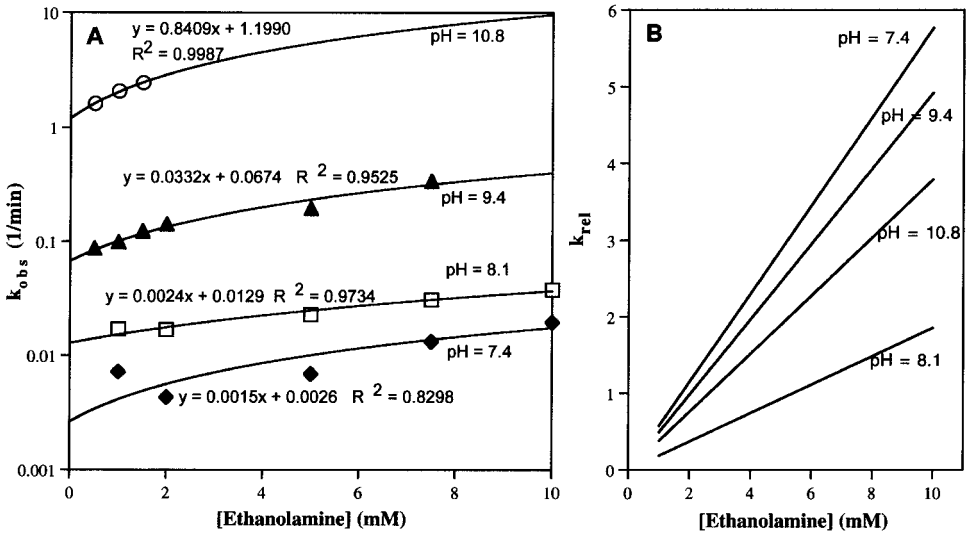

Figure 4 Aminolysis of NiPAM/NASI polymer (#8) as a function of pH. (A) k_{obs} versus [ethanolamine]. (B) Relative reactivity (k_{rel}) versus [ethanolamine]. k_{am} and k_{hyd} was increased at higher pH values but the highest k_{rel} was at pH 7.4.

esters. Such polymers can couple proteins without the need for additional crosslinking agents. The succinimide ester content of the polymers were restricted to < 5% (based on feed ratio), since a small fraction of NASI groups was considered sufficient for protein conjugation. Additionally, we wanted to minimize any changes in the CPT of NASI-containing polymers from that of NiPAM homopolymer. A photopolymerization scheme was used because of the short reaction time necessary to obtain the polymers. The feasibility of NiPAM polymerization by photoinitiators was previously demonstrated,[16] but the reported methodology was for the preparation of three-dimensional NiPAM gels in an aqueous environment (using $H_2O_2/K_2S_2O_8$ as initiators). We used an organic solvent-based polymerization so that NASI could be incorporated into the polymers without hydrolysis. The concentration of the photoinitiator was relatively high (5% w/v) compared to the conventional free radical generator AIBN.[9] Polymerization attempts with lower initiator concentrations were not successful.

Polymer Properties

A desired characteristic of any free radical polymerization process is the ability to control polymer composition by the monomer feed ratio. For monomers with the same reactivity ratios, the final polymer composition is identical to the monomer feed ratios. This enables one to control the polymer composition simply by the adjusting the relative ratios of monomers added to a polymerization solution. For the polymers prepared for this study, the feasibility of controlling final

Table II Conjugation Efficiency as a Function of Reaction Time and pH

Reaction Time	pH 7.4			pH 8.1			pH 9.2			pH 10.8		
	Lactalbumin	Albumin	IgG	Lactalbumin	Albumin	IgG	Lactalbumin	Albumin	IgG	Lactalbumin	Albumin	IgG
24 h	++	+	++	++	+	++	±	±	+	−	−	−
8 h	++	±	++	++	+	++	+	±	+	−	−	−
4 h	+	±	++	+	±	++	±	±	+	−	−	−
2 h	++	±	+	±	±	+	+	±	+	−	−	−
1 h	+	±	++	±	±	X	+	±	+	−	−	−
0.5 h	±	±	+	±	±	+	+	±	+	−	−	−

Polymer (#8) = 10 mg/mL, protein = 0.5 mg/mL. X, unreadable lane.

Table III Conjugation Efficiency at Different Temperatures

Polymer (#8) Concentration	Lactalbumin			Albumin			IgG		
	4°C	22°C	37°C	4°C	22°C	37°C	4°C	22°C	37°C
20 mg/mL	+	++	++	±	+	+	++	++	+
10 mg/mL	±	+	+	±	+	+	+	++	+
5 mg/mL	−	+	+	−	±	±	±	++	+
1 mg/mL	−	−	−	−	−	±	−	±	−

Time = 6 h, pH 7.4.

polymer composition by the monomer feed ratios was demonstrated. The NASI composition of polymers was similar to the initial feed ratio in NiPAM/NASI copolymers. Others also have obtained a similar reactivity between NASI and acrylamide[12] and NASI and NiPAM,[17] using the AIBN-initiated polymerization. We have additionally prepared terpolymers of NiPAM, NASI, and methyl methacrylate and the composition of the terpolymers also was effectively controlled by the monomer feed ratio (not shown).

