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Modification of albumins by grafting poly(amido amine) chains

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Bovine serum albumin and human serum albumin have been modified by grafting poly(amido amine) chains via a hydrogen-transfer addition reaction involving the available amino groups of the protein, in which the aminic character of the latter is preserved. The chemical processes involved are probably of general application for grafting multifunctional polymeric chains on to proteins under very mild, non-denaturing conditions.

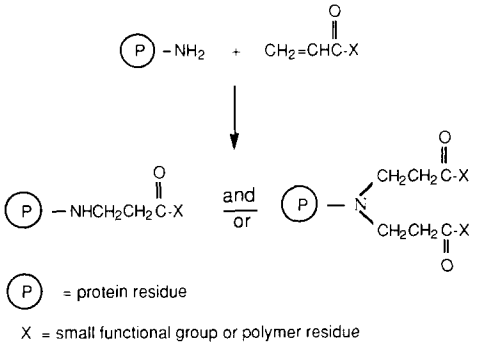
(Keywords: albumins; poly(amido amine)s; grafting)

INTRODUCTION

A well established technique for modifying the physico-chemical properties of proteins, as well as their localization and permanence in biological environments, is the grafting on to them of polymeric chains[1].

Broadly speaking, the grafting methods reported for this purpose in the literature may be divided into two main categories: the growing of polymeric chains with the participation of chemical groups, either originally present in the protein, or purposely introduced; or grafting of preformed polymeric chains on to the protein by means of a coupling reaction.

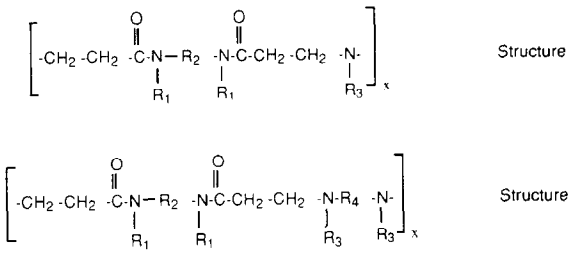
In both cases, the initial step is most often an acylation-type coupling reaction involving the available amino groups of the protein with the formation of amide or urethane bonds. This procedure has been employed, for instance, in the modification of enzymes by suitably activated poly(ethylene glycol)s or poly-(ethylene glycol) derivatives[2–6], as well as by other amphiphilic oligomers[7,8].



Scheme 1

Relatively little attention has been paid so far to protein modifications, still involving $-NH_2$ groups, but making use of hydrogen-transfer addition reactions to carbon–carbon double bonds, activated by electron-attracting groups in the α-position, such as, for instance, acrylamido derivatives (*Scheme 1*). It is apparent that this type of reaction, if compared with acylation-type reactions, maintains the aminic character of the $-NH_2$ groups that are involved, and therefore induces less alteration in the protein. Moreover, it usually takes place in aqueous media under very mild conditions (see below). Among previous work on this subject, the use of 1,4-bis(acryloyl)piperazine as a vinylating agent for graft copolymerization purposes deserves to be mentioned[9].

Hydrogen-transfer addition reactions of compounds bearing mobile hydrogens to activated double bonds has been utilized for many years by our group for preparing several families of multifunctional polymers[10–12]. Among these, poly(amido amine)s (PAAs) (*Scheme 2*) have received particular attention, owing to their hydrophilicity and ability to form stable complexes with heparin in aqueous media[13]. Moreover, PAA-grafted materials can be heparinized, acquiring non-thrombogenic properties[14], while several PAA-based crosslinked hydrogels act as selective heparin absorbers from plasma or blood without exerting significant side



Scheme 2

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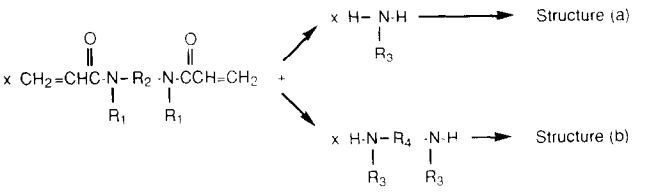


Scheme 3

effects on normal blood components[13,15]. Recently, it has also been found that some PAAs, including the one considered in this present work (see below), show little or negligible cell toxicity, a feature unusual for water-soluble aminic polymers, and therefore are being considered as cationic soluble drug carriers[16].

