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Preparation and characterization of new biospecific adsorbents with fatty acids as ligands, usable to retain bovine serum albumin

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The property of albumin to bind long-chain fatty acids was exploited for the development of adsorbents containing them as ligands to isolate that protein. The synthesis of gels containing oleic or ricinoleic acids bonded to a synthetic matrix and the capacity to retain albumin are presented. The probable retention of other proteins (gelatin and lysozyme) was also tested. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

In the plasma, the proteins contribute to maintaining the volume of circulating fluid, transport substances relatively insoluble and act in the inactivation of toxic compounds and in defence against invader agents[1]. Normally, albumin is the more abundant protein in the plasma, representing more than 60% of the total. One of its more important functions is to transport long-chain unesterified fatty acids (C_{12}–C_{20}), generated in blood from adipose cells, which in free form are insoluble in aqueous media. Also, other lipophilic ligands from plasma and interstitial liquid such as retinol, vitamin D and steroids can be transported by albumin[2]. The acids bonded to albumin are non-toxic and usable for different tissues in the organism; in contrast, the free fatty acids have deep detergent properties, denaturalize proteins and cause problems in organelles[1].

The property of the albumin to bind fatty acids has been used in different studies for the development of specific adsorbents that contain such compounds as ligands to retain them since purification is very important for therapeutic purposes[3]. There exists a commercial gel obtained by the cyanogen bromide method[4] whose matrix (agarose) contains linolenic acid bonded covalently through a spacer. There are antecedents of coupling of alkylsuccinic acids[5], oleates and palmitates[6] to Sepharose gel through a diamine spacer, usable to retain albumin from plasma. The selectivity of the adsorbent allows it to eliminate quantitatively the albumin present in biological liquids in cases where its presence prevents chemical analysis or isolation of other proteins[1].

This work describes the synthesis and characterization of fatty acid-like structures coupled to a synthetic matrix[7], obtained in our laboratories, through epichlorhydrin (ECH) as spacer and activator. The fatty acids, oleic and ricinoleic, were covalently bonded by coupling under different reaction conditions. In addition, we compared the mechanism of affinity in the retention of bovine serum albumin (BSA) using such adsorbents, and the probable retention of other non-specific proteins such as gelatin and lysozyme was examined.

EXPERIMENTAL

Preparation of adsorbents

The polybutadienic hydroxylated-*co*-hydroxyethylmethacrylate (PB-HEMA) solid matrix was obtained through a crosslinking reaction using a polybutadienic hydroxylated resin (PB) and hydroxyethylmethacrylate (HEMA) as reactives in a 1:2 weight ratio, respectively. The reaction was carried out under reflux in benzene with stirring for 2.5 h using benzoyl peroxide (1 wt%) as initiator[7]. The PB-HEMA matrix was purified by exhaustive washes in a Soxhlet with water, ethanol, benzene and methanol for 8 h with each solvent and dried *in vacuo*. Their hydroxyl equivalents were determined. It was then activated by reaction with ECH and NaOH (10 N) in a 1:16:1 ratio of equivalents, respectively, at room temperature for 28 h with stirring, yielding a poly(butadienic hydroxylated-*co*-hydroxyethylmethacrylate-epichlorhydrin) (PB-HEMA-ECH) product[7]. The epoxy-activated gel was purified by washes in a batch system with water, methanol and acetone and dried. Their epoxy equivalents and swelling index (Sw) were then determined.

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Table 1 Comparison of different reaction conditions among PB-HEMA-ECH, fatty acids and NaOH

Product	PB-HEMA-ECH (equiv)	Fatty acid (equiv)	NaOH (equiv)	Time (h)	Amount of carboxyl groups (meq g^{-1})	Yield of reaction (%)	Sw (ml g^{-1})
I	1	1.0[a]	0.1[c]	4	–	28.8	4.2[e]
II	1	1.0[a]	0.1[c]	6	–	35.9	3.1[e]
III	1	1.0[a]	0.1[c]	8	–	32.9	2.6[e]
IV	1	1.5[a]	0.1[c]	4	–	62.5	2.6[e]
V	1	1.5[a]	0.1[c]	6	–	48.2	3.0[e]
VI	1	1.5[a]	0.1[c]	8	–	42.9	3.4[e]
VII	1	1.5[b]	1.0[d]	8	0.127	23.5	8.1[f]
VIII	1	1.5[b]	2.0[d]	8	0.140	31.2	10.8[f]
IX	1	1.5[b]	5.0[d]	8	0.174	34.1	16.7[f]