Two types of polymerization solvents were used because of the earlier literature, which indicated a distinct difference in the MW of final products (using AIBN as the initiator). THF, being an efficient chain-transfer agent, was shown to yield low-molecular-weight polymers, whereas benzene gave high-molecular-weight polymers.[9] As expected, the MW of the NiPAM/NASI synthesized in THF was relatively low (~ 10 kD), but the use of benzene/THF mixture did not increase the polymer MW (only a 2-fold increase was seen, whereas Chen et al.[9] obtained a 50-fold increase). The high concentration of photoinitiator might be the reason behind this observation.

The CPT of our NiPAM homopolymer (~ 30°C) was in agreement with previously reported values of 30–32°C by several other investigators.[17–21] The CPT of NASI-containing NiPAM was not different from the homopolymer since polymer NASI content was kept to a minimum. We anticipate insignificant NASI hydrolysis in aqueous buffers during LCST measurements since an introduction of carboxyl groups (i.e., NASI hydrolysis product) would have changed the CPT drastically (up to a 10°C with 5% NASI hydrolysis.[19] In serum, however, we anticipate a significant fraction of NASI groups to be reacting with serum components (both small molecules and proteins), but this did not result in a significant CPT change. This is consistent with previous work, which indicated no changes in CPT as a result of protein conjugation[17] but inconsistent with results that indicated CPT changes as a result of charge introduction to NiPAM backbone.[19] A more detailed study investigating the exact components of serum conjugating to polymers might help to resolve this issue.

Polymer Reactivity

Hydrolysis of succinimide esters in an aqueous environment will compete with protein conjugation to decrease the effectiveness of protein coupling. As shown for NASI monomer here, succinimide[22] and sulfosuccinimide esters[23] of small organic entities undergo accelerated hydrolysis at higher pHs. The relatively short half-life of NASI ester bond ($=1/k_{obs}$, ranged from ~ 166 min at pH 7.4 to ~ 2 min at pH 9.4 for the monomer) makes aqueous polymerization not practical for our purposes. NASI hydrolysis in polymers was also pH-dependent, but it was significantly reduced, possibly because of aggregation of a relatively hydrophobic succinimide ring, which can exclude water molecules.

Small molecular esters of NHS are readily reactive with amine-containing reagents, with a reactivity higher than hydrolysis rate (see Fig. 1B). In the absence of hydrolysis (i.e., in organic solvents)[24] and in aqueous environment,[15] the reaction rate is directly correlated with the basicity (pK_b) of the reacting species. For some substrates, steric constraints on amine moiety can play a significant role, lowering aminolysis rates by as much as an order of magnitude and even higher.[15] No information is available about the role of species attached to leaving NHS group. In our case, a polymer attached to the succinimide provides a considerable hindrance for aminolysis (compare

the k_{am} for NASI monomer and polymerized NASI). The aminolysis rate is expected to be dependent on the reacting amino compound, but this issue has not been explored in detail in this study. Our observation that polymer NASI units had a more pronounced decrease in aminolysis rate (compared to hydrolysis) is consistent with the larger size of ethanolamine compared to water (i.e., ethanolamine will experience a higher steric hindrance). The size of the proteins, however, did not seem to affect the conjugation since the smallest (α-lactalbumin: 14.2 kD) and the largest protein (IgG: 150 kD) reacted to the same extent with the NASI polymers.

As expected, the polymer underwent an increased aminolysis rate at higher pHs. However, the relative reactivity of NiPAM/NASI (using ethanolamine as the model amine compound) was highest at pH 7.4 and, consistent with this, the protein reactivity also was better at this pH. The HEPES buffer at pH 8.1, on the other hand, had a low k_{rel} but was effective in polymer conjugation. This is in contrast to carbonate buffer at pH 10.8, where a high k_{rel} was obtained without any protein conjugation. One would expect a better protein reactivity at a higher pH due to a lower degree of lysine protonation.[17] Since no reaction was obtained for all three proteins at the highest pH, it is likely that a polymer-related phenomena, such as rapid hydrolysis of succinimide esters, might be the underlying reason for no reaction. Ideally, one would determine the reaction constant of the polymers with proteins using the same set-up used for ethanolamine reactivity. However, the presence of proteins did not allow us to quantitate NHS release at 260 nm (due to high Abs. background). A new technique that will separate the released NHS from polymer/protein mixture (such as an HPLC method) will be needed to quantify NHS release and calculate the "true" protein reactivity.