PAAs are synthesized by stepwise polyaddition reactions of primary monoamines or bis(secondary amine)s with bisacrylamides, according to *Scheme 3*[17]. Mixed structures, i.e. (a) + (b), can also be obtained from mixtures of amines. The above polymerization reactions require very mild conditions, as they easily take place in water or alcohols, at room temperature, and without added catalysts. Moreover, a large variety of structures have been obtained, since almost every aliphatic or cycloaliphatic primary monoamine or bis(secondary amine), as well as every bisacrylamide, can be used as monomers. Among the latter group, the same 1,4-bis(acryloyl)piperazine proposed for enzyme modification (see above) had long ago been synthesized and used as a monomer for PAA synthesis[18,19].

We considered it interesting to report here on the chemical results of our studies on the modification of albumins by the grafting of PAA chains, because they provide new and probably general procedures for protein modification. This work is a part of a larger European research project which is aimed at obtaining new degradable materials to be used as components of drug delivery systems.

EXPERIMENTAL

Measurements

Gel permeation chromatograms were obtained by using Bio Rad TSK40 and TSK30 columns connected in series, with 0.1 M phosphate buffer (pH 8.0 in 0.1 M NaCl) as eluent; the flow rate was maintained at 1.0 ml min^{-1} by an ERMA model ERC-8710 pump, while the samples were checked by a Knauer UV detector operating at 230 nm. The retention times reported are those corresponding to the peak maximum.

Fourier transform infra-red (*FT*i.r.) spectra were run by a Jasco 5300 FTIR spectrophotometer, equipped with a diffusive reflection model DR-81 attachment, which allowed us to obtain spectra from films produced by casting from water on the aluminium surface of the sample holder.

Potentiometric titrations were carried out by an Orion model SA 720 pH meter, equipped with an Ingold 405-S7/120 glass electrode, using the same concentration in all experiments (250 mg of sample dissolved in 5.0 ml of water).

Intrinsic viscosities were measured in 0.1 M NaCl at 30°C by an Ubbelohde viscometer. Ultrafiltrations were performed in water by an Amicon model 8400 cell using Amicon XM50 (cut-off 50 000) and Y1 (cut-off 1000) membranes. Freeze-drying was carried out by means of an Edwards Modulyo freeze-dryer.

Raw materials

Piperazine (anhydrous) (II), triethylamine, morpholine and 1 M HCl were purchased from the Fluka Co., and used without further purification. Bovine serum albumin (BSA) was purchased from the Sigma Chemical Co., while human serum albumin (HSA) (Albutein, 25% albumin solution) was purchased from the Alpha Therapeutic Co. 1,4-Bis(acryloyl)piperazine (I) was prepared as previously described[18,19].

PAA-grafted BSA by process (a) (III)

BSA (2.003 g, 0.003 mmol) and I (1.326 g, 6.83 mmol) were dissolved in distilled water (20 ml); the pH was then raised up to 8 by addition of a few droplets of triethylamine. The reaction mixture was left at 25°C in the dark, under stirring, for 2 days; II (0.647 g, 7.51 mmol), previously dissolved in 1 M HCl (7.1 ml), in order not to change the pH of the BSA solution, was then added and the reaction mixture allowed to stand for a further 4 days under the same conditions. Afterwards, the solution was diluted with water and ultrafiltered through a membrane (cut-off 50 000). After freeze-drying, 2.56 g of III were recovered (gel permeation chromatography (g.p.c.) retention time = 860 s).

PAA-grafted HSA by process (a) (IV)

This was prepared by the same procedure as used for III, by substituting HSA (2.032 g, 0.003 mmol) for BSA. Yield = 2.24 g; g.p.c. retention time = 870 s.

PAA prepared for comparison purposes in process (a) (V)

I (3.492 g, 17.98 mmol) and II (1.704 g, 19.78 mmol) were dissolved in distilled water (30 ml). The reaction mixture was then left at 25°C, under stirring and in the dark for 4 days and finally freeze-dried. G.p.c. retention time = 1030 s; intrinsic viscosity = 0.10 dl g^{-1}.

