Polymer 51 (2010) 2503–2507

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Bovine serum albumin as chain transfer agent in the acrylamide polymerization. Protein-polymer conjugates

A. Valdebenito, P. Espinoza, E.A. Lissi, M.V. Encinas*

Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

ARTICLE INFO

Article history:
Received 18 January 2010
Received in revised form
1 April 2010
Accepted 9 April 2010
Available online 24 April 2010

Keywords:
Chain transfer constants
Polymer-protein conjugates
BSA-poly(acrylamide)

ABSTRACT

Acrylamide photopolymerization at 25 °C, using as chain transfer agent the single exposed cysteine residue of bovine serum albumin (BSA), resulted in a conjugate where a single poly(acrylamide) chain is bound to the cysteine residue of the protein. Studies of the intrinsic fluorescence of the protein and of the extrinsic probe, 1-anilino-8-naphthalene-sulfonic acid, indicate that the protein mostly maintains its native structure in the conjugate. Kinetic studies showed that the chain transfer efficiency of thiols depends on the microenvironment where the –SH group is located. The single exposed cysteine residue of BSA is a more efficient chain transfer agent of the acrylamide polymerization than the free cysteine or glutathione tripeptide. Other potentially reactive amino acids, such as tryptophan, tyrosine and histidine, are two orders of magnitude less efficient than the protein as chain transfer agents.

1. Introduction

Bioconjugates formed by the covalent attachment of proteins to polymer chains are widely used in medicine and biotechnology, in particular with respect to drug delivery [1,2]. The conjugation of proteins and peptides to synthetic or natural polymers has been demonstrated to improve their therapeutic uses [3,4]. Early works on protein-polymer conjugate synthesis were based on the reaction of polymer chains bearing functional groups suitable to react with specific amino acids [5–9]. These reactions present some inherent difficulties and limitations with respect to their direction and control. Frequently, the post-polymerization modification of a polymer chain requires several steps to form the reactive group.

Recently, living radical polymerization techniques have been used to overcome these difficulties. In particular, atom transfer radical polymerization (ATRP) and reversible-addition fragmentation transfer (RAFT) are well-established methods to obtain well-defined protein-polymer conjugates [10–16]. A strategy to obtain conjugates is the use of the protein as macroinitiator of the radical living polymerization of the monomer. Maynard, Haddleton, Russell, and others have employed the protein modified with chain transfer agents of ATRP as macroinitiators [17–20]. More recently, Bulmus, Davis et al. prepared polymer-BSA conjugates using as macroinitiator the protein functionalized with RAFT chain transfer agents [12,21]. These authors also demonstrated the utility of the piridyl disulfide functionality introduced at the protein to obtain the polymer-BSA conjugate. Sumerlin et al. have reported the synthesis of stable and low molecular weight conjugates prepared by RAFT polymerization of *N*-isopropylacrylamide using BSA functionalized with maleimide as chain transfer agent [22]. Living polymerization also has been employed in the synthesis of polymers with end groups suitable for the conjugation to proteins [15,23–25]. Recently, Maynard et al. have reported the conjugation of vinyl sulfone and maleimide end functionalized polymers to cysteine residues of proteins [26,27].

The synthesis of polymers conjugated to proteins employing functionalized mercaptans as classical chain transfer agents in free radical polymerizations also has been evaluated [28,29]. Thiol compounds are known to be effective chain transfer agents in the polymerization of several polymers [30–33]. These compounds allow the control of polymer size, besides introducing functional groups at the end of growing polymeric chain.

In this work we take advantage of the single exposed cysteine residue of BSA to use it as chain transfer agent in the free radical photopolymerization of acrylamide in aqueous medium at 25 °C. This method allowed the binding of the protein to the polymer chain during the polymerization process. The kinetic behavior of the protein as chain transfer agent was quantified, and compared to that obtained using free amino acids.

* Corresponding author. Departamento de Quimica, Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Correo 33, Santiago, Chile. Fax: +56 2 6812108.
E-mail address: maria.encinas@usach.cl (M.V. Encinas).

