Covalent Immobilization of Bovine Serum Albumin onto (Maleic Anhydride–*alt*-Methyl Vinyl Ether) Copolymers

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ABSTRACT: The covalent immobilization of bovin serum albumin (BSA) onto maleic anhydride-*alt*-methyl vinyl ether copolymers (MAMVE) was successfully achieved under aqueous conditions. The grafting reaction was shown to be controlled by attractive electrostatic interactions and so took place at a low salt concentration. Under these conditions, the covalent binding reaction was quite efficient, reproducible, and complete within 20 min. The maximum loading capacity of the polymer was of 20 BSA molecules per polymer chain. This preliminary study demonstrated that the immobilization of proteins in an aqueous medium could be an efficient process, despite the existing hydrolysis of the functional polymer by water molecules. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 72: 1565–1572, 1999

Key words: maleic anhydride copolymers; bovine serum albumine; immobilization; aqueous medium

INTRODUCTION

The conjugation of biological molecules to polymers has long been used in a great variety of applications. Conjugates of proteins with soluble polymer were synthetized with polyethylene glycol in order to induce immunotolerance for particular therapies.[1] Conjugates obtained with thermo-responsive polymers were used for extraction and purification.[2–5] With a polymer bearing a multiplicity of reporter molecules, such as europium chelates, for instance, the resulting protein polymer conjugates were a means of signal amplification.[6]

We have been interested in polymer/biological molecule conjugates for a few years,[7–10] and we demonstrated that polymer bound oligonucleotides (ODN) could improve the sensitivity of the diagnostics of the DNA of the Hepatitis B virus.[11]

In our pursuing effort to improve the sensitivity of diagnostics tests in immunoassays, we designed a similar strategy as that used for oligonucleotides and genetic diagnostics. It consisted in using protein/polymer conjugates to increase the efficiency of the tests. The first step of this strategy was to develop a methodology to graft proteins onto polymers in solution. Here, we wish to report on preliminary results obtained on the covalent immobilization onto poly(maleic anhydride-*alt*-methyl vinyl ether) of bovine serum albumin (BSA), used as a model protein.

EXPERIMENTAL PROCEDURES

Materials and Methods

Polymer Samples

Copolymers of maleic anhydride and methyl vinyl ether P(MAMVE) were supplied by Polysciences,

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Inc. (Warrington, USA) [samples P(MAMVE) 1 $\overline{M}_n$ = 67,000 g mol, and P(MAMVE) 1A $\overline{M}_n$ = 20,000 g mol). The detailed physicochemical characterization of these polymers has already been published.[12] Other chemicals were from Aldrich (L'isle d'Abeau, France) and were used as received, unless stated otherwise.

Bovine Serum Albumin

BSA, from Sigma (L'isle d'Abeau, France) was used as such. Its molecular weight was approximately 60,000 g/mol, and the isolelectric point close to pH 4.9. It was constituted of a single polypeptide chain containing 584 amino acid residues. Its ellipsoïdal shape had a diameter of 38 Å and a length of 150 Å.[13] According to the manufacturer, the water content was 5.8%, the nitrogen content was 14.6%, and the solubility in water was 50 g L.

Coupling Proteins to Copolymers

The appropriate amounts of polymers were dissolved in anhydrous dimethylsulfoxide (DMSO) at 37°C. To 100 μL of a protein solution in a buffer was added 5 μL of polymer dissolved in anhydrous DMSO. The reaction vessels were placed under stirring in a thermomixer (Eppendorf) at 37°C for 3 h.

Kinetics of the Coupling Reaction

In order to follow the course of the coupling of BSA onto polymers, 50 μL of the reaction mixture were pipetted off, and the reaction was quenched by addition of 5 μL of 15% ammonium hydroxide solution. The coupling yields were determined by high-performance liquid chromatography (HPLC), as reported below. In the presence of ammonia, the conjugates were stable as no protein release was observed with time in this medium.

Coupling with Hydrolyzed Copolymer

Two copolymer samples were hydrolyzed, one in a 0.1*M* phosphate buffer pH 4.20, and the second one in a 0.1*M* carbonate buffer pH 10.70, during 2 h at 67°C. Then, the coupling reaction was carried out as described above.