Preformed PAA oligomer (VI)

This was prepared by the same procedure as used for V, but in this case starting from 2.928 g (15.07 mmol) of I and 1.098 g (12.74 mmol) of II. After 4 days, one half of the solution was separated and employed for the synthesis of VII, whereas the other half was freeze-dried, thus providing 2.013 g of VI.

PAA prepared for comparison purposes in process (b) (VII)

This was prepared by adding morpholine (2.03 ml, 2.33 mmol) to one half of the reaction mixture of VI. After 2 further days at 25°C, under stirring and in the dark, the solution was diluted with water and ultrafiltered through a membrane (cut-off 1000). After freeze-drying, 1.54 g of PAA were recovered. G.p.c. retention time = 960 s; intrinsic viscosity = 0.12 dl g^{-1}.

PAA-grafted BSA by process (b) (VIII)

BSA (2.003 g, 0.003 mmol) and VI (2.013 g) were dissolved in distilled water (20 ml). The pH was lowered to 8–8.5 by the addition of 1 M HCl and the reaction

mixture left for 4 days at 25°C, under stirring and in the dark. Then morpholine (2.03 ml, 2.33 mmol), previously neutralized with 1 M HCl (2.3 ml), was added and the reaction mixture allowed to stand under the same conditions for a further 2 days. After dilution with water and ultrafiltration through a membrane (cut-off 50 000), the solution was freeze-dried. Yield = 2.45 g; g.p.c. retention time = 860 s.

PAA-grafted HSA by process (b) (IX)

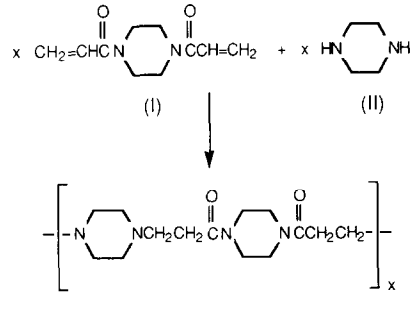
This was prepared by the same procedure as used for VIII, by substituting HSA (2.032 g, 0.003 mmol) for BSA. Yield = 2.34 g; g.p.c. retention time = 870 s.

RESULTS AND DISCUSSION

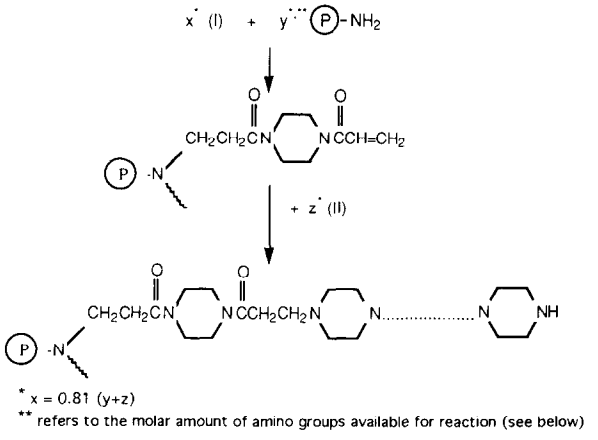
Chemistry

The grafting of PAA chains on to bovine serum albumin (BSA) and human serum albumin (HSA) involved a hydrogen-transfer addition reaction of the available $-NH_2$ groups of the protein to acrylamido bonds. A similar reaction would take place with –SH groups, but it is known that as a rule no such groups are available in albumins. The PAA selected for this present study was that derived from 1,4-bis(acryloyl)piperazine (I) and piperazine (II) as monomers[18] (*Scheme 4*). This structure was selected on the basis of our previous study on the cell toxicity of PAAs[16].