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doi:10.1016/j.polymer.2010.04.015

2. Experimental section

Acrylamide from Aldrich (>99%) was used as received. 4,4′-Azobis(2-amidinopropane) dihydrochloride (AAPH, Polysciences) was used without further purification. All amino acids and crystalline lipid free bovine serum albumin (BSA) (A7511) were obtained from Sigma.

Free radical polymerizations were carried out under N_2 atmosphere in aqueous solutions at pH 5.8 and 25 °C, using AAPH as the photoinitiator. Amino acids and BSA were used as chain transfer agents. The samples were irradiated in a Rayonet photochemical reactor equipped with a 360 nm irradiation source. Irradiation times were fixed to obtain conversions below 40%. The acrylamide concentration was kept constant at 0.4 M and the AAPH concentration was 0.01 M. Cysteine and reduced glutathione concentrations were in the 0.02–2 mM range, and polymers were isolated by precipitation of the reaction solution into methanol. Polymerizations in the presence of BSA were done with protein concentration in the 10–80 μM range. In these experiments the polymers were isolated from the low molecular weight compounds by successive precipitations from methanol. Polymerization rates (Rp) were determined dilatometrically. Viscosity measurements were carried out in aqueous solutions with an Ubbelohde-type viscometer. The temperature of the sample was 20 ± 0.3 °C.

Average molecular weights of polymers obtained in the presence of the amino acids or the protein were determined by size-exclusion chromatography (HP 1100), using water as the mobile phase. The apparatus was equipped with a TSK-G-3000 (TOSOH) column. Monodisperse polyethylene oxide polymers (TSK standard, TOSOH) were used for calibration. Differential refractometry and fluorescence at 340 nm were used to detect the polymers.

Protein-polymer conjugates were purified in a Superdex 200 10/300 GL column eluted with 40 mM sodium phosphate, pH 7, plus 100 mM NaCl, at a flow rate of 0.4 mL/min.

UV measurements were performed using a HP spectrophotometer. Fluorescence spectra were recorded using a Spex spectrofluorimeter. Excitation and emission monochromator slits were adjusted to 1.25 nm. The presence of the protein in the isolated conjugate was determined spectroscopically from the UV absorption and fluorescence of the tryptophan residue of the protein, and employing the Bradford assays [34]. In all these experiments the native protein was used as standard.

3. Results and discussion

3.1. Incorporation of BSA to the polymer

The addition of millimolar concentrations of cysteine or glutathione to the polymerization of acrylamide in aqueous medium at pH 5.8 reduced markedly the molecular weight of the polymer. For both sulfur compounds, over all the considered ranges of concentrations (0–0.8 mM), polymerization rates were similar to those measured in the absence of additives. On the other hand, when other potentially reactive amino acids (tryptophan, histidine, or 4-ethylphenol as tyrosine model) were used as chain transfer agents, the decrease of the polymer molecular weight was much smaller. These results can be explained in terms of a simple reaction scheme comprising steps (1–3),

$$M_n\bullet + M \rightarrow M_{n+1}\bullet \quad (1)$$

$$M_n\bullet + RSH \rightarrow \text{polymer} + RS\bullet \quad (2)$$

$$RS\bullet + M \rightarrow RSM\bullet \quad (3)$$

where the only reactive fate of the RS• radical is to re-initiate the chain polymerization process. Furthermore, in this scheme it is considered that the reactive species is the protonated thiol [32]. The acid dissociation of the aliphatic sulfur compounds considered in this study has a pK_a lying in the 8–10 range. Hence, at pH 5.8, only a negligible amount of the thiol anion is present.

The polymerization of acrylamide was also carried out in the presence of micromolar concentrations of BSA. The formed polymers were isolated from the low molecular weight compounds by repetitive precipitations in methanol and dissolutions in water. The intrinsic viscosity of these polymeric materials decreased with respect to that of the poly(acrylamide) polymerized in the absence of the protein. This effect is larger than that obtained with free cysteine or glutathione, Fig. 1. These results suggest that the reactive cysteine residue of the protein acts as an efficient chain transfer agent of acrylamide polymerization. In fact, when acrylamide polymerization was carried out in the presence of BSA previously treated with ethylmaleimide, the intrinsic viscosity of the polymer solution did not change. This can be related to the blockage of the thiol group since ethylmaleimide reacts selectively with cysteine protein residues.