Analysis of the Coupling Reactions

The crude coupling mixture was analyzed by size exclusion chromatography (SEC) using a Waters Ultra-Hydrogel 500 column, a Kontron HPLC 422 pump, a Kontron HPLC autosampler 465, and a Kontron ultraviolet (UV) diode array detector on line. Purifications were run in a 0.1*M* phosphate buffer pH 6.8 with 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 has no absorption). 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. The extinction coefficient of the bound protein was similar to that of the unbound BSA. This was checked by comparing the areas of the peaks obtained by HPLC, detected at 280 nm, for bound and unbound BSA.

RESULTS AND DISCUSSION

The grafting reaction investigated in this work occurred by nucleophilic attack of an anhydride moiety in the polymer by a primary amino group of the protein, as depicted in Figure 1. The immobilization of BSA onto maleic anhydride was described by Brissova et al.[14] They added the copolymer under the solid form to a protein solution, probably relying on the anhydride group hydrolysis to achieve dissolution in the phosphate buffer. For reasons of reproducibility, our strategy was based on a covalent coupling in solution, using water miscible organic solvent to ensure the solubility of the polymer in the reaction medium.

Determination of the Coupling Conditions

Salomon et al.[15] used a 10% *w/v* solution of polymer in acetone, but the addition of the protein solution led to the formation of a precipitate, which required a few hours to dissolve. Goldstein et al. avoided the precipitation by adding up to 70% of organic solvent.[16] Few proteins can sustain dissolution in so much organic solvent without loss of their molecular recognition properties. Since this work aimed at developing a somewhat standard method of covalent immobilization of proteins onto the copolymer, experiments had to be run in a medium susceptible to comply with most kinds of proteins. Therefore, the coupling mixture composition was limited to 5% DMSO (*v/v*) in an aqueous buffer. These conditions were quite different from those developed for the covalent grafting of oligonucleotides,[10] which con-

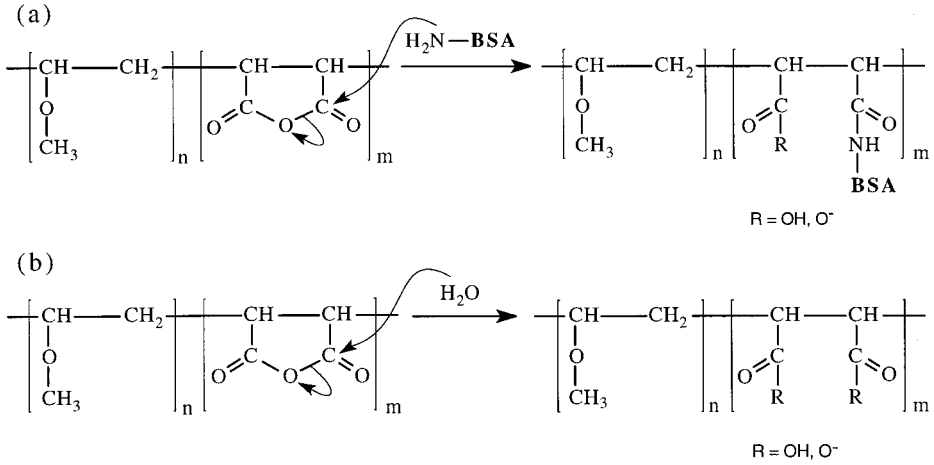

Figure 1 Coupling reaction between the BSA molecule and the copolymer P(MAMVE) (a) and the hydrolysis reaction of polymer (b).

sisted of 95% DMSO and 5% buffer (*v*/*v*), designed to disfavor the competing hydrolysis of the anhydride moieties of the copolymer, to the profit of the grafting process. DMSO had already been used by Isosaki et al.,[17] who used poly(methyl vinyl ether–maleic anhydride) polymer solutions to functionalize, by physical adsorption, the inner walls of the wells of microtitre plates. The adsorbed polymer was then used to immobilize proteins for immunoassays.

A series of buffers, as coupling media, was tested as reported in Table I, and, as a general trend, it appeared that the coupling yields of BSA onto the P(MAMVE) polymer were lower than those obtained with oligonucleotides, which could reach 90%.[10] This lower efficiency of the grafting reaction was probably due to an elevated rate of exhaustion of anhydrides via hydrolysis, under these experimental conditions, in which the reaction medium is 95% (*v*/*v*) aqueous. Out of the eight buffers tested in Table I, only two allowed moderate immobilization yields. In a phosphate buffer, at pH 5.5, the hydrolysis of the reactive anhydride moieties was slower than in any other more basic medium, which could explain the fair yield obtained under these conditions. Another favorable factor for the immobilization at a pH close to the isoelectric point of BSA was that the effect of electrostatic interactions should be reduced since the global net charge of BSA at pH 5.5 was close to zero or slightly negative.