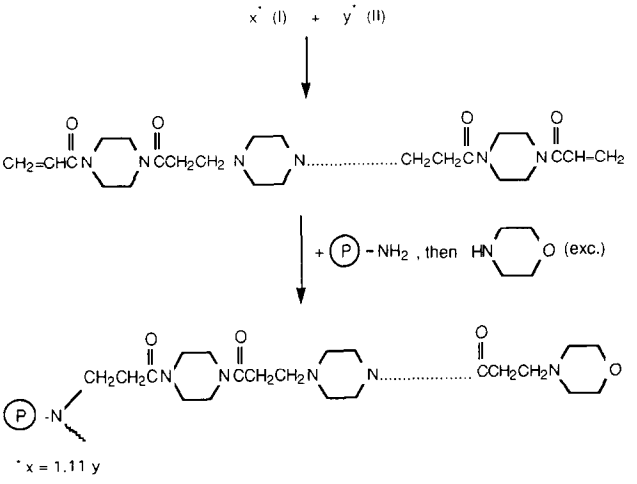
Two processes were followed for the grafting of PAA on to albumins. Process (a) involved the preliminary activation of the albumin by addition of excess I, followed by direct copolymerization of the activated albumin, and compounds I and II, caused by adding the last component as the final step and allowing the resulting mixture to remain at room temperature with occasional stirring for 4 days. In order to avoid any residual double bonds in the end product, an excess of the amine (over the bisacrylamide) was employed (*Scheme 5*). Process (b) involved grafting of an acrylamido-terminated PAA prepolymer on to the albumins. The acrylamido-terminated PAA, in turn, was prepared by performing the polyaddition reaction in the presence of excess bisacrylamide. The grafting reaction was simply performed by mixing an aqueous solution of the acrylamido-terminated PAA with albumin and allowing the system to react for 4 days at 25°C. As a final step, morpholine was added in order to saturate all of the residual double bonds that were present (*Scheme 6*). It may be observed that the acrylamido-terminated PAA bears two reactive functions at both ends, and therefore some risk of protein



Scheme 4



Scheme 5



Scheme 6

crosslinking could not be excluded *a priori*. However, no evidence of crosslinking was ever found.

Even if the two synthetic processes appear considerably different, they have in common several aspects:

(1) The reactions involving hydrogen-transfer from the albumin $-NH_2$ groups to the acrylamido double bonds were all performed at pH 8–8.5, since, if on the one hand the reaction rate increases with pH, on the other hand higher pH values would have increased the risk of albumin denaturation.

(2) The weight ratio of albumin to the sum of the monomers (process (a)), as well as to the prepolymer (VI) (process (b)), was ~1.

(3) In both processes, after completion of the reaction, the products were isolated by ultrafiltering through a membrane with a *MW* cut-off of 50 000, followed by freeze-drying. Preliminary experiments were performed separately with virgin BSA and HSA, V (a PAA obtained under the same stoicheiometric conditions as in the grafting process (a)) and VII (the same acrylamido-terminated PAA used in process (b), but finally end-saturated with morpholine, see Experimental section). These experiments showed that under the above ultrafiltration conditions only the albumins were retained by the membrane. Furthermore, by ultrafiltering mixtures

of albumins and the same PAA samples under the same conditions, a complete separation occurred.

The relative amounts of I and II used in processes (a) and (b) were selected in order to produce polymeric chains with approximately the same average lengths. In this connection, the Flory theory of stepwise polymerizations was employed, according to which the number-average degree of polymerization, $\bar{x}_n$, at the completion of the reaction is given by the following[20]:

$$\bar{x}_n = \frac{1+r}{1-r} \quad (1)$$

with r being the molar ratio of the minor component to the major one. In previous work we have already demonstrated that the polymerization reaction leading to PAAs follows this theory[21]. Thus $\bar{x}_n$ for prepolymer VI, prepared by using $r = 0.845$, was, according to equation (1), ~11.9; we think it reasonable to assume that this value was generally maintained after grafting of VI on to the albumins. Calculations of $\bar{x}_n$ for chains grafted following process (a) can be made bearing in mind that the Flory theory assumes equal reactivity of functional groups and, consequently, the order in which these react does not affect the final results. Hence, referring to *Scheme 5*, we can suppose that the second stage may be divided, ideally, into two steps: (a) reaction of excess I with II, giving an amino-terminated oligomer, and (b) addition of the latter to the acrylamido groups of the activated albumin. In calculating the moles of unreacted I we considered that the molecular weights of BSA and HSA were 66 000 and 67 000, respectively. Furthermore, we have supposed that each protein chain contained 30 out of about 65 amino groups available for reaction and that each $-NH_2$ group might react with two molecules of I. Thus, the acrylamido-terminated oligomer of step (a) may be supposed to develop with $r = 0.67$ and, according to equation (1), its $\bar{x}_n$ to be ~5.1. Since, under our assumptions, each available $-NH_2$ group has already reacted with two molecules of I, the approximate $\bar{x}_n$ of the grafted chains is expected to be ~12.2. On the other hand, calculations performed assuming transfer of only one hydrogen atom, as well as a different number of amino groups available for reaction (from 20 to 40), lead to $\bar{x}_n$ values ranging from 9 to 15, which are still, however, in qualitative agreement with that expected for process (b).

