Synthesis and Characterization of Poly(*N*-vinyl pyrrolidone-*alt*-maleic anhydride): Conjugation with Bovine Serum Albumin

L. VERON, M. REVOL, B. MANDRAND, T. DELAIR

Unité Mixte CNRS—bioMérieux, UMR 103, ENS—Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France

Received 13 July 2000; accepted 11 November 2000

ABSTRACT: Alternate *N*-vinyl pyrrolidone/maleic anhydride (NVPMA) copolymers were obtained by radical solution polymerization in dioxane with various MA contents in the monomer feed. The conversion of each monomer was monitored by proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and the kinetics investigation showed that both monomers had identical polymerization rates if both monomers were present in the reaction mixture. The presence of excess NVP in the polymerization medium increased the kinetics of the polymerization and the molar masses of the resulting polymers. This increase was attributed to a cosolvent effect due to NVP, which is a better solvent for the polymer than dioxane. The hydrolysis rate constant of the polymers increased with pH, and NVPMA copolymers were more prone to hydrolysis (by a factor 10) than the methyl vinyl ether ones. Finally, the immobilization of bovin serum albumin (BSA) was investigated. A 25 m*M* phosphate buffer (pH 5.5) was the best medium to covalently bind 5 BSA molecules onto a 29 kDa NVPMA copolymer and 13 BSA molecules onto a 58 kDa sample, with grafting efficiencies $> 90\%$. Noncovalent interactions with the hydrolyzed form of the polymer and BSA occured at pHs lower than the isoelectric point of BSA, and the resulting complexes were insoluble in water.
 J Appl Polym Sci 81: 3327–3337, 2001

Key words: bovine serum albumin; poly(*N*-vinyl pyrrolidone-*alt*-maleic anhydride); kinetics by ^{1}H NMR; charge transfer complex radical polymerization; light scattering

INTRODUCTION

Conjugation of proteins to polymers has many applications, in particular in diagnostics in which better efficiency of immobilization is required. In a preceding work we demonstrated that the covalent immobilization of recombinant HIV-1 core protein P24 could take place in an aqueous medium in very high yields. High grafting efficiencies were obtained in low ionic strength buffers with a recombinant protein, RH24K, bearing a six-lysine residue tag at the amino terminus. In a continuing effort to improve the quality of polymer–protein conjugates, we envisioned using *N*-vinyl pyrrolidone (NVP)-based maleic copolymers because poly(NVP) is known to be quite biocompatible and to improve adhesion.[1]

Alternating copolymers of NVP and maleic anhydride (MA) have already been obtained in benzene by various authors[2–4] or in tetrahydrofuran (THF).[5] To our knowledge, in most of the works just cited, characterizations were incomplete in terms of yields, molar mass, polymerization kinetics, and rate of hydrolysis in aqueous media. The latter is of paramount importance for use the polymers for the covalent immobilization of pro-

Correspondence to: T. Delair (Thierry.delair@ens-lyon.fr).
Journal of Applied Polymer Science, Vol. 81, 3327–3337 (2001)

teins in aqueous buffers. Furthermore, in our hands, some experimental conditions failed, in particular when trying to dissolve the monomers in THF at a molar concentration of 2.

In this paper we report the synthesis of (*N*-vinyl pyrrolidone-*alt*-maleic anhydride) copolymers and their chemical and physicochemical characterizations. The covalent and noncovalent interactions of the synthesized polymers with bovine serum albumin (BSA) as a model protein are also described.

EXPERIMENTAL

Chemicals

Maleic anhydride (49.5 g) was recrystallized from chloroform (300 mL), with a yield of 61% after drying under vacuum. *N*-Vinyl pyrrolidone was distilled under reduced pressure in the presence of hydroquinone as inhibitor. The monomers were store at +4°C. Azobis isobutyronitrile (2.5 g) was recrystallized from ethanol (450 mL). After 48 h at −20°C, the product was filtered, and the recrystallization yield was 38.4%. Dioxane was distilled over aluminium hydride and stored under nitrogen before use.

Proteins

Bovine serum albumin (BSA; 67,000 g/mol) was used as received from Sigma. The isoelectric point of BSA is close to pH 4.9, and it is constituted of a single polypeptide chain containing 584 amino acid residues. Its ellipsoidal shape had a diameter of 38 Å and a length of 150 Å.[6] According to the manufacturer, the water content was 5.8%, nitrogen content 14.6%, and solubility in water 50 g $\cdot$ L^{-1}.

Polymer Synthesis

Polymerization reactions were run in anhydrous dioxane. The reaction mixture was deaerated under nitrogen and warmed at 60°C, and then a dioxane solution of initiator (AIBN) was added so that the amount of initiator corresponded to 1% molar (referring to the total amount of monomers). After cooling the medium, the polymer was precipitated in diethyl ether and dried under vacuum.

Polymerization Kinetics

Kinetics experiments were run as described at a total monomer concentration of 2 mol $\cdot$ L^{-1} in anhydrous dioxane, in the presence of trioxane as an internal reference for monitoring by proton nuclear magnetic resonance spectroscopy (^{1}H NMR). Aliquots (0.3 mL) were taken via a syringe, and the polymerization was stopped with hydroquinone and by immersion of the samples in ice cold water. Then, 0.3 mL of deuterated dimethylsulfoxide (DMSO-d_6) was added to the aliquots for ^{1}H NMR analysis (see Figure 1).

