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Preparation of new gels derived from poly(sucrose acrylate) with immobilized Cibacron Blue and their application in affinity chromatography

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Summary

New pseudoaffinity chromatography supports were prepared and characterized from poly(sucrose acrylate) crosslinked with MDI or TDI and Cibacron Blue dye as ligand. Blue gels were useful in the retention of albumin and the results are reported.

Introduction

The isolation of proteins from blood plasma or serum often turns complicated by the presence of albumin as a contaminant. Conventional purification procedures are not reliable for the production of albumin free fractions [1].

It is well established that immobilized sulphonated aromatic dyes of the chlorotriazine type such as Cibacron Blue F3GA are effective ligand adsorbents for the isolation and purification of hundreds of enzymes and other proteins, such as albumin [2-3].

Travis and Pannell [4] reported the interaction of human serum albumin with these dyes and developed an efficient procedure of affinity chromatography for the removal of albumin from other serum proteins.

The interactions between ligand-protein play an important role in the technique of affinity chromatography and depend strongly on the properties of the matrix [5] and on the mode of covalent attachment of the dye to the insoluble support [6]. In this way, triazine dyes have been immobilized to a wide variety of support matrices in the search for an ideal system. Some of the supports that have been examined include agarose, dextrans, agarose-polyacrylamide copolymers, cellulose and glass [5-7-8].

This aspect of the affinity technique has been extensively developed in the last few years in response to a greater demand. Thus, the need of new polymeric supports that improve the qualities of the matrix, the activation chemistry, ligand selection and the purification format itself have all been reexamined in the light of these new requirements.

In this paper we describe the synthesis and characterization of new affinity materials with Cibacron Blue as ligand and report its utility in the biospecific retention of albumin.

The polymeric base support is a poly(sucrose acrylate) (PAS), previously synthesized in our lab [9], and crosslinked with toluene diisocyanate (TDI) or methyldiphenyl diisocyanate (MDI).

Experimental

Materials and analysis: the following chemicals were commercially acquired and used: sucrose (Mallinckrodt); acrylic acid (BASF Argentina); thionyl chloride (Merck); TDI

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(Petroquimica Rio III); MDI (Merck) and Cibacron Blue F3GA (Ciba Geigy); human serum albumin (supplied by Laboratorio de Hemoderivados. Univ. Nacional de Córdoba).
IR spectra were recorded on a Nicolet 5-SXC FTIR spectrophotometer.
Column chromatography of monomers was obtained by silica gel column separation (Kiesegel Gnach Stahl Merck AG) using a mixture of propanol and water (7:1) as solvents. The main products were isolated and identified by ^{1}H-NMR.
Scanning electron microscopy (SEM) was performed on a Phillips SEM 501 B instrument at the laboratories in the Centro de Investigaciones de Materiales y Metrologia (CIMM).
Swelling indexes (Sw) were determined in water as the ratio of the swollen (Vs) and dried (Vd) volumes of samples, respectively (Sw=Vs/Vd).
Albumin was measured using a modified version of the Lowry method [10] and UV-Vis spectra were measured with a Shimadzu UV 260 recording spectrophotometer.
Synthesis of poly(sucrose acrylate) (PAS)
The sucrose acrylate was obtained by reaction between 6.10^{-2} eq. of acryloyl chloride 0.5M and 3.10^{-2} eq. sucrose 0.3M both in DMF with an acyl chloride: sucrose molar ratio of 2:1.
Table I: Cibacron Blue (CB) covalently bonded to PAS-TDI and PAS-MDI.

Contact time (h)	PAS-TDI µmol CB/g dry gel	PAS-MDI µmol CB/g dry gel
1	7.14	11.35
2	10.95	13.40
3	24.20	16.74
4	24.30	18.45
5	21.60	19.90
72	17.10	17.20
3 (a)	13.29	10.00