The SEC chromatograms of polymers obtained in the polymerization of acrylamide in the absence of chain transfer agents showed only one peak. Polymers obtained in the presence of cysteine and glutathione also showed one peak at higher retention times (Fig. 2). However, the chromatogram detected by refraction index when the polymerization is carried out in the presence of the protein showed three well-resolved peaks, Fig. 3. On the other hand, SEC chromatograms using fluorescence detection showed that only peak I is fluorescent, Fig. 3. The comparison of peak I with that obtained for the unmodified BSA (Fig. 3) indicates that peak I corresponds to the protein-polymer conjugate and/or to the free protein. The lack of fluorescence of peaks II and III shows that these peaks correspond to protein free polymers. The retention time of peak II barely changes with the protein concentration. However, peak III shows a notable increase in retention time when the BSA concentration increases.

3.2. Kinetics of protein binding to the polymer

The propagation and termination reactions (Eqs. 4 and 5) in the classical chain mechanism for free radical polymerization in the absence of chain transfer agents must be modified to take into account the chain transfer to a protein molecule (PS-H) (Eqs. 6–10).

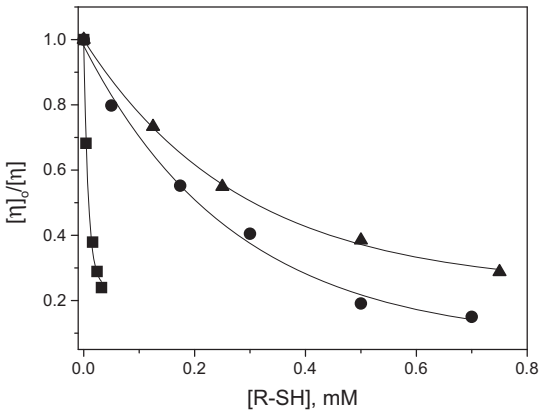

Fig. 1. Intrinsic viscosity of the polymers obtained in the polymerization of acrylamide in the presence of different concentrations of (■) BSA; (●) glutathione; (▲) cysteine. $[\eta]_o$ and $[\eta]$ stand for the intrinsic viscosity in aqueous solutions of polymers obtained in the absence and presence of chain transfer agents, respectively.

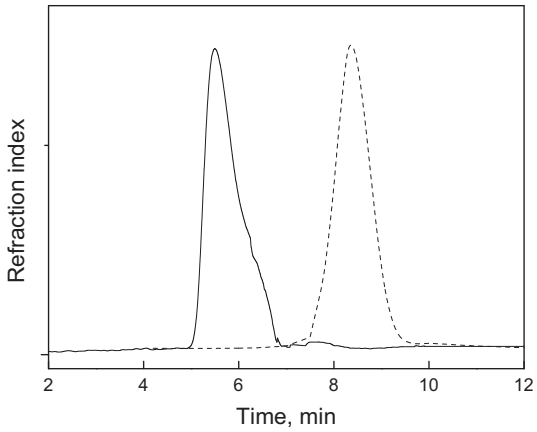

Fig. 2. SEC chromatograms detected by the refraction index signal of poly(acrylamide) polymerized in: (—) absence, and (- - -) presence of 0.8 mM Cys. Column TSK-G (TOSOH).

$$M_n\bullet + M \rightarrow M_{n+1}\bullet \quad (4)$$

$$M_n\bullet + M_m\bullet \rightarrow M_{n+m}\,(M_n + M_m) \quad (5)$$

$$M_n\bullet + \text{PS-H} \rightarrow M_nH + \text{PS}\bullet \quad (6)$$

$$\text{PS}\bullet + M \rightarrow \text{PSM}_m\bullet \quad (7)$$

$$\text{PSM}_m\bullet + \text{PS-H} \rightarrow \text{PSM}_mH + \text{PS}\bullet \quad (8)$$

$$\text{PSM}_m\bullet + M_n\bullet \rightarrow \text{PSM}_{m+n}\,(\text{PSM}_m + M_n) \quad (9)$$

$$\text{PSM}_m\bullet + \text{PSM}_n\bullet \rightarrow \text{PSM}_{m+n}\text{P}\,(\text{PSM}_n + \text{PSM}_m) \quad (10)$$

According to this mechanism, polymer-protein conjugates can be produced in steps (8–10). The intensity of the fluorescent peak (I), as expected, increases when the protein concentration employed in the polymer synthesis increases. On the other hand, its retention time is very little dependent upon the employed protein concentration, and is close to that of the unmodified BSA, in spite of the considerably higher molecular weight of the conjugate. This insensitivity of the peak I position to the experimental conditions can be due to the fact that the retention of the conjugate is strongly dominated by the protein moiety.