Polymer Hydrolysis for Average Molar Mass Determination

To analyse the molar mass distribution, the polymers were hydrolyzed prior to injection in the size exclusion chromatography (SEC) columns. One-hundred milligrams of sample were dissolved into 20 mL of bi-distilled water and then hydrolyzed by adding two equivalents of sodium hydroxide per anhydride. The resulting solution was diluted with 80 mL of 50 m*M* borate buffer to give a polymer solution of 1 mg $\cdot$ mL^{-1}. The pH of the final solution was $\sim$ 10, which gave an equilibrium between the acid and carboxylate forms of the anhydride units. Therefore, the polymer composition was estimated, using the titration curves, as being comprised of $\sim$ 43% COONa moles and 7% COOH moles. The molar mass of a single repeat unit was calculated as followed:

$$M = (0.5 \times 111) + (0.43 \times 160) + (0.07 \times 116) = 132 \text{ g·mol}^{-1} \quad (1)$$

From the number average molar mass, M_n, obtained experimentally by SEC on line with a multiangle light scattering system (SEC-MALS), we calculated the polymerization degree of polymers (DP_n) with the following expression:

$$DP_n = 2 \times M_n \text{ hydrolyzed}/132 \quad (2)$$

The M_n value of the nonhydrolyzed polymer was

$$M_{n\,\text{nonhydrolyzed}} = DP_n \times 104.6 \quad (3)$$

Hydrolysis Kinetics

We used the following procedure: The polymer was dissolved into 1 mL of dimethylsulfoxide, and then this solution was poured onto 19 mL of a stirred buffer solution of known pH. The decrease of pH with time was followed by a pH-metric method. The concentration range of the polymer

in the medium was 1–8 mg · mL^{-1}. Two polymers, of 20 kDa M_n, were used for this work. Phosphate and borate buffers were prepared to cover the pH range from 5.6 to 10.

Polymer Titration

Polymer (100 mg) was dissolved in 10 mL of milli Q grade water (Millipore) at 67°C and allowed to stay at this temperature for 30 min. After cooling to room temperature, the polymer solution was titrated with a 0.1*M* sodium hydroxide solution (Merck) using a Minisis 800 pH-meter (Tacussel) fitted with a Xerolyt electrode (Ingold).

Molar Mass Distribution Analysis

Hydrolyzed polymer samples were lyophilized and then dissolved in 0.1 N borate buffer (pH 10). The molar mass distribution was determined by SEC-MALS. The SEC setup consisted of two columns, Ultra Hydrogel 500 and 2000 (Waters), and a high-performance liquid chromatography (HPLC) pump (Waters 510), with 0.1*M* borate buffer (pH 10) as a mobile phase (flow rate of 0.5 mL · min^{-1}). The refractive index increment of the polymers in the buffer, determined with a Brice-Phoenix differential refractometer equipped with a 633-nm wavelength laser, was 0.175 mL · g^{-1}, taking into account that after lyophilization, the polymers contained 15% water in weight. The apparatus was calibrated with aqueous KCl solutions as standards. For the detection part, a Waters 484 UV absorbance detector, a Waters 410 differential refractometer, and a three-angle MINI DAWN F detector (Wyatt Technology) at 690 nm were used on-line.

NMR

^{1}H NMR spectra were recorded at 27°C on a Bruker AC 200 spectrometer working at 200 MHz or a Varian Unity-*plus* operating at 500 MHz; tetramethylsilane (TMS) was used as internal standard (δ = 0 ppm).

^{13}C NMR spectroscopy was carried out at 27°C with a Bruker AC200 apparatus working at 50.3 MHz. In the organic solvent (DMSO-d_6), TMS was used as internal standard (δ = 0 ppm) and in the aqueous solvent (deuterated water), TMS was replaced with trimethyl silyl-3 propionic acid (δ = −2.35 ppm).

Elemental Analysis

Elemental analyses were performed at Service Central d'Analyses (CNRS—Vernaison).

Chemical Coupling of Proteins to Copolymers

The appropriate amounts of polymers were dissolved in anhydrous dimethylsulfoxide (DMSO) at 37°C. To a protein buffer solution at the appropriate concentrations was added the required volume of polymer solution in DMSO. The reaction mixture was stirred for 3 h at 37°C. The pH of the buffers was measured and monitored with an Ingold HA405-DXK-58/120 electrode and a Minisis 8000 pH-meter (Taccussel-Radiometer, France).

Analyses of the Coupling Reactions

The crude coupling mixture was analyzed by SEC with a Waters Ultra-Hydrogel 500 column, a Kontron HPLC 422 pump, a Kontron HPLC autosampler 465, and a Kontron UV diode array detector on line. Purifications were run in a 0.1*M* phosphate buffer (pH 6.8) at a flow rate of 0.5 mL · min^{-1}. Detection was achieved by measuring the absorbance at 280 nm, corresponding to the BSA (at the concentrations used, the polymer did not absorb). The ratio of the peak area corresponding to the polymer-bound BSA *versus* the sum of the two peaks corresponding to the unbound and to the bound BSA (i.e., the total amount of protein involved in the reaction) gave the coupling yield, *Y*. The extinction coefficient of the bound protein was similar to that of the unbound BSA. This result was checked by comparing the areas of the peaks obtained by HPLC, observed at 280 nm, for bound and unbound BSA.