[a] Oleic acid was used
[b] Ricinoleic acid was used
[c] The NaOH used was 0.1 N
[d] The NaOH used was 0.4 N
[e] The Sw was determined in water
[f] The Sw was determined in 0.1 N NaOH

The PB-HEMA-ECH previously swollen in dimethylformamide (DMF) (15% w/v) was reacted with oleic acid, 0.1 N NaOH and triethylamine (TEA) (5 wt%) as reagent, in a water bath at 50–60°C. In this way, several reactions under different conditions of time and ratio of equivalents, described in *Table 1*, were carried out. The products, poly(butadienic hydroxylated-*co*-hydroxyethylmethacrylate-epichlorhydrin-oleic I–VI) (PB-HEMA-ECH-Oleic I–VI), were purified by several washes in a column with benzene, 50% ethanol, ethanol–0.075 M sodium phosphate (1 : 1) (termed 'acid–alcohol'), ethanol–0.05 N NaOH (1 : 1) and ethanol.

After coupling of oleic acid to the epoxy-activated matrix, the remaining epoxide reactive groups in each product (I–VI) were quantified in order to determine the yield of the reaction. The Sw of the products I–VI were determined in water (*Table 1*).

On the other hand, the PB-HEMA-ECH matrix was reacted with ricinoleic acid using different alkaline conditions. The PB-HEMA-ECH matrix previously swollen in DMF (15% w/v) reacted with ricinoleic acid in a ratio of equivalents of 1 : 1.5, respectively and with different amounts of equivalents of 0.4 N NaOH, as can be seen in *Table 1*. The reactions were carried out in a water bath at 50–60°C for 8 h, yielding poly(butadiene hydroxylated-*co*-hydroxyethylmethacrylate-epichlorhydrin-ricinoleic VII–IX) (PB-HEMA-ECH-Ric VII–IX), which were purified following the technique described for products I–VI.

The free carboxyl groups and remaining epoxide reactive groups in PB-HEMA-ECH-Ric VII–IX were quantified and these data were used to determine the yield of the reaction. Their Sw were determined in 0.1 N NaOH (*Table 1*). Products II, VI and IX were studied by i.r. spectroscopy.

Retention assays of BSA

The activated matrix PB-HEMA-ECH and final products (I–IX) were previously weighted (100 mg) and were incubated in a batch system with 5 ml of a solution of protein (0.30% w/v) in a phosphate-buffered saline solution (PBS) (0.15 M NaCl containing 0.02 M sodium phosphate at pH 7.55) for 24 h at 5°C and for 48 and 72 h at room temperature with stirring in order to determine the ability to retain BSA. The retained albumin was determined in duplicate, measuring the quantity of protein present in the supernatant, before and after the experiments were carried out, using a previously established calibration curve (absorbance vs. concentration). *Table 2* shows the results of retention of BSA. After each experiment of retention of BSA, the products were washed in a batch system with 'acid–alcohol' and with ethanol–0.05 N NaOH (1 : 1) until no more protein was present in the supernatant, followed by u.v.-visible spectrophotometry (λ: 280 nm).

Table 2 Retention of BSA (mg g^{-1} of dry gel)

Product	Fatty acid (ligand)	24 h incubation	48 h incubation
PB-HEMA-ECH	–	0.00	0.00
I	Oleic	6.25	26.15
II	Oleic	6.25	11.21
III	Oleic	6.25	5.00
IV	Oleic	0.00	1.20
V	Oleic	0.00	7.36
VI	Oleic	10.00	10.00
VII	Ricinoleic	32.30	34.80
VIII	Ricinoleic	44.60	45.50
IX	Ricinoleic	53.30	53.30

Retention assays of gelatin and lysozyme

The activated matrix and final products (I–IX) were previously weighted (50 mg) and were incubated in a batch system with 3 ml of a solution of gelatin 0.08% w/v) in PBS at pH 7.50 for 48 h at room temperature with stirring. Another set of weighed samples (50 mg) were incubated with 5 ml of a solution of lysozyme (0.10% w/v) in the same conditions previously described for gelatin. All experiments were carried out in duplicate. In all cases, the quantity of protein present in the supernatant was determined before and after the assays were carried out.