Table I Immobilization of Bovine Serum Albumin (BSA) onto MAMVE Copolymer ($\overline{M_n}$ = 67,000 g mol)

Composition and pH of Buffer	Immobilization Yield (%)
0.1*M* Sodium phosphate pH 5.5	18
0.05*M* Tris pH 7	6
0.05*M* Tris pH 7.6	8
0.1*M* Sodium phosphate pH 8	7
0.1*M* Sodium carbonate pH 9.1	5
0.1*M* Sodium borate pH 9.2	17
0.05*M* Tris pH 9.3	1
0.1*M* Sodium carbonate 10.75	BSA degradation

Experimental conditions: 5 μL of a 1 g/L solution of MAMVE copolymer are added to 100 μL of a 1 g/L BSA solution in an appropriate buffer and stirred 3 h at 37°C.

The role of the borate anion in the coupling mixture at pH 9.2 is not understood yet, and it was surprising to get a 17% immobilization yield at such an elevated pH value, which should favor the hydrolysis reaction, rather than the coupling reaction. Nevertheless, these results were repeated several times as seen in Table II.

Two kinds of information can be obtained from Table II, as follows: first, the reproducibility of experiments run on the same day (i.e., several runs carried out with identical stock solutions of protein and polymer), or on different days with differing protein and polymer solutions. From our results, it appeared that with identical reactant solutions, experiments run on the same day, the reproducibility was quite satisfactory. But, when differing reagent stock solutions were used, the day to day reproducibility was only fair, probably because a slight variation in the preparation of

Table II Reproducibility Assessment of the Immobilization Reaction of BSA onto MAMVE Polymer ($\overline{M_n}$ = 67,000 g/mol)

Series No.	Borate Buffer pH 9.2 Coupling Yields (%)	Phosphate Buffer pH 5.5 Coupling Yields (%)
1	17	18
2	9, 10, 12	19, 22, 24
3	15, 16, 16	35, 35, 32
4	9, 11	24, 25
Average yield	13 (±4)	26 (±8)

Experimental conditions: 5 μL of a 1 g/L solution of MAMVE copolymer are added to 100 μL of a 1 g/L BSA solution in an appropriate buffer and stirred 3 h at 37°C.

Table III BSA Concentration Effect on the Course of the Reaction ($\overline{M_n}$ = 67,000 g/mol)

[BSA][a] g L	Borate Buffer (pH 9.2)		Phosphate Buffer (pH 5.5)	
	% Y[b]	$\bar{N}$[c]	% Y[b]	$\bar{N}$[c]
0.95	9	2.0	26	5.7
2.86	3.5	2.3	13	8.7
4.76	4.5	5.0	8	9.5
9.52	2.5	5.6	4	10.0

[a] Final concentration.
[b] Coupling yield (average of two experiments).
[c] Average number of BSA molecules per polymer chain $\bar{N} = n.\ Y/n'$ where n and n' correspond, respectively, to the number of protein molecules and the number of polymer chains in the reaction mixture.

the stock solutions had a greater impact on the course of the reaction since coupling yields were moderate.

The stabilities of the conjugates were monitored over eight days of storing at +4 and +37°C in a coupling medium (a phosphate buffer pH 5.5). No degradation of the protein–polymer conjugates was detected by HPLC, at both temperatures.

Factors Controlling the Amount of Immobilized Proteins

For diagnostics applications, it is essential to be able to vary the amount of bound proteins onto the polymer. Hence, we investigated various factors that could have an impact on the course of the grafting reaction of BSA onto the MAMVE copolymer, such as the reactant concentration, the ionic strength of the buffer, and the molar mass of the polymer.

Role of the Protein Concentration

In Table III, the BSA concentration was increased in the reaction medium; for two buffer composition conditions, the polymer concentration remained at 0.048 g/L. The data reported in Table III are (1) the coupling yield, representing the efficiency of the covalent binding of BSA; (2) $\bar{N}$, the average number of bound BSA molecules per polymer chain. This value was calculated assuming that each polymer chain reacted according to the same pattern and that no crosslinking arose from the immobilization reaction. $\bar{N}$ reflects an experimental measurement of loading of the polymer.