The average amount $\bar{N}$ of protein molecules bound per polymer chain was assessed according to eq. 4:

$$\bar{N} = n \cdot Y/n' \tag{4}$$

where n and n' correspond, respectively, to the number of protein molecules and the number of polymer chains in the reaction mixture. As a first approximation, we assumed that each polymer chain reacted according to the same pattern (that is the case for oligonucleotide immobilization).[7]

Noncovalent Protein/Polymer Interactions

Pure electrostatic interactions between the polymers and the proteins were investigated by mixing an aqueous solution of hydrolyzed polymer with a BSA solution at the respective concentrations of 0.15 and 1 g · L^{-1}, with subsequent addition of 1*M* or 0.1*M* hydrochloric acid solutions to lower the pH. For each pH value, samples were

analyzed by HPLC as already described. The protein was detected by measuring the absorbance at 280 nm, whereas the polymer could only be detected at 220 nm. Blanks containing only BSA or the polymer were run for control.

RESULTS

Free Radical Copolymerization of Maleic Anhydride and N-Vinyl Pyrrolidone

Preliminary experiments showed that at a concentration of 2 mol · L^{-1}, the monomers were not soluble in either benzene or toluene. Dioxane was chosen for its ability to yield clear monomer solutions. At lower monomer concentrations, the polymers obtained had a molar mass too low for the desired applications. At monomer concentration > 2 mol · L^{-1}, solubility problems were very important, in particular during the polymerization.

Polymerization Kinetics

The polymerization kinetics were obtained from the analysis of the ^{1}H NMR spectra, recorded at 500 MHz in the presence of trioxane as internal reference. The conversions of each monomer were deduced from the peak area measurements corresponding to the vinylic protons of MA (H_{MA}) and NVP (H_{NVP}) (singlet at 7.4 ppm, doublet of doublet centered at 6.95 ppm for NVP, see Figure 1), using the following equations:

$$\% \text{ MA}_{(t)} = 1 - \frac{(H_{MA}/H_{Ref})_t}{(H_{MA}/H_{Ref})_{t0}}$$

$$\% \text{ NVP}_{(t)} = 1 - \frac{(H_{NVP}/H_{Ref})_t}{(H_{NVP}/H_{Ref})_{t0}} \quad (5)$$

^{1}H NMR allowed independent monitoring of the consumption with time of both monomers, as seen in Figure 1 for a 24% MA content. Using these representations for various monomer compositions, it was possible to obtain the polymerization rate for each monomer at each composition. Reporting the variation of the polymerization rate versus the maleic anhydride content in the monomer feed (f_a), Figure 2, demonstrated that both monomers have the same polymerization rate over the investigated monomer composition range. Furthermore, as the NVP content increased (decrease of f_a), the polymerization kinetics increased.

NMR

^{1}H and ^{13}C NMR were used to characterize the polymers and, apart from C_7 and C_8, every other carbon atoms were unambiguously attributed (Figure 3 and Table I).

Molar Mass Distribution

The determination of the molar mass distributions was achieved by SEC-MALS using a borate buffer (pH 10) as the mobile phase. The results are reported in Table II.

Interestingly, the M_n increases with decreasing MA content in the monomer feed (Figure 4) in a similar way as the polymerization rate (Figure 2).

Elemental Analyses

The compositions of some copolymers were checked by elemental analysis. Whatever the initial mixture composition, polymer compositions were close to 50/50, as can be seen in Table III. The weight percentages of nitrogen and oxygen for entries B and C (initial compositions of 35/65 and 15/85, respectively) clearly demonstrate the synthesis of alternating copolymers.

Polymer Hydrolysis and Base Titration

The kinetics of hydrolysis of the anhydride groups of the copolymers were investigated at various pHs by monitoring the pH variation with time in different buffers. The hydrolysis rate can be expressed as

$$r_H = k\,[\text{polymer}]^{\alpha} \quad (6)$$

The hydrolysis rate constants and orders of the reaction were determined by plotting ln r_H versus ln[polymer] over the investigated pH range and are reported in Table IV.

The titration of hydrolyzed polymers by 0.1M sodium hydroxide showed two equivalence points (data not shown), as reported by Nagasawa et al.[4] and by Ladavière et al. for (maleic anhydride-alt-methyl vinyl ether) (MAMVE) copolymers.[8] This acid/base titration method was a means of checking the overall amount of MA in the polymers obtained at various monomer feed compositions, as reported in Table V.

Using data acquired by the titration of the hydrolyzed polymers, it was possible to determine, for the first acidity, the pK_{ao} by plotting the