(a): heating 1 h at 60 C and 2 h at 80 C

The reaction was carried out for 1 h at room temperature and then for 30 min. at 40°C. Finally, the product was precipitated with 50 ml of toluene, washed with 50 ml of acetone and dried until constant weigth. The identification and quantification of the relation of monomers gave 40% of monoesters, 34% of diesters and 17% of unreacted sucrose as well as other unidentified products.
The homopolymer was obtained by reaction of 10 g of sucrose acrylate monomers with 1% w/w of benzoyl peroxide as initiator. Polymerization in bulk was carried out in a nitrogen atmosphere with illumination from a mercury lamp (350 nm) for 4 h. Then the reaction mixture was heated at 35°C under vacuum for 2 h. A solid yellowish product (PAS)(9.5 g), soluble in water, ethanol and DMF, was obtained [9].
Crosslinking with TDI and MDI: a) Synthesis of PAS-TDI: to 0.5 g of PAS previously dissolved in 10 ml of dry dioxane at 40°C, were dropped different aliquots of TDI between 30 and 50% w/w and maintained in these conditions for 2 h. The products were purified by repetitive washes with water and methanol, alternatively.
b) Synthesis of PAS-MDI: it was carried out under the same conditions as those described in a). The concentration of MDI was used between 18 to 63% w/w.

Table II: Retention of Albumin from PAS-TDI-CB and PAS-MDI-CB.

Time (min.)	PAS-TDI-CB mg albumin/ g gel	PAS-MDI-CB mg albumin/ g gel
15	25.47	30.63
30	26.20	31.89
45	30.47	32.40
60	31.52	33.36
75	34.90	33.50
90	35.54	33.67
105	35.26	39.67
120	35.16	39.14

Coupling of Cibacron Blue (CB): different reaction conditions were used to obtain the blue gels. 5 ml of a solution of CB 6.10^{-3} M in water was added with stirring to a suspension of 0.5 g of the gels at room temperature. After stirring for 30 min, 0.5 ml of NaCl 2 M was added and the stirring was continued for 45 min. After, to the mixture was added 0.5 ml of Na_2CO_3 1.10^{-2} M and kept for different times (See Table I). Then the gels were filtered by suction and washed with water and NaCl 1 M until the filtrate became colourless.
Cibacron Blue bonded to the support was calculated from the difference between the initial and final concentrations of the mother solution before and after the reactions were carried out by UV spectroscopy using an extinction coefficient of 18.51 at 610 nm.
Pseudoaffinity chromatography: the obtained blue gels were used in a batch system in order to determine the ability to retain albumin. 0.5 g of blue gels were putting in contact with 2ml of a solution of human albumin (20g/100ml) in a buffer Tris-HCl 0.2M (pH=8) at room temperature. Aliquots were run from a remaining solution every 15 min to know the concentration of albumin present (See Table II).

Results and discussion

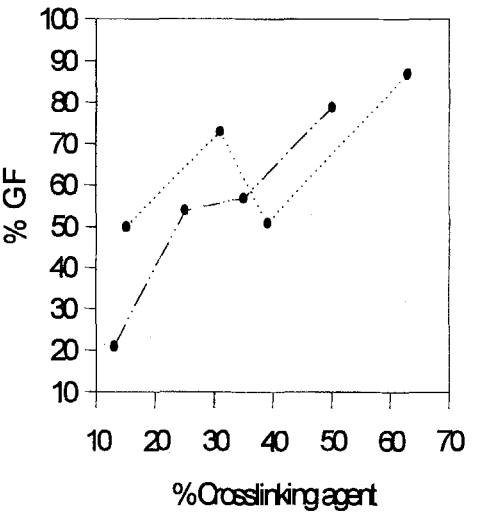
Preparation of pseudo- biospecific affinity adsorbents: PAS is a viscous colourless liquid soluble in water, methanol, ethanol, THF and DMF.
Synthesis and characterization of monomer and polymer have been previously reported by us[9].
PAS was crosslinked with different concentrations of crosslinking agent with the aim of finding the best ratio between the yield of gel fraction (%GF) and swelling indexes (Sw). Figures I and II show the relation to %GF and Sw with percent of crosslinking agent used, respectively. It can be seen in Fig. I and II that PAS-MDI shows better %GF and Sw than PAS-TDI. In the same way, we can observe that by increasing the amount of added crosslinking agent, the %GF increases while Sw decreases. This is an expected behaviour if we take into account that when an increased of MDI and TDI is added a more compact crosslinked network is formed. The crosslinked products were a yellowish solid powder.
The IR spectra of PAS-MDI and PAS-TDI show the characteristic bands corresponding to the presence of urethane signals: PAS-MDI, 3289 cm^{-1} (OH); 1703 cm^{-1}, (-C=O ester); 1643 cm^{-1} (amide I); 1604 cm^{-1}(phenyl); 1545 cm^{-1} (phenyl and amide II); 1512 cm^{-1} (phenyl).