We did not observe any striking differences between the behaviour of BSA and HSA towards both grafting processes. Moreover, according to our data, both processes gave similar results, as far as the amounts of grafted PAA were concerned. However, as a standard procedure, we preferred process (a), because it was more straightforward, and not requiring the preparation of a PAA prepolymer and the addition of morpholine. All characterizations data reported below refer to products obtained by using process (a).

Characterizations of the albumin–PAA adducts and determination of their PAA contents

Gel permeation chromatography. Chromatograms of native BSA, PAA oligomer, a mixture of native BSA and PAA oligomer, and PAA-grafted BSA, are reported in *Figure 1*. It may be observed that the grafted product

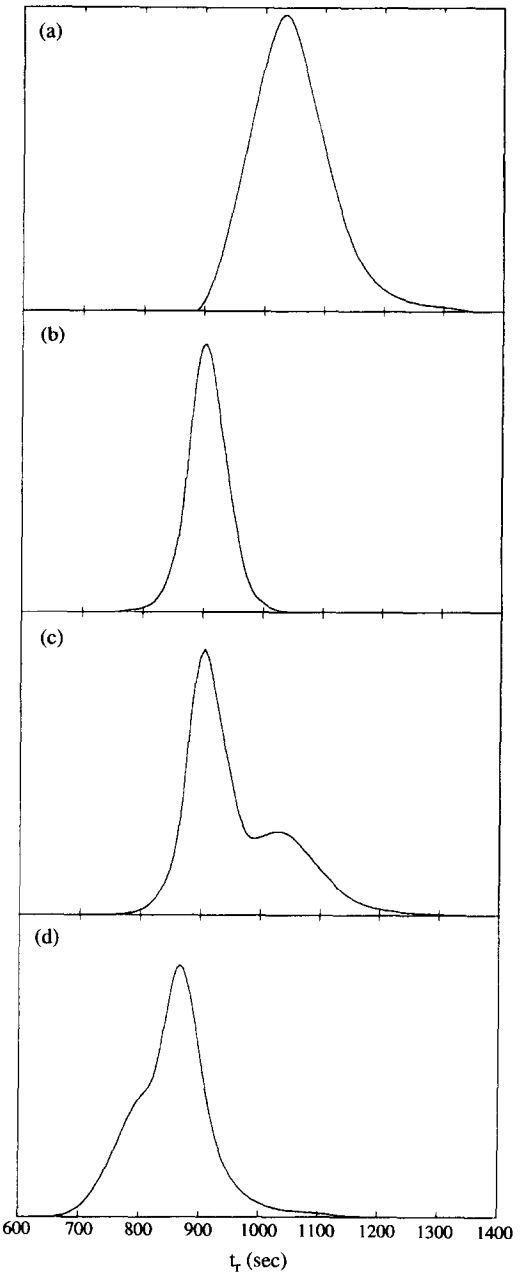


Figure 1 Gel permeation chromatograms of: (a) PAA oligomer; (b) virgin BSA; (c) a mixture of BSA and PAA; (d) PAA-grafted BSA

does not contain appreciable amounts of free PAA oligomer, while its chromatogram is displaced towards lower retention times with respect to BSA (860 instead of 905 s), thus indicating an increase of molecular weight, which is consistent with a successful grafting reaction. The corresponding g.p.c. tracings for HSA are reported in *Figure 2*. It is apparent that the same observations as in the case of BSA hold true for this system.