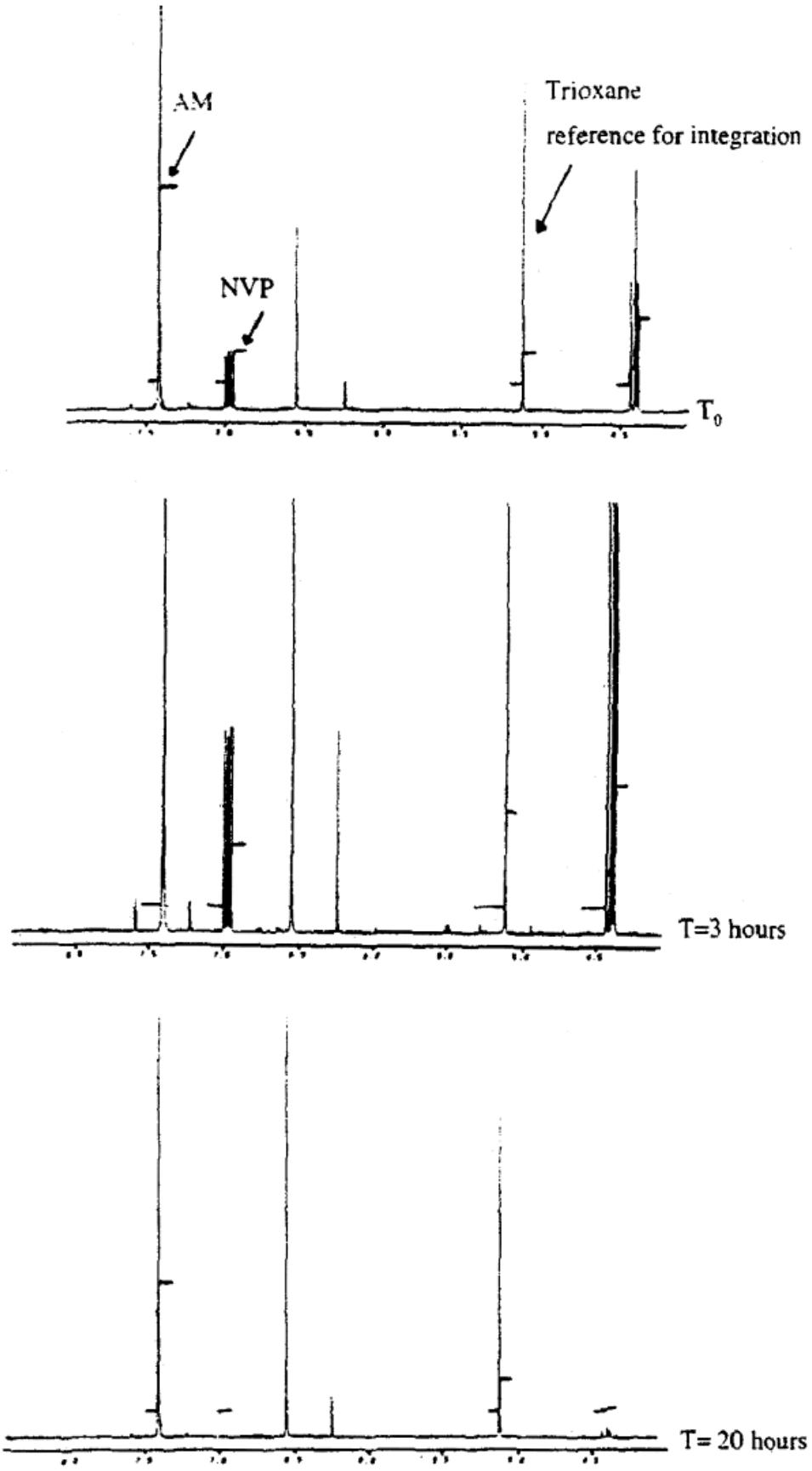

Figure 1 Polymerization kinetic by ^{1}H NMR in DMSO-d_6 for a NVPMA mixture (25/75).