Materials and methods

The following chemicals were commercially acquired

and used: ECH (Riedel-de Haen), TEA (Sintorgan), oleic and ricinoleic acids (Carlo Erba), BSA (Riedel-de Haen), gelatin 225 bloom from calf skin (Aldrich), lysozyme from chicken egg white (Sigma) and bromocresolsulphonephthalein (BCF) and EDTA/Cu reagent (Wiener).

The determinations of hydroxyl equivalents of PB-HEMA and oxirane equivalents of activated matrix and final products were carried out using the acetyl and pyridinium chloride methods, respectively[8]. The quantification of carboxyl groups was performed through volumetric titration with a NaOH solution and phenolphthalein as indicator.

The Sw were determined from the volume (ml) reached by gram samples after swelling them in the solvent for 64 h at room temperature.

The quantification of BSA, gelatin and lysozyme was determined by u.v. spectrophotometry. In the case of BSA, it was carried out using an excess of BCF (3.1 ml) as reactive in bufferized medium (pH 3.8) and 0.1 ml of sample (supernatant of incubation) with stirring. After this, the samples were left for 10 min at 25°C before performing the determinations in the spectrophotometer. The increase of absorbance at $\lambda = 625$ nm against a reagent blank of reactive was proportional to the quantity of albumin present in the sample. These experiments were carried out using a calibration curve (absorbance vs. concentration).

In contrast, for gelatin and lysozyme, 1 ml of the supernatant of incubation from all experiments was reacted with 3 ml of EDTA/Cu alkaline reagent at 37°C during 15 min, followed by reading of the absorbance at 540 nm, in duplicate, against a reagent blank.

The i.r. spectra were recorded on a Nicolet 5-SXC Fourier Transform Infrared spectrometer.

The u.v.–visible spectra were recorded with a Shimadzu recording spectrophotometer UV-260.

RESULTS

Preparation of the adsorbents

The activation of the PB-HEMA matrix (3.93 meq. of hydroxyl groups per gram of dry gel), carried out on hydroxyl groups in alkaline medium with ECH, yielded the epoxy-activated PB-HEMA-ECH product which contained 0.85 meq. of oxirane groups per gram of dry product and had a Sw of 2.1. Such oxirane groups were covalently bonded at the oleic-carboxyl group or at the C_{12} hydroxyl group of ricinoleic acid. With oleic acid, the esterification reaction was favoured by the presence of TEA used as catalyst, which confers specificity to the epoxide–acid reaction[8] and eliminates side reactions between oxirane and hydroxyl groups existing in the matrix or those in formation.

After the reactions were produced in different experimental conditions to obtain products I–VI (*Table 1*), the quantification of oxirane groups was carried out to estimate the percentage of reaction produced by the ratio of the quantity of remaining groups before and after the experiments were carried out.

The increase of the reaction time to obtain products I–III produced a higher percentage of reaction of the epoxy groups followed by its subsequent diminution probably due to hydrolytic reactions. The increase of the percentage of reaction could indicate greater access of ligand to the activated matrix with subsequent diminution in Sw because of steric hindrance to the diffusion of water[1] due to the presence of the hydrocarbon chain. This was correlated with a diminution of its hydrophilicity.

The excess of oleic acid used to perform the reactions to obtain products IV–VI involves a higher binding of ligand with shorter time of reaction and later decrease, probably due to hydrolytic reactions. This behaviour involves an increase in the swelling of products in water.

Products VII–IX, carried out by an etherification reaction, were obtained with excess of ricinoleic acid in different alkaline media. In these cases, the larger the quantity of NaOH in the reaction, the higher its percentage of reaction and the amount of milliequivalents of free carboxyl groups per gram of dry gel. The Sw in NaOH solution are increased because of the presence of carboxylate groups in such media (*Table 1*).

The i.r. spectra of products II and VI show a relative increase of the bands at 1729 and 1275 cm^{-1} (carbonyl of ester group) and 1163 and 1084 cm^{-1} (ether group) in comparison with the i.r. spectrum of the epoxy-activated matrix[7]. In contrast, a band appears at 900 cm^{-1} corresponding to the deformation of C–H in the double bond C–C from oleic acid.