FT *i.r. spectroscopy.* The spectra of native BSA, PAA oligomer, their mixture, and PAA-grafted BSA, are shown in *Figure 3*. It can be observed that in the grafted product the bands typical of the spectra of both BSA and PAA are recognizable. As anticipated, the same results were obtained for HSA. By comparing in the spectrum of the BSA–PAA conjugate the relative

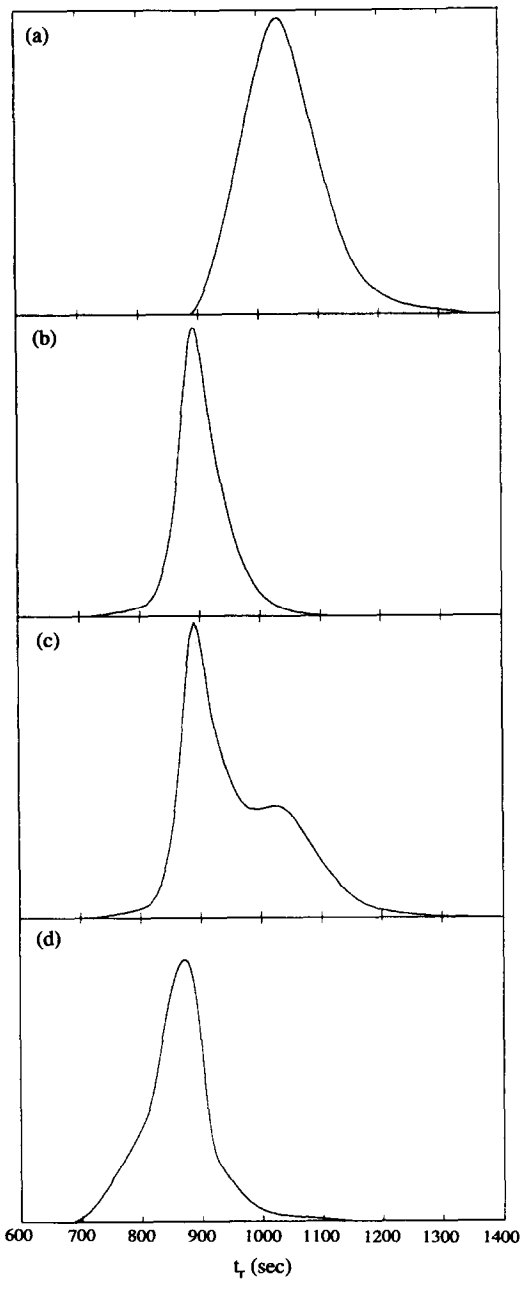


Figure 2 Gel permeation chromatograms of: (a) PAA oligomer; (b) virgin HSA; (c) a mixture of HSA and PAA; (d) PAA-grafted HSA