The amount of polymer bound protein increased with increasing protein concentrations up to an average plateau values, $\bar{N}$, of 5.6 in the borate buffer and 10 in the phosphate buffer. This result means that during the immobilization process, BSA molecules adopted a conformation that depended on the chemical composition of the buffer. Under conditions where the coupling reaction was the least favored, the more readily available amino groups of BSA would preferentially react with the polymer, binding the protein molecules under a particular orientation. When more efficient coupling conditions were used, other amino groups on the proteins could react, yielding conjugates in which the BSA molecules adopted such an orientation that allowed more molecules to be bound to the polymer chain. The observed plateau values resulted from the steric hindrance of globular-shaped BSA molecules, whose large specific area could limit the amount of protein bound on a polymer chain. The low efficiency of the grafting reaction could account for the rather low observed plateau values.

As a consequence of the increase in protein concentration, the coupling yield decreased down to 4% in the phosphate buffer. Therefore, coupling had to be improved so as to improve the immobilization efficiency.

Role of the Polymer Concentration

In Table IV, the polymer concentration was increased in the reaction medium for two buffer composition conditions, the BSA concentration remained at 0.95 g/L.

Table IV P(MAMVE) Concentration Effect on the Course of the Reaction (M_n = 67,000 g/mol)

[P(MAMVE)][a] g/L	Borate Buffer (pH 9.2)		Phosphate Buffer (pH 5.5)	
	% Y[b]	$\bar{N}$[c]	% Y[b]	$\bar{N}$[c]
0.048	11	2.5	26	5.7
0.143	27	2.0	69	5.05
0.238	35	1.55	83	3.75
0.476	56	1.25	87	1.95

[a] Final concentration.
[b] Coupling yield (average of two experiments). [BSA] = 0.95 g/L.
[c] Average number of BSA molecules per polymer chain $\bar{N} = n.\ Y/n'$ where n and n' correspond, respectively, to the number of protein molecules and the number of polymer chains in the reaction mixture.

Results from Table IV show that on increasing the anhydride moiety concentration in the coupling mixture, the coupling yield raised up to 87%. In the mean time, a regular decrease of the number of immobilized BSA molecules per polymer chain was observed, as more polymer chains were added.

This series of experiments demonstrated that when sufficient functional groups were available to compete with the hydrolysis reaction, the coupling yields could be high.

Effect of the Ionic Strength of the Coupling Buffer

Due to the formation of carboxylate groups resulting from the hydrolysis of some of the anhydride moities, the copolymer acquires a polyelectrolyte character during coupling. Therefore, since at pH 5.5, BSA bears a negative net charge, electrostatic repulsive forces could develop and prevent the two macromolecules from getting close to one another for the chemical reaction to take place. So, a high salt buffer concentration was used, in order to shield the charges borne by both macromolecules and reduce the assumed electrostatic repulsive forces. Trying to improve the coupling efficiency, the buffer salt concentration was increased, but a decrease in coupling yields was observed, as reported in Table V. Therefore, in contrast to what was expected, repulsive electrostatic forces were not involved in the coupling process of BSA, as they were in the case of oligonucleotides, which are negatively charged polyelectrolytes.[10] Another explanation for the lack of coupling at high ionic strength could be that the presence of excess salt in the coupling medium would entail a collapse of the macromolecules into a more compact conformation, in which the reactive groups would be too embedded to react.

Table V Salt Concentration Effect on the Course of the Reaction (M_n = 67,000 g/mol)

[NaCl] (mol/L)	Coupling Yields (%)
0	33
0.25	10
0.5	7
1	5

0.1*M* sodium phosphate coupling buffer; pH 5.5; [BSA] = 0.95 g/L; [P(MAMVE)] = 0.048 g/L; 3 h at 37°C.

So, though at pH 5.5 the net charge of BSA is slightly negative, it only means that positive charges on the protein almost offset the negative charges. Hence, from the results in Table V, the idea was to actually lower the buffer ionic strength in order to take profit of the positive charges on the BSA molecules to create attractive electrostatic forces. These forces would bring together the two macromolecular reactants and, as a consequence, improve the efficiency of the grafting reaction.