The i.r. spectrum of product IX shows signals at 1717, 1650 and 1099 cm^{-1} attributable to the stretching vibrations of the carbonyl of the acid group, double bond C–C and ether bonding, respectively.

Retention assays of BSA

Table 2 shows results for the retention of BSA from a solution prepared in PBS at pH 7.55, which in products I–VI increases with time of incubation, temperature and stirring. Hence, for 48 h of incubation, the retention of BSA can be observed to be directly related to the Sw of the products I–VI in water, since when the Sw increases, the retention is increased also, as can be seen in *Figure 1*, probably due to the best accessibility through diffusion of the biological macromolecule.

The behaviour of products III and IV that present the same Sw, but different retention of BSA, could be due to the fact that these products reached different percentages of reaction (32.9 for product III and 62.5 for product IV), with the probable consequence of association of hydrocarbon tails from the ligand (for product IV, which retains a lesser quantity of BSA with respect to product III), which begin to play a certain role at some critical content of the ligand[1]. On the other hand, a high concentration of coupled ligand is likely to increase steric hindrance, reducing in this way binding efficiency of the adsorbents[9].

In contrast, the epoxy activated matrix PB-HEMA-ECH did not retain BSA under the experimental conditions used.

For products VII–IX, the retention of BSA is independent of time and temperature of incubation, although it is increased with the amount of free carboxyl groups and Sw. This can be seen in *Figure 2* (data of 48 h of incubation).

On the other hand, denaturation of the protein was noticed when the retention experiments were carried out for 72 h.

Retention assays of gelatin and lysozyme

For the epoxy-activated PB-HEMA-ECH matrix and

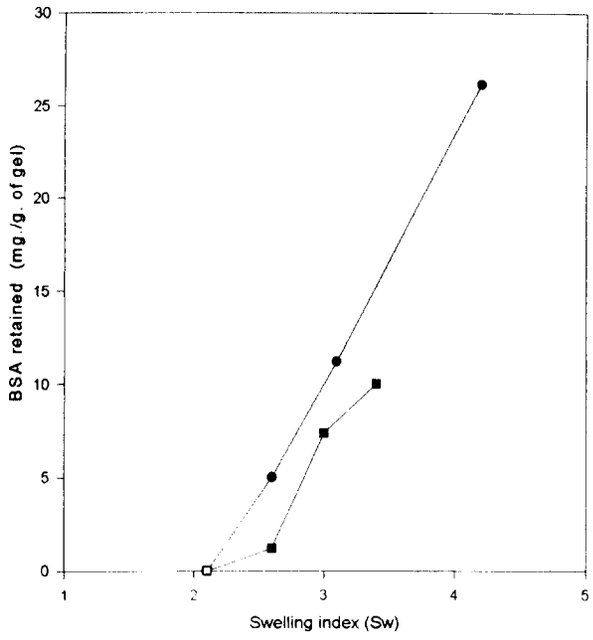

Figure 1 BSA retained vs. Sw: (□) unmodified PB-HEMA-ECH; (●) products I–III and (■) IV–VI; fatty acid bound: oleic

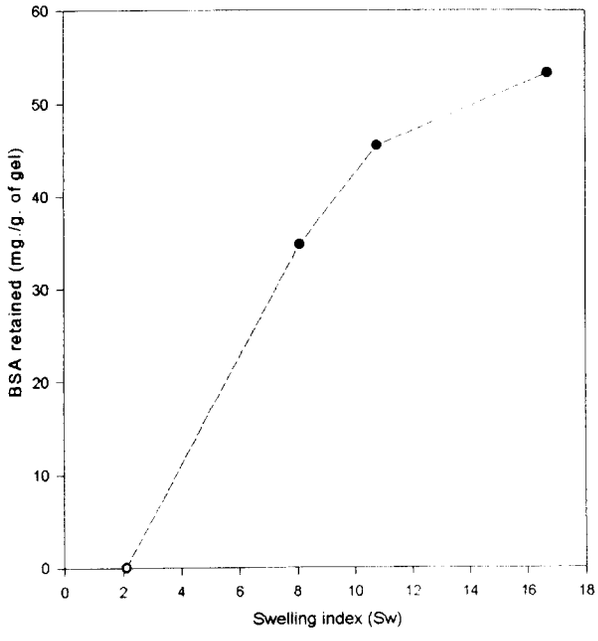

Figure 2 BSA retained vs. Sw (○) unmodified PB-HEMA-ECH; (●) products VII–IX; fatty acid bound: ricinoleic

products I–IX, the retention of gelatin and lysozyme from solutions prepared in PBS at pH 7.50 was zero.