The protein–polymer conjugates so prepared had a broad *MW* range, as indicated by a long "tail" on SDS-PAGE gels. This heterogeneity is likely to stem from (i) polydispersity of the prepared polymers (polydispersity > 2 is expected but was not quantitated in this study) and (ii) different conjugation amount (i.e., different no of polymer chains conjugated per protein). The relative contribution of the above two factors to overall heterogeneity has not been determined and is the subject of future studies. The LCST of the protein conjugates also was not determined, but it is expected to be similar to CPT of the polymers.[17,20] It will also be important to compare the reactivity of NASI-containing polymers with polymers containing other reactive groups. Based on qualitative observations from the literature, the reactivity of NASI polymers appear to be similar to that of maleic acid polymers, but faster than epoxide polymers, which had to be incubated for 72 h for an efficient conjugation.[2]

In conclusion, the results of the present study indicate that, compared to NASI monomer, a polymerized NASI exhibits a significant reduction in hydrolysis rate and especially the aminolysis rate. Although both hydrolysis and aminolysis rates were higher at a higher pH, the relative aminolysis rate was found to be the highest at pH 7.4. The protein conjugation was accordingly better at this pH and diminished at the higher pHs for all proteins tested. It is likely that one will desire to engineer the properties of NASI-containing polymers for particular applications (e.g., to manipulate polymer CPT). The methodology outlined in this study will be beneficial to design novel polymers for protein conjugation and elucidate the role of different monomer units on the polymer reactivity.

This study was supported by a special grant provided to H. Uludag by University of Alberta. Expert technical assistance of Mr. Japheth Noah is appreciated.

REFERENCES

1. Ding, Z.; Chen, G.; Hoffman, A. S. J Biomed Mater Res 1988, 39, 498.
2. Hoshino, K.; Taniguchi, M.; Kawaberi, H.; Takeda, Y.; Morohashi, S.; Sasakura, T. J Ferm Bioeng 1997, 83, 246.
3. Chen, J. P.; Hoffman, A. S. Biomater 1990, 11, 631.
4. Hoffman, A. S. Art Org 1992, 19, 458.
5. Yang, Z.; Williams, D.; Russell, A. J. Biotech Bioeng 1995, 45, 10.
6. Kodera, Y.; Tanaka, H.; Matsushima, A.; Inada, Y. Biochem Biophys Res Comm 1992, 184, 144.
7. Kaneda, Y.; Yamamoto, Y.; Tsunoda, S.-I.; Kamada, H.; Tstsumi, Y.; Hirano, T.; Mayumi, T. Biochem Biophys Res Comm 1997, 239, 160.
8. Yoshioka, H.; Mikami, M.; Mori, Y.; Tsuchida, E. J Macromol Sci-Pure Appl Chem 1994, 31, 113.
9. Cole, C-A.; Schreiner, S. M.; Priest, J. H.; Monji, N.; Hoffman, A. S. in Reversible Polymeric Gels and Related Systems; Russo, P., Ed.; American Chemical Society: Washington, 1987, p 245.
10. Stayton, P. S.; Shimoboji, T.; Long, C.; Chilkoti, A.; Chen, G.; Harris, J. M.; Hoffman, A. S. Nature 1995, 378, 472.

11. Stayton, P. S.; Fisher, M.; Sligar, S. G. J Biol Chem 1988, 267, 13544.
12. Pollack, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. J Am Chem Soc 1980, 102, 6324.
13. Miron, T.; Wilchek, M. Anal Biochem 1982, 126, 433.
14. Jencks, W. P. Catalysis in Chemistry and Enzymology; McGraw Hill: New York, 1969, p 568.
15. Cline, G. W.; Hanna, S. B. J Org Chem 1988, 53, 3583.
16. Kubota, H.; Fukuda, A. J Appl Polym Sci 1997, 65, 1313.
17. Chen, J. P.; Yang, H. J.; Hoffman, A. S. Biomat 1991, 11, 625.
18. Schild, H. G. in Water-Soluble Polymers; Shalaby, S. W.; McCormick, C. L.; Butler, G., Eds., American Chemical Society: Miami Beach, 1991, p 249.
19. Feil, H.; Bae, Y. H.; Feijen, J.; Kim, S. W. Macromolecules 1993, 26, 2496.
20. Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. Bioconj Chem 1993, 4, 42.
21. Heskins, M.; Guillet, J. E. J Macromol Sci Chem 1968, 1441.
22. Schnaar, R. L.; Lee, Y. C. Biochemistry 1975, 14, 1535.
23. Anjaneyulu, P. S. R.; Staros, J. V. Int J Pept Prot Res 1987, 117.
24. Cline, G. W.; Hanna, S. B. J Am Chem Soc 1987, 109, 3087.