In Figure 2, the coupling yield of the grafting reaction versus the ionic strength of the phosphate buffer is plotted, and we can observe a monotonous increase of the coupling yield as the ionic strength of the immobilization medium decreases. The best coupling condition were ob-

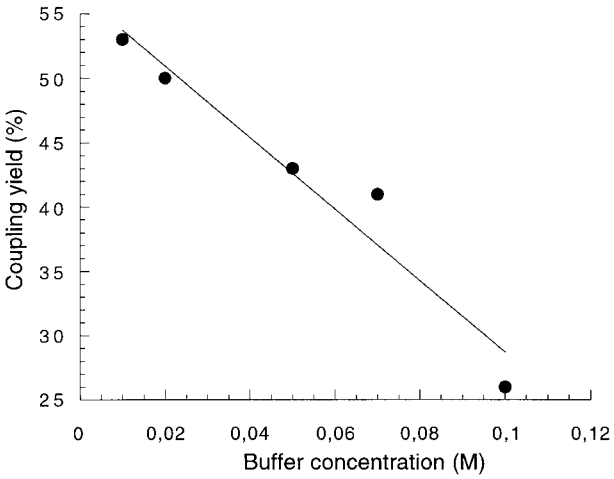

Figure 2 BSA coupling yields onto P(MAMVE) 1 as a function of the phosphate buffer concentration: [BSA] = 0.95 g/L; [P(MAMVE)] = 0.048 g/L.

Table VI Reproducibility Assessment of the Immobilization Reaction of BSA at Low Ionic Strength onto MAMVE Polymer ($\overline{M_n}$ = 67,000 g/mol)

[BSA] (g/L)	Day 1 (% Y)	Day 2 (% Y)	Day 3 (% Y)	Average (%)
		55		
0.95	63	53	62	58 ± 5
4.76	19	18	17	18 ± 1
9.52	9	9	10	9 ± 1

10 m*M* sodium phosphate coupling buffer; pH 5.5; [P(MAMVE)] = 0.048 g/L; 3 h 37°C.

tained in 10 m*M* phosphate buffer (53% coupling yield). Reducing the ionic strength was a means to improve the reproducibility of the reaction, as shown by the results reported in Table VI. An important result was that the day to day reproducibility was much better when the reaction was run at low ionic strength (compare with results in Table II).

The kinetics of the reaction was investigated in a phosphate buffer, pH 5.5 at 2 ionic strengths: 100 and 10 m*M*. As shown in Figure 3, the maximum coupling yield was obtained within 20 min of reaction in 100 m*M* buffer and within 40 min in 10 m*M* buffer. This difference could be explained by the higher amount of protein bound per polymer chain in the 10 m*M* buffer, approximately 13, than in the 100 m*M* one, only 5. One other possible explanation was that the buffer, at low ionic strength, could be exhausted by the hydrolysis of the polymer reactive groups, leading to acidification of the mixture with increased protonation of amines and subsequent loss of reactivity. Monitoring the pH of the reaction mixture showed that within 1 h, the pH dropped of the 0.3 pH unit, which was too limited a drop of pH to account for the observed difference in Figure 3.

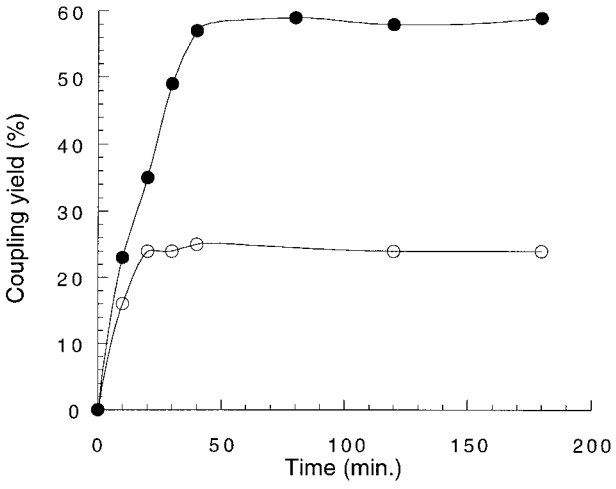

Figure 3 Kinetics of BSA coupling reaction onto the P(MAMVE) 1 sample: buffer, (○) 100 and (●) 10 m*M* sodium phosphate pH 5.5; [BSA] = 0.95 g/L; [P(MAMVE)] = 0.048 g/L.