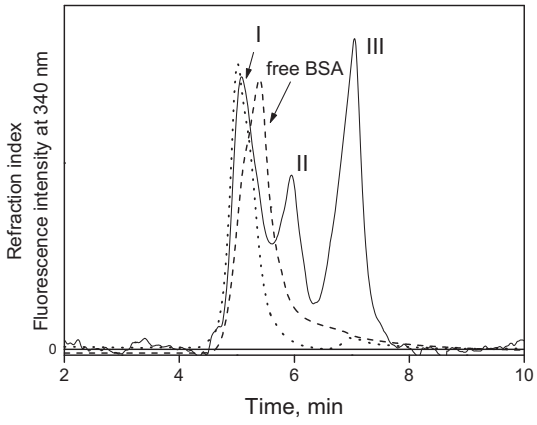

Fig. 3. SEC chromatograms of polymers obtained after repetitive precipitation and dissolution of acrylamide polymerized in the presence of 80 μM BSA, detected by: (—) refraction index, and (…) fluorescence at 343 nm; (- - -) corresponds to the free protein. Column TSK-G (TOSOH).

Peaks II and III of the SEC chromatogram (Fig. 3) do not fluoresce and must be then devoid of protein. They can be assigned to the dimer formed in process (5) (peak II) and to the polymer formed in the chain transfer process (6) (peak III). This peak could have also a contribution from the polymer formed by disproportionation in processes (5) and (9). This could be particularly important if macroradicals bearing protein tend to terminate by disproportionation, a behavior contrary to that of unsubstituted poly(acrylamide) macroradicals. However, a closer analysis of the molecular weight of the polymers present in peaks II and III shows some peculiarities (Table 1). In particular, these data show that: (i) the ratio between the molecular weights of the polymers present in peaks II and III is not constant; and (ii) the molecular weight of the polymer present in peak II barely changes with the protein concentration. These results indicate that the decrease of the molecular weight of peak III with the protein concentration is due to the behavior of the Cys-34 residue of BSA as chain transfer agent.

Considering that chain transfer to the polymer and initiator are negligible [35], that transfer to the monomer is much lower with respect to the thiol, and that chain transfer and termination processes are independent on the chain length of the growing macroradicals, the chain transfer constant (C_{tr}) can be determined from the experimental chain length value at different concentrations of the chain transfer agent by the classical Mayo equation [36],

$$\frac{1}{DP_n} = \frac{1}{(DP_n)_O} + C_{tr}\frac{[CTA]}{[M]} \quad (11)$$

where $(DP_n)_O$ and (DP_n) are the polymerization degrees in the absence and in the presence of the chain transfer agent ([CTA]), respectively, and [M] stands for the monomer concentration. Since amino acids and the protein decrease the molecular weight with negligible changes in the polymerization rates, C_{tr} is given by the ratio of the chain transfer rate constant (k_{tr}) to the propagation rate constant (k_p), Eq. (12)

$$C_{tr} = \frac{k_{tr}}{k_p} \quad (12)$$

Values of C_{tr} evaluated from Eq. (11) are shown in Table 2. The chain transfer constant for the −SH group of BSA was determined by the decrease of the molecular weight of peak III of the SEC chromatograms. Since, Cys-34 is partially oxidized [37], the concentration of free thiol was quantified by titration with 5,5′-dithiobis(2-nitrobenzoate) (DTNB). These experiments show that only 40% of the cysteine-34 residue is available, rendering a chain transfer constant value of 4.4, Table 2. This large chain transfer efficiency value is compatible with the notable effect of the protein upon the polymer intrinsic viscosity, as evidenced in Fig. 1. Data of Table 2 indicate that the −SH group in the protein is the most efficient chain