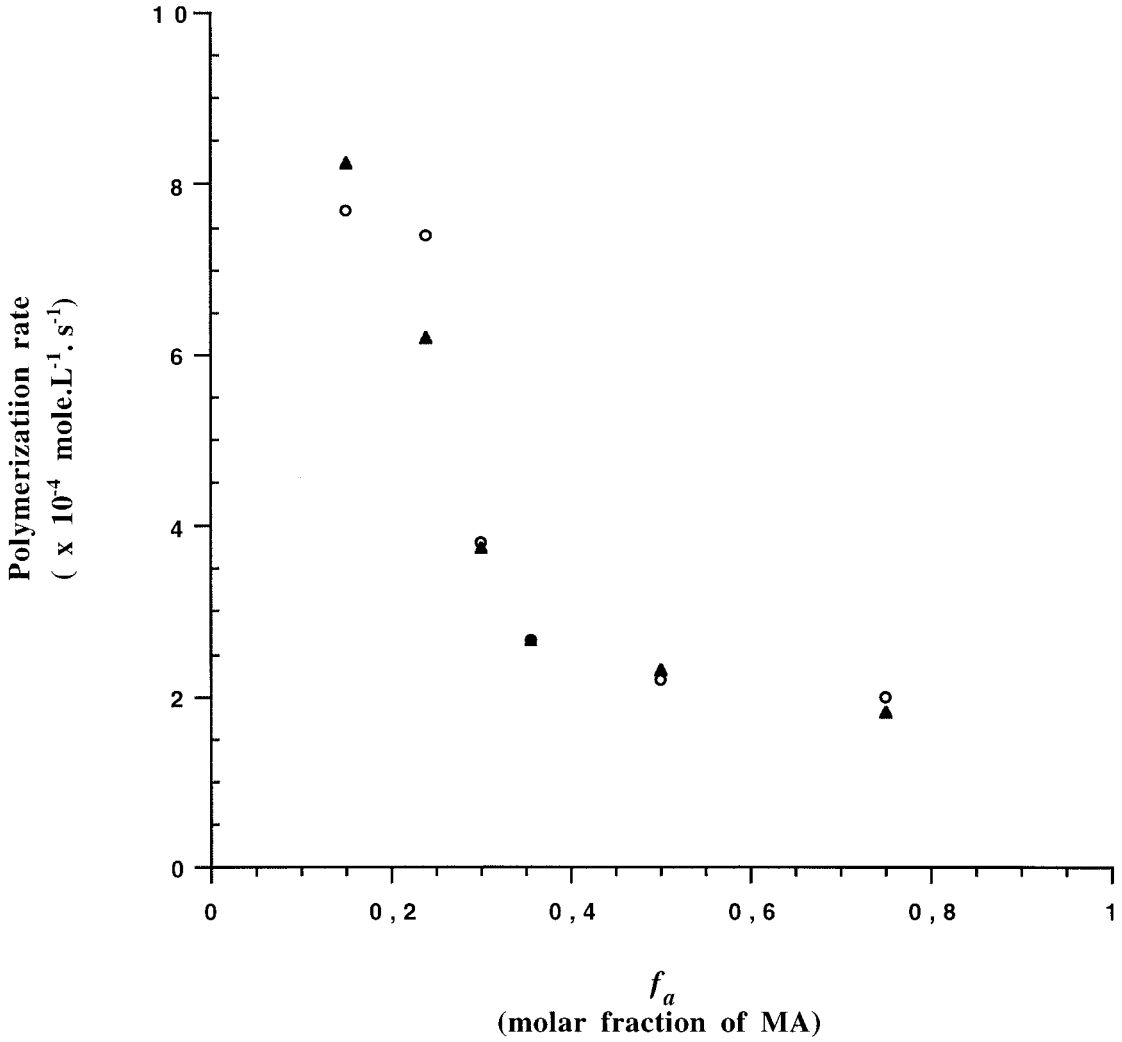

Figure 2 Polymerization rate versus initial molar fraction of MA in the medium. (○) MA; (△) NVP.

variation of pK_a as a function of the dissociation degree α' (i.e., the ionization of the polymer). Extrapolation to $\alpha' = 0$ afforded the value of pK_{a1}

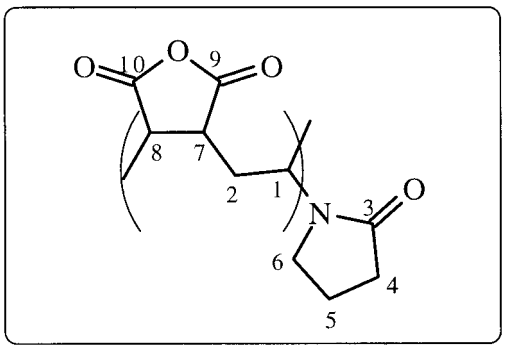

Figure 3 NVPMA formula and ^{13}C labels.

Table I ^{13}C NMR Chemical Shifts[a]

^{13}C	δ (ppm)
1	42.41
2	30.95
3	177.75
4	30.95
5	15.74
6	44.10
7	49–50
8	49–50
9	173.69
10	171.92

[a] In acetone d_6; temperature = 27°C; carbon numbering according to Figure 3.

Table II Molar Mass Distributions[a]

Entry	Code	Initial % AM	M_w (10^3 g · mol^{-1})	M_n (10^3 g · mol^{-1})	Ip[b]	M_n[c] (10^3 g · mol^{-1})	Yield[d] (%)	Time (h)
1	31	15	122	73.3	1.7	58.1	24	1.5
2	33	24	134	73.6	1.8	58.4	55	1.0
3	32	30	129	66.8	1.9	52.9	33	3.0
4	30	35	113	50.5	2.2	40	37	4.0
5	29	50	90.1	34	2.6	26.9	50	3.0
6	28	75	47.8	17.7	2.9	13.2	83	19.0

[a] All polymers were obtained by AIBN (1% molar)-initiated free-radical copolymerization at 60°C in dioxane. Total monomer concentration was 2 mol · L^{-1}. The reactions were stopped prior to NVP homopolymerization for entries 1–5.
[b] Polydispersity index.
[c] Calculated molar mass of the polymer under the polyanhydride form.
[d] Percent yield of precipitated polymer in ether, referring to the theoretical amount of 1/1 alternating copolymer, deduced from the monomer composition.

= 1.6. The second equivalent point could not easily be unambiguously determined.

Protein Covalent Immobilization

Based on results obtained by Ladavière et al. with copolymer MAMVE 1A,[9] BSA was immobilized in a phosphate buffer (pH 5.5) at varying ionic strengths. The immobilization efficiency was increased with decreasing salt concentration, as reported in Figure 5. Therefore, the immobilization isotherms of BSA (Figure 6) were obtained for two polymer samples of different M_n values; that is, 29 and 58 kDa at a salt concentration of 25 m*M*. From Figure 6, the maximal amount of immobilized BSA per polymer chain appears to be proportional to the M_n of the polymer; that is, ~ 5 BSA molecules can be immobilized on a 29 kDa NVPMA sample and 13 on a 58 kDa polymer.