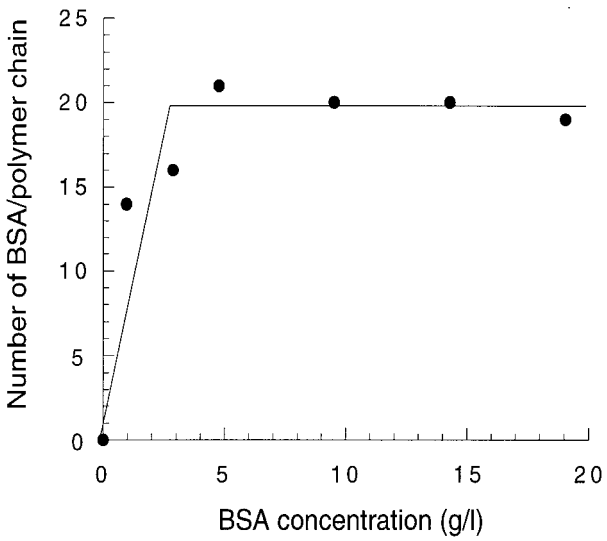

Figure 4 The average number of BSA molecules per polymer chain versus the BSA concentration: 10 m*M* phosphate buffer pH 5.5; [P(MAMVE)] = 0.048 g/L.

Interestingly, reducing the ionic strength of the borate buffer pH 9.2 failed to increase the immobilization yields of BSA. This suggested that the reaction pathways in the two buffers might be quite different. In a phosphate buffer pH 5.5, the approach of both macromolecules should be controlled by electrostatic attractive forces, which could not be the case in a borate buffer at pH 9.2 (at this pH value, the quantity of negative charges on BSA is more important). Hence, the reaction could also efficiently be run at elevated ionic strength.

In order to determine the maximal amount of bound BSA per copolymer chain, we increased the BSA concentration (Fig. 4). The maximum immobilized amount was 20 BSA molecules per polymer chain, which corresponded to approximately 1 BSA molecule every 21 maleic anhydride group. For oligonucleotides, one ODN molecule was bound to the polymer every 4 maleic anhydride group. It is worth noting that with a hydrolyzed polymer, in which all the anhydride moieties were converted to the corresponding diacids, no covalent immobilization of BSA was observed.

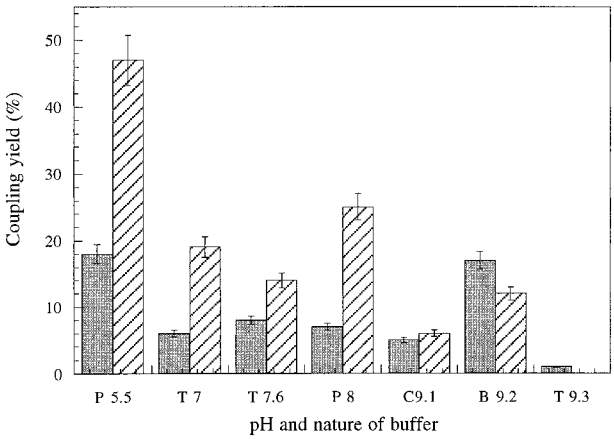

Figure 5 BSA coupling yields onto MAMVE 1 polymer sample (filled bar) and MAMVE 1A polymer sample (hatched bar) at 37°C: [BSA] = 0,95 g/L; [P(MAMVE)] = 0,048 g/L; P, 0.1*M* phosphate buffer; T, 0.05*M* Tris buffer; C, 0.1*M* carbonate buffer; B, 0.1*M* borate buffer pH 9.2.

Effect of the Polymer Molar Mass Distribution

Finally, the effect of the length of the polymer chain, as described by the molecular weight, on the course of the immobilization reaction was investigated. A second polymer sample was tested whose $\bar{M}_n$ was 20,000 g mol. As shown in Figure 5, the grafting reaction was more efficient with the polymer of lower molecular weight [P(MAMVE) 1A sample] over the range of buffer tested, excepted for the borate buffer, in which the coupling yield was better for the higher molecular weight sample. These results reflected the differences in the conformations of the polymer molecules in solution. We demonstrated[10,12] that the P(MAMVE) 1 sample, of higher molecular weight, was aggregated, and the P(MAMVE) 1A was not. Therefore, in the former case, the polymer reactive groups were less available for the covalent grafting of the protein, and they were more hindered, especially for molecules as large as BSA. As a consequence, coupling yields were lower. In the latter case, the polymer reactive groups were more readily available for coupling because of the reduced steric hindrance due to a more expanded conformation of the synthetic macromolecule. That might account, as well, for the lower yield obtained at pH 9.2 in a borate buffer for the P(MAMVE) 1A sample. In such a buffer, the expanded conformation of P(MAMVE) 1A could favor the hydrolysis of the polymer reactive groups, which would become predominant over the grafting reaction, at this high pH value.