DISCUSSION

A preliminary report[10] indicates that the BSA contains six high-energy binding sites for long-chain fatty acids resolved into two distinct classes, each containing three sites and a large number of weak binding sites. This model is comparable with results of studies with short-chain, anionic dyes and detergents, which indicated that BSA has the capacity to bind organic-like ligands. The observations reported by Spector *et al.*[10] indicated that 6–13 mol of long-chain fatty acids can be bound per mole of albumin, although such binding is limited by the solubility of the fatty acids in aqueous solutions and not by the number of sites of binding of the protein. Such a model assumes that each site of binding of albumin competes independently with fatty acids; nevertheless, there exists the possibility that some or all weak sites are formed from an alteration in the conformation of the albumin arising from the union of fatty acids to sites of high energy.

Such association of fatty acids with albumin involves, at pH 7.6, electrostatic interaction of the carboxyl groups with cationic sites of the protein (guanidinium group of arginin residue and ϵ-ammonium group of lisin residue of the albumin), whose union decreases when those groups are modified or removed[10]. Besides, there is significant hydrophobic interaction between the non-polar side chains of the fatty acids and lateral sites of the protein products by penetration of the hydrophobic fragment of the ligand into the hydrophobic cavity of the protein by a number of short-range non-specific van der Waals interactions.

In the retention of BSA of PB-HEMA-ECH-Oleic I–IV (26.15 mg g^{-1} of dry gel), the acid was bonded to the activated matrix through the carboxyl group whereas the hydrophobic chain is responsible for the union with the exposed non-polar amino acid sites of the albumin. In contrast, in the case of PB-HEMA-ECH-Ric VII–IX, the free carboxyl groups in anionic form are the principals responsible for the fixed site retaining the larger quantity of the protein (53.30 mg g^{-1} of dry gel) together with the hydrophobic interactions previously described. Strong interactions between carboxylate groups of fatty acids bonded to intestinal fatty acid binding proteins (FABP) appear to be located in an inner region of the folded macromolecule and are probably involved in ion-pair electrostatic interactions. For other FABP, the interactions are lower[11].

In order to check the specificity of retention of BSA from products I–IX, some assays to prove the possible retention of gelatin (fibrilar hydroxylysine-containing protein without lipophilic centre) and lysozyme (globular protein that contains a lipophilic centre) were performed under the same experimental conditions as for BSA. In both cases, the retention was null, confirming the specific interaction of the new fatty acid-containing products with BSA.

On the other hand, the quantity of BSA retained with the synthetic products presented in this work was comparable to that attained using gels obtained from macromolecules from natural sources[6].

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REFERENCES

1 Plate, N. A. and Valuev, L. I. *J. Polym. Sci.: Polym. Symp.* 1979, **66**, 149
2 Godovac-Zimmerman, *J. Trends Biochem. Sci.* 1988, **13**, 64
3 Peters, T. *Adv. Clin. Chem.* 1970, **13**, 37
4 'Sigma Catalog', Sigma Chem. Co., St. Louis, MO, 1993, p. 1646
5 Wichman, A. and Andersson, L. O. *Biochim. Biophys. Acta* 1974, **372**, 218

6 Peters, T., Tanluchi, H. and Anfinsen, C. B. *J. Biol. Chem.* 1973, **248**, 7, 2447
7 Alvarez, C., Bertorello, H. and Strumia, M. *J. Appl. Polym. Sci.* 1994, **54**, 1887
8 Lee, H. and Neville, K. 'Handbook of Epoxy Resins', McGraw-Hill, New York, 1967, Ch. 4, p. 17
9 'Affinity Chromatography. Principles and Methods', Pharmaca Fine Chemical, Uppsala, Sweden, 1979, Ch. 6, p. 84
10 Spector, A. A., John, K. and Fletcher, J. E. *J. Lipid Res.* 1969, **10**, 56
11 Veerkamp, J. H., van Kuppevelt, T. H. M. S. M., Maatman, R. G. H. J. and Prinsen, C. F. M. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 1993, **49**, 887