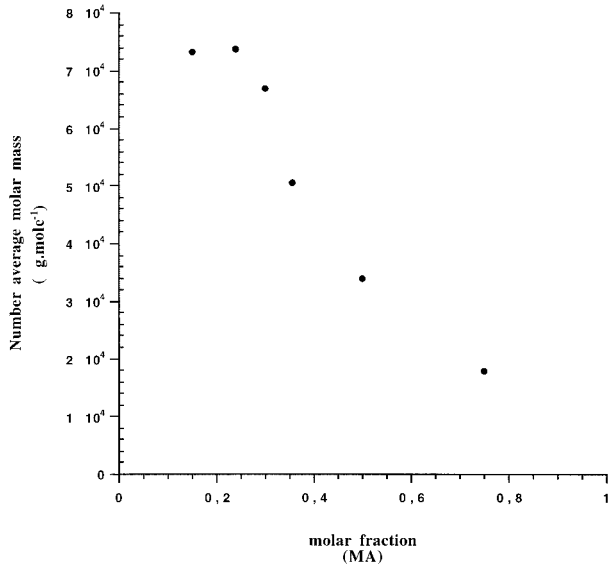

Figure 4 Number average molar masses of the final polymers versus initial molar fraction of MA in the medium.

BSA/Polymer Noncovalent Interactions

To check whether the proteins were effectively bound onto the polymer via covalent interactions, several series of experiments were conducted. First, the anhydride moieties of the polymer were hydrolyzed to the corresponding dicarboxylates, and these samples were mixed with BSA solution under identical experimental conditions as for covalent coupling. In HPLC traces, only the peak corresponding to free BSA could be detected, with no loss of proteic material. Then, in a second set of experiments, the BSA and polymer solutions were mixed and the pH was lowered by successive addition of 1 or 0.1*M* hydrochloric acid. For each pH value, the solution was analyzed by HPLC, and the variations in the amounts of free polymer and free protein were monitored, as described in the *Experimental* section, by integration of the areas of the peaks corresponding to the polymer at 220 nm (~ 13.7-min elution time) and to the protein (at 17.1-min elution time). No disappearance of either the free polymer or the protein was observed until the pH dropped down to 4.55. A precipitate was clearly witnessed visually, and no residual free BSA nor free polymer could be detected by HPLC.

Table III Elemental Analyses for Several Copolymers Obtained from Three Different Initial AM/NVP Mixtures[a]

%	50/50 Theoretical	Feed Composition 50/50	35/65 Theoretical	Feed Composition 35/65	15/85 Theoretical	Feed Composition 15/85
C	**57.41**	56.58	**59.7**	55.92	**62.7**	56.24
H	**5.3**	5.72	**6.2**	5.74	**7.3**	5.90
N	**6.7**	6.34	**8.5**	6.65	**10.9**	6.87
O	**30.59**	31.97	**25.5**	31.93	**19.1**	31.07

[a] The characters in bold print are the theoretical values for copolymers of composition identical to that of the initial mixture.

DISCUSSION

In our hands, dioxane proved the best solvent to allow dissolution of monomers up to 2 mol · L^{-1}. Using these conditions, we investigated the effect of NVP molar fraction in the monomer feed on the initial polymerization rate and the average molar mass. For both monomers, whatever the MA content, the initial polymerization rates were identical, confirming that MA and NVP copolymerized as a 1 : 1 complex (Figure 2).

The increase in polymerization rate with increasing NVP mole fraction, observed in Figure 2, could be related to what Fujimori et al.[10] observed with maleic anhydride and isobutyl vinyl ether. The authors interpreted this result in terms of charge transfer complex concentration, which was the highest close to the ideal 1 : 1 composition, in particular at high total monomer concentration.

In our case, the maximum kinetic rate was observed far from the ideal 1 : 1 composition, but rather at the lowest MA molar fraction. This result could be explained by considering the complex formation to be dependent on a mass action law and that an excess of one monomer would favor the formation of the complex, hence increasing the polymerization rate. But if that was true, the same effect should be observed in the presence of excess MA, which was not the case. This acceleration effect should actually be attributed to a solvent effect, with NVP acting as a better solvent for the growing chains than dioxane from which the polymer precipitated during the polymerization process. As an experimental proof of this solvent effect, a polymerization reaction was run at 30% molar fraction of MA and 70% molar fraction of a mixture of NVP (30%) and *N*-methyl pyrrolidone (40%) used to mimick the solvent properties of NVP. Compared with the reference experiment (MA 50%/NVP 50%), the initial polymerization rate was increased from $2.2 \cdot 10^{-5}$ to $3.6 \cdot 10^{-5}$ mole · $L^{-1} \cdot s^{-1}$, which was similar to the value obtained when a 70% molar fraction of NVP was used in the polymerization mixture.

This solvent effect could also explain the increase, in Figure 4, of the number average molar masses observed along with the acceleration of the polymerization rate for low MA molar fractions.

^{1}H NMR monitoring of a polymerization with a 25% MA molar fraction showed a similar rate of consumption when the two monomers were present in the medium. After the total disappearance of MA, the polymerization rate of NVP was less rapid. The polymerization rate of the complex

Table IV Hydrolysis Rate Constants and Order of the Reaction Determined at 25°C as a Function of pH

Polymer Sample (M_n)	Buffer pH (Composition)	k (s^{-1})	α
NVPAM(20600)	5.6 (phosphate)	$33.4 \cdot 10^{-3}$	1.11
NVPAM(20600)	6.8 (phosphate)	$7.9 \cdot 10^{-3}$	1.19
NVPAM(20600)	8.0 (phosphate)	$8.3 \cdot 10^{-3}$	1.03
NVPAM(19000)	10.0 (borate)	$1.5 \cdot 10^{-2}$	1.27

Table V Bulk Copolymer Composition Determined by Acid/Base Titration[a]

Polymer Code	28	29	30	31
Initial AM ratio in the monomer feed	75	50	35	15
AM ratio in the copolymer	52	53	47	45
	53	51	50	

[a] Results of duplicate experiments.

is much higher than that of NVP in homopolymerization (data not shown). This phenomenon would make it possible to obtain pure alternate copolymers from a mixture containing excess of NVP if the polymerization is stopped before the homopolymerization of the latter starts.