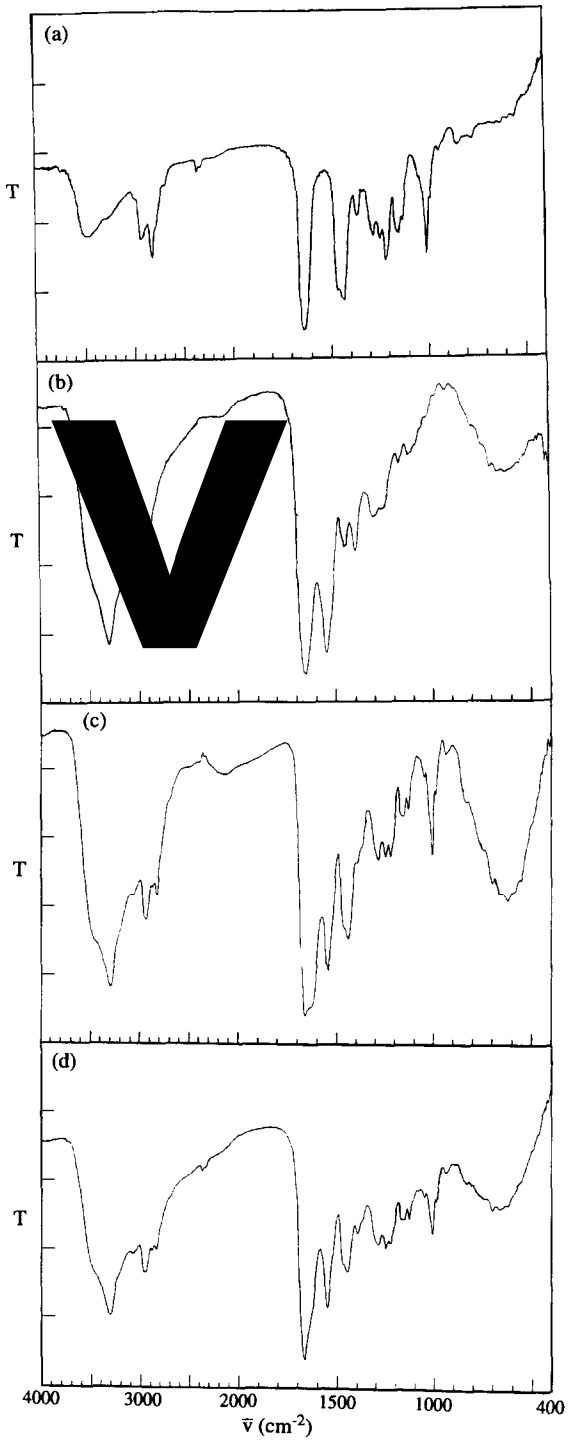


Figure 3 *FT*i.r. spectra of: (a) PAA oligomer; (b) virgin BSA; (c) a mixture of BSA and PAA; (d) PAA-grafted BSA

intensities of two bands, i.e. a typical one for both PAA and BSA (C=O stretching, 1618–1766 cm^{-1}) and the other for PAA only (CH_2 scissoring of the piperazine ring, 1413–1487 cm^{-1}), with those found in the spectra of a series of BSA–PAA mixtures of known composition, the PAA content of the conjugate was roughly estimated to be 23% by weight. The results obtained with HSA were very similar, with the PAA content in the adduct being estimated as 18%.

Titration experiments. The titration curves of native BSA, PAA and BSA–PAA mixtures of known composition are reported in *Figure 4*. In the same figure, the titration curve of the grafted product is also reported. Considering that the aminic character of the albumin $-NH_2$ groups involved in the grafting reaction is preserved, we may reasonably suppose that PAA–albumin mixtures are good models for PAA-grafted albumin, as far as titration is concerned. Therefore, from the family of titration curves reported in *Figure 4*, the PAA content in the grafted product can be determined as 20% by weight, a value which is very similar to that approximately estimated by *FT*i.r. spectroscopy. A similar procedure performed on HSA gave a PAA content in the grafted products of 19%, which is again a value consistent with that obtained by spectroscopy.

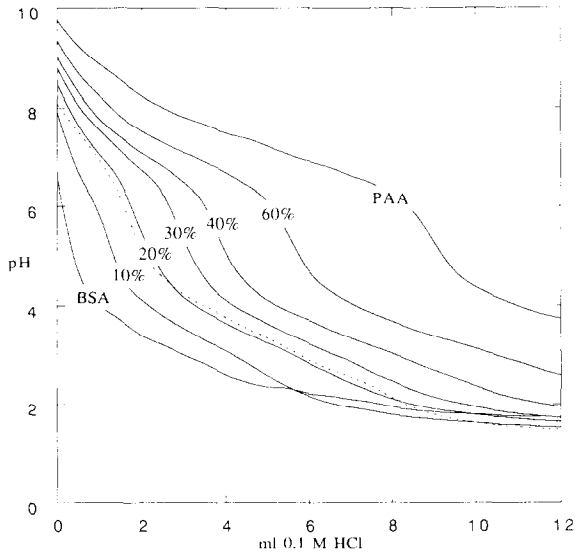


Figure 4 Titration curves of BSA–PAA mixtures of known composition and PAA-grafted BSA (····)

CONCLUSIONS

The following conclusions may be drawn from the above data:

(1) At least part of the aminic groups of BSA and HSA are available for grafting procedures involving polymeric chains obtained by hydrogen-transfer stepwise polyadditions.

(2) The grafting reaction occurs under very mild, non-denaturing conditions.

(3) It is unlikely that the grafting procedures described in this present paper are limited to albumins and the particular PAA employed here. On the contrary, we may reasonably assume that many proteins and many PAA structures can be combined by the same procedures, which should be considered as being general ones. To support this point, we may observe that in addition various hydrogen-transfer stepwise polyaddition polymers other than PAA can be grafted on to proteins by similar techniques. In fact, we have already been successful in grafting poly(thioether amido acid)s[22] and poly(thioether amido acid)–poly(ethylene glycol) block copolymers on to HSA. The latter will be the subject of a forthcoming paper[23].

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