Table 1
Molecular weights of polymers present in peaks II and III at different BSA concentrations

BSA concentration, μM	Molecular weight, $\times 10^{-3}$	
	Peak II	Peak III
–	750	–
10	695	413
40	659	290
60	618	203
80	610	158

Table 2
Chain transfer constants for the polymerization of acrylamide in aqueous medium at 25 °C

Chain transfer agent	C_{tr}
BSA	4.4
Cysteine	0.32
Reduced glutathione	0.9
Tryptophan	0.0082
4-Ethylphenol	0.003
Histidine	0.0004

transfer agent among the three compounds bearing the −SH group, and that cysteine is three times less efficient as chain transfer agent than the tripeptide glutathione. Chain transfer constants vary with the thiol structure and its environment [28,32,33,38]. Lu et al. [28] have shown that electron-drawing groups increase the reactivity of thiols towards the *N*-(2-hydroxypropyl)methacrylamide derived macroradicals. Recently, Costioli et al. [33] have reported that chain transfer efficiency of thiols in the polymerization of *N*-isopropylacrylamide decreases when the polymerization is carried out in solvents able to form hydrogen bonds or present dipole–dipole interactions with the monomer. Considering that $C_{tr} = k_{tr}/k_p$, the enhancement of C_{tr} can be consequence of an increase of k_{tr} and/or a decrease of k_p. It is well known that the propagation rate constant of acrylamide and derivatives is much higher in aqueous medium than in organic media [39,40]. The lower accessibility of the −SH group to the aqueous medium in the protein, as compared with the free cysteine, could decrease the propagation rate constant, and then increases C_{tr}, as shown in Table 2. Furthermore, the microenvironment where the cysteine residue is located in the protein can increase the reactivity of the −SH group. In fact, highly reactive cysteine residues are found in several proteins [41,42]. On the other hand, the high reactivity of the thiyl radical formed by hydrogen abstraction from the thiol by the macroradicals explains its exclusive behavior as chain transfer agent. Tryptophan, phenol and histidine are markedly less reactive than cysteine. These differences can be ascribed to the low dissociation energy of the S–H bond [43].

Also, it is interesting to note that thiol titration in the polymerizated mixture, at the highest protein concentration used, showed only 10% of free thiol. This evidences a high incorporation efficiency of the protein to the conjugate, which is in agreement with the high efficiency of the Cys-34 residue as chain transfer agent of the acrylamide polymerization.

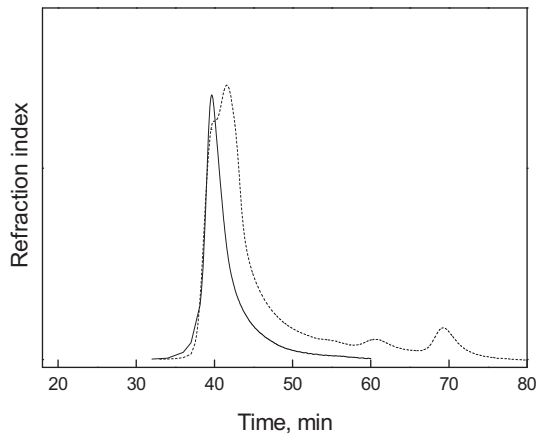

Fig. 4. SEC chromatograms of refraction index signal using the Superdex 200 column. Polymers obtained: (····) after the precipitation and dissolution of the polymerization mixture; (—) the polymer isolated by elution from the Superdex column.

3.3. Characterization of BSA-acrylamide conjugates

The isolation of the conjugate from the free protein and the non-conjugated polymers was accomplished by preparative SEC chromatography. In this case, a Superdex 200 column was used, which gives a better resolution for proteins than for lineal polymers. The SEC chromatogram of the polymers isolated from the polymerization mixture by precipitation and dissolution, and the conjugate isolated by a preparative column (Superdex 200) are shown in Fig. 4. It can be observed that the elution of the polymeric material isolated from the Superdex column shows only one peak, which is superimposed to that obtained using the fluorescence at the maximum emission wavelength of the BSA (342 nm) as detection. These results, and the lack of conjugate formation when the protein previously treated with ethylmaleimide was used as chain transfer agent, confirm the incorporation of BSA to the polymer through the Cys-34 residue. Furthermore, the fact that the retention time of the free BSA is higher than that of the conjugate (Fig. 3) shows that the protein is bound to the polymer.