The physicochemical characterization of the products demonstrated that all the polymers obtained had a 1 : 1 composition, irrespective of the initial composition of the medium. The hydrolysis rate constant in aqueous media of the polymer functional groups was determined at 25°C, and found to increase with increasing pH, which was expected from our previous works.[8] Interestingly, the hydrolysis rate for a NVPMA copolymer was higher than that of a MAMVE sample of similar M_n. NVP being highly hydrophilic, the hydrolyzing water molecules may have an easier access to the reactive groups in a NVP copolymer than in a methyl vinyl ether one. Closely related is the determination of the first acidity, with $pK_{ao} = 1.6$, a

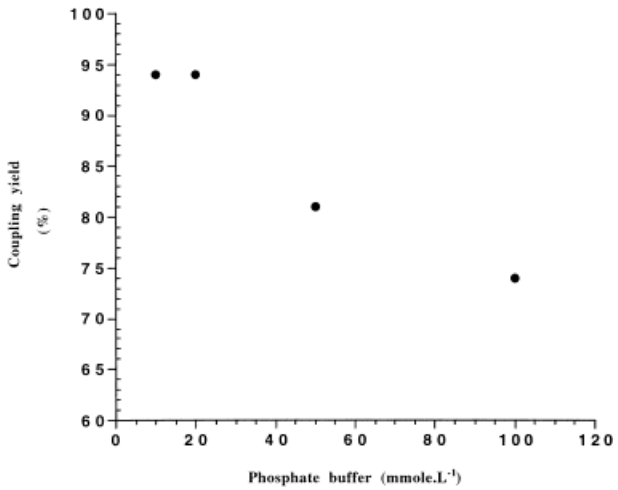

Figure 5 Effect of the buffer ionic strength on the course of the immobilization reaction: [BSA] = 1 mg · mL^{-1}; [NVPMA] = 0.2 mg · mL^{-1}.

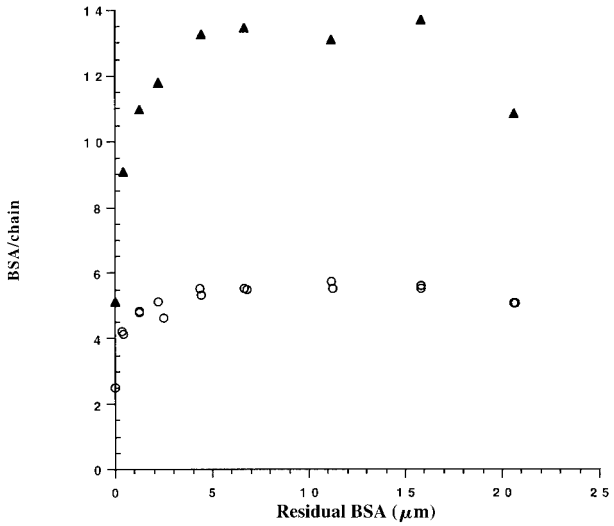

Figure 6 Immobilization isotherms at 37°C of BSA on NVPMA samples (25 m*M* phosphate buffer, 3 h, [polymer] = 0.05 mg · mL^{-1}). (○) 29 kDa; (△) 58 kDa.

value much lower than for the MAMVE copolymer ($pK_{ao} = 3.59$).[8]

The immobilization of BSA took place in an acidic phosphate buffer. All other conditions, for instance a 50 m*M* Tris buffer (pH 7) or 0.1*M* borate buffer (pH 9.2), only yielded very small amounts of conjugates, probably because of the high propensity of the polymer toward hydrolysis.

The grafting efficiency of the protein was dependent on the salt concentration of the buffer; the lower the ionic strength, the more efficient the reaction (Figure 5), which shows that physicochemical factors play a determinant role in the course of the immobilization process. For the chemical reaction to take place, the macromolecules should be able to get in close vicinity to one another, at a distance inferior to the length of a chemical bond, to allow the chemical reaction. The fact that coupling yields increase with decreasing ionic strength demonstrates that attractive electrostatic interactions favor the approach of the macromolecules. BSA is an amphoteric molecule whose isoelectric point, zero charge point (IP), is at pH 4.9; the polymer, in the phosphate buffer pH 5.5, can have some anhydride moities hydrolyzed to partially dissociated carboxylate groups. Therefore the interactions involve the carboxylate groups on the polymer with some patches of positive charges carried by the protein, because the charges on BSA are unevenly distributed.[11]

The immobilization process is influenced as well by the proteins bound onto the polymer,