PAS-TDI, 3302 cm^{-1} (-NH and -OH); 1722 cm^{-1} (-C=O ester); 1650 cm^{-1} (amide I); 1604 cm^{-1} (phenyl); 1545 cm^{-1} (amide II); 1512 cm^{-1} (phenyl).

The gels selected for the study of coupling of dye were PAS-MDI crosslinked with 31% of MDI and Sw= 4.5 and PAS-TDI with 25% of TDI and Sw= 3.0.

Coupling of CB to PAS-MDI and PAS-TDI through the reaction between the -OH of PAS and chlorine of triazine ring of the dye, were performed as shown in Table I. The best experimental conditions were those using room temperature and a contact time of 5 h for PAS-MDI and 3 h for PAS-TDI. Although the concentration of ligand bonded to the support was great when the temperature was raised and the contact times were longer, it was observed a 10 % loss weight of the support, attributed to the starting chemical degradation. On the other hand, it is wellknown that high concentration of ligand bonded to the support would not be advantageous for the captation assays [10].

Figure I: Yield gel fraction (%GF) vs. %MDI (.....) and TDI (..).



Blue gels revealed by IR spectroscopy the characteristic bands to CB coupling to the supports: 3600 and 3500 cm-1 (N-H); 3387 cm-1 (H-N-H); 2500-2300 cm^{-1} (C-C aromatic ring); 1700 cm^{-1} (ester); 1650 cm^{-1} (C=C conjugated, C=N, C-O-C); 821 cm^{-1} (C-C out of plane).

Scanning electron microscopy (SEM) studies of PAS-MDI-CB and PAS-TDI-CB show wrinkled and irregular size particles with a porous surface of variable pores sizes.

Pseudoaffinity chromatography: the blue gels chosen for the captation experiments were PAS-MDI with 19.90 μm of dye/g dry gel and PAS-TDI with 24.20 μm of dye/g dry gel. Experimental conditions used for albumin captation are detailed in Table II. The best conditions that showed the highest albumin concentration retained were obtained at 90 min for PAS-TDI and 105 min for PAS-MDI, respectively.

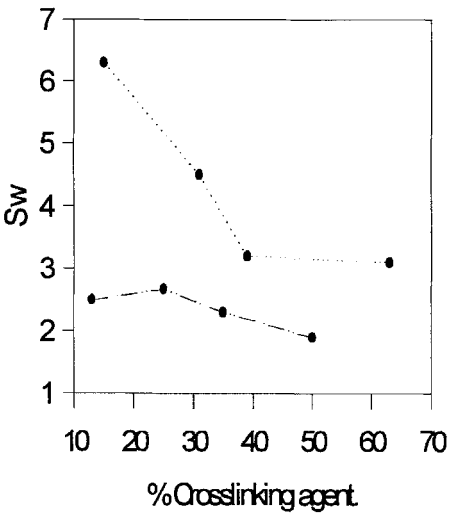
The absence of non-specific interactions was corroborated by using the matrices without

dye covalently bonded, whose capacity to retain albumin were zero. The maximum capacity of retention from the supports was of 39.67 mg albumin/g dry gel and 35.54 mg albumin/g dry gel for PAS-MDI and PAS-TDI, respectively.This value was comparable with the results obtained with commercial gels [5,11] using naturally modified macromolecules, whose captations are in the order of 30 mg albumin/ml of CB containing gels.

Conclusions

The synthesis of supports for affinity chromatography is an important subject of research in order to obtain new adsorbents applicable in the purification or isolation of proteins. The results herein reported, contribute to new supports which could be used for the retention of albumin through Cibacron Blue as ligand. These supports derived from Poly(sucrose acrylate) crosslinked with TDI or MDI, containing Cibacron Blue covalently bonded (24.20 μm/g gel and 19.90 μm/g gel) to its and with good stability properties, were able to retain

Figure II: Swelling Indexes (Sw) vs. % MDI (...) and TDI (..).



35.54 mg and 39.67 mg of albumin, respectively. These values were comparable or better than other commercial hydrogels used at present.

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