Considering that the polymer is bound to the single Cys-34 residue exposed to the surface of the globular protein molecule, only negligible disorganization of the protein structure could be

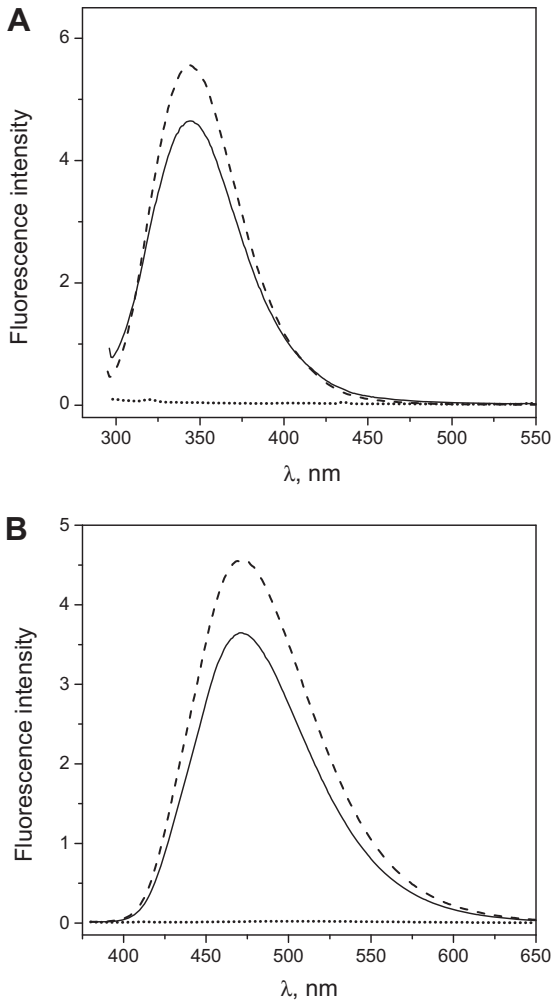

Fig. 5. Fluorescence spectra of (A): tryptophan residues of: (- - -) native protein; (—) conjugate isolated from the Superdex column; (…) poly(acrylamide), excitation wavelength at 290 nm. (B) Fluorescence of ANS (excitation at 360 nm) in the presence of: (- - -) native BSA; (—) polymer isolated by the Superdex column; (…) poly (acrylamide).

expected in the conjugate. In agreement with this, we observed that the fluorescence spectrum of the conjugate overlaps to that obtained with the free protein, Fig. 5A. This indicates that the tertiary structure of the protein is not markedly modified by its incorporation into the polymer chain.

To gain further insight on the BSA conformation in the conjugate, 1-anilino-8-naphthalene-sulfonic acid (ANS) fluorescence was evaluated in a sample of native protein, and in a sample of the isolated conjugate. It is well established that ANS binds with high affinity to hydrophobic domains in proteins [44]. In aqueous solutions, ANS presents a very weak fluorescence with a maximum at 520 nm that is markedly increased and blue shifted in non-polar environments. Fig. 5B shows that the fluorescence spectrum of ANS incorporated to the conjugate and to the native protein are almost identical. On the other hand, when ANS was added to the free BSA, previously denaturated by urea or guanidinium hydrochloride (Gnd) addition, the fluorescence intensity was two orders of magnitude lower than that measured in the native protein, and the maximum emission wavelength was shifted 25 nm to the red (data not shown). All these results suggest that the hydrophobic domain of the protein remains unaltered in the polymer-protein conjugate.

In conclusion, the exposed cysteine residue (Cys-34) of BSA presents a chain transfer constant markedly higher than the −SH group in the free cysteine amino acid or the −SH in glutathione tripeptide. This high reactivity allowed modification of the protein to a single polymer chain, without drastic changes in its conformation.

Acknowledgements

We thank FONDECYT (# 1070123), and DICYT (Universidad de Santiago de Chile) for the support of this research. Also, we thank Dr. Cardemil, University of Santiago, Chile, for his valuable help in the purification of the protein-polymer conjugate.

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