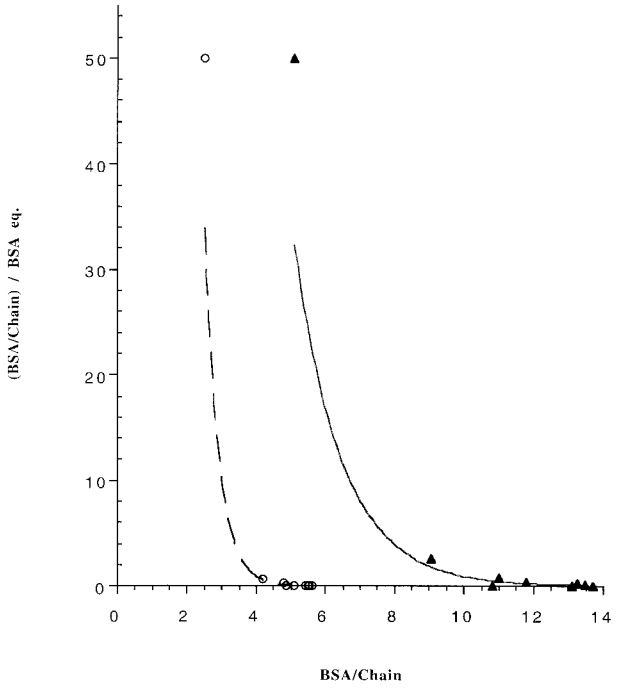

Figure 7 Scatchard plots derived from the immobilization isotherms at 37°C of BSA on NVPMA samples (25 m*M* phosphate buffer, 3 h, [polymer] = 0.05 mg · mL^{-1}). (○) 29 kDa; (△) 58 kDa.

which can prevent the binding of others either via steric hindrance or by modification of the electrostatic environment of the macromolecular chain. The influence of these factors can be assessed by plotting the ratio $N/[BSA]_{eq}$ versus N (N is the number of BSA bound per polymer chain), which is known as the Scatchard plot.[12] Efficient grafting processes will give high $N/[BSA]_{eq}$ values, whereas poor reactions will lead to low values of this ratio. As seen from Figure 7, high N values can be obtained, but with a drastic reduction of the $N/[BSA]_{eq}$ ratio. The sharp decrease observed in the Scatchard plots is quite representative of a behavior, designated as negative cooperativity, as reported by Jennissen.[12]

If electrostatic interactions are involved in the approach of the macromolecules prior to chemical reaction, no polyelectrolyte complex was ever evidenced on mixing the polymer, under the hydrolyzed form, and the protein in the experimental conditions used for the covalent immobilization. The HPLC traces only featured free BSA. Specific experimental conditions to obtain polyelectrolyte–BSA complexes consisted of lowering the pH of a solution containing both the polymer and the protein. The formation of an insoluble complex was evidenced in HPLC by consumption of the polymer and the protein and it required a pH inferior to the IP of BSA. Furthermore, the complexes were insoluble in water.

These results confirm that the conjugates obtained under the experimental condition for covalent binding result from a chemical grafting and that the electrostatic interactions are of sufficient energy to allow the macromolecules to get close to each other, making possible the chemical reaction, but are too weak to yield a stable complex.

CONCLUSIONS

By free radical polymerization in dioxane, 1 : 1 *N*-vinylpyrrolidone/maleic anhydride copolymers were obtained via the polymerization of the corresponding charge transfer complex. NMR kinetics, elemental analysis, and base titrations ascertained the 1 : 1 composition of copolymers. Kinetics experiments pointed out that the complex polymerizes faster than NVP homopolymerizes, which allows use of NVP as a co-solvent for polymerization, to increase the kinetics of the reaction and the average molar masses of the products. The hydrolysis of the anhydride group of NVPMA copolymer was a faster process than for the methyl vinyl ether/MA counterpart because the former is more hydrophilic than the latter.

The covalent formation of polymer–BSA conjugate was obtained at low pH (5.5) and low ionic strength without the formation of purely polyelectrolytic complexes, which required a pH lower than the BSA isoelectric point. As a prerequisite for the formation of a covalent bond between the two reactants, BSA and NVPMA molecules need to get in close contact, to about the length of a chemical bond. The formation of this transition state was demonstrated to rely on the existence of attractive electrostatic interactions. This transition state was formed according to a process in which cooperativity is negative, with already bound molecules preventing the binding of others.

This preliminary study with BSA was valuable to assess the grafting capacity of copolymers. Further use of these copolymers, with HIV-1 P24 capside protein, is currently under investigation.

REFERENCES

1. Tomalia, A. D. Functional monomers, 2. New York: Marcel Dekker; 1974.
2. Georgiev, G.; Konstantinov, C.; Kabaivanov, V. Macromolecules 1992, 25, 6302.
3. Morner, R. R.; Longley, R. I. U.S. Patent 2,676,949 (1954).
4. Nagasawa, M.; Rice, S. J Am Chem Soc 1960, 82, 5070.
5. Reddy, B. S.; Arshady, R.; George, M. H. Polymer 1984, 25, 115.
6. Lehninger, A. L. Principles of biochemistry. New York: Worth Publishers Inc.; 1982.
7. Ladavière, C.; Veron, L.; Delair, T.; Domard, A.; Pichot, C.; Mandrand, B. J Appl Polym Sci 1997, 65, 2567.
8. Ladavière, C.; Delair, T.; Domard, A.; Pichot, C.; Mandrand, B. J Appl Polym Sci 1999, 71, 927.
9. Ladavière, C.; Delair, T.; Domard, A.; Pichot, C.; Mandrand, B. J Appl Polym Sci 1999, 72, 1565.
10. Fujimori, K.; Organ, P. P.; Costignan, M. J.; Craven, I. E. J Macromol Sci Chem A23 (5) 1986, 647.
11. Gao, J. Y.; Dubin, P. L.; Muhoberac, B. B. Anal Chem 1997, 69, 2945.
12. Jennissen, H. P. Biochemistry 1976, 15, 5683.