Characterization of the Conjugates

BSA–P(MAMVE) conjugates were characterized by size exclusion chromatography (SEC). As seen from Figure 6, the peak corresponding to the conjugate consists of two subsections: the excluded peak (EP = peak 1 in Fig. 6) corresponding to the higher molar mass conjugates, and the broad peak (BP = peak 2 in Fig. 2) corresponding to lower molar mass conjugates. The ratio that excluded peak area versus the broad peak area remained constant over eight days at 37°C, in the coupling medium (DMSO to H_2O, 5 : 95). Hence, we can assume that no aggregation process took place on storing in the reaction mixture. This result was opposite to what was observed with ODN, for which an aggregation process occurred on storing in the coupling medium (DMSO to H_2O, 95 : 5).

CONCLUSION

This work allowed us to establish the experimental conditions for an efficient covalent immobilization of bovine serum albumin (BSA) onto maleic anhydride derivatized polymer, in a medium consisting mainly of water.

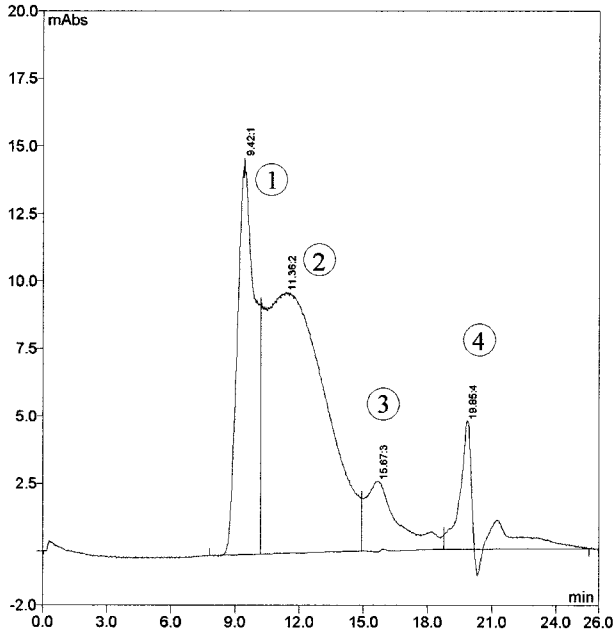

Figure 6 SEC trace of the coupling reaction mixture showing four kinds of peaks corresponding to (1) the excluded peak, (2) lower molecular weight conjugates, (3) unreacted BSA molecule, and (4) DMSO.

The grafting reaction best occurred at a pH close to neutrality in order to limit the competing hydrolysis reaction. The best coupling medium was found to be a sodium phosphate buffer, pH 5.5, close to the isoelectric point of the protein. Ionic strength had a drastic effect on the course of the binding reaction. A decrease in the salt concentration down to 10 m*M* increased the coupling yields higher than 50 % and, interestingly, greatly improved the reproducibility of the immobilization reaction. At low ionic strength, positive charges borne by the BSA were used to generate attractive electrostatic forces which improved the coupling efficiency.

In the phosphate buffer pH 5.5, kinetics studies showed that the grafting reaction was achieved in 20 min for the 100 m*M* salt concentration and 40 min for the 10 m*M* concentration, a difference due to the higher amount of BSA bound in the latter conditions. The maximum amount of BSA loaded on the polymers was approximately 20 BSA molecules per polymer chain, close to five times less than for nucleic acids, which is proportional to the difference in molar masses of the two model biomolecules.

In addition, we pointed out that the conformation of the polymer in solution played a role on the course of the reaction. An aggregated macromolecule was less reactive than a nonaggregated one, in which the reactive sites were more available.

Finally, this work allowed us to demonstrate on a model protein, BSA, that covalent binding onto P(MAMVE), a maleic anhydride copolymer, was achievable, despite the use of a large amount of an aqueous buffer. We established that the main factor ruling the grafting reaction was the presence of electrostatic forces attracting the macromolecules close enough to chemically react. Currently, these results are being used in the lab for the immobilization of proteins having more biological relevance.

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