Preparation of New Synthetic Hydrogels Containing Galactose, Applicable in Affinity Chromatography

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SYNOPSIS

New biospecific affinity chromatography adsorbents were prepared from epoxy reactive matrices with subsequent coupling of galactose as the ligand and were afterward characterized. Their usefulness in the sorption of peanut lectin, a protein specifically retained by the galactose residue, is reported. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

An important subject of the macromolecular chemistry involves the preparation of materials with specific properties. These compounds that contain functional reactive groups in polymer chains, in branches pending from it, or in the cross-linking agent used were obtained by conventional methods of synthesis or, preferentially, by modification of natural polymeric materials able to work as gels.[1-3] The reactive functional groups on the supporting gel were attached covalently, in many examples, to carbohydrates, coenzymes, nucleotides, etc.,[4-6] in order to work as ligands.

Many types of affinity adsorbents having carbohydrates as ligands have been used in the isolation and purification of cell-agglutinating proteins called lectins.[5-9] These proteins bound in a specific form to sugar residues[10] and are widely used in chemical and biological research.

Few reports are available about adsorbents such as polyacrylamide entrapped guar beads and polyacrylamide copolymerized with allyl α-D-galactopyranoside,[7] Sepharose-ε-aminocaproyl-β-galactopyranosylamine,[11] and N-1-(1-deoxylactilol) aminoethyl Bio Gel P-150,[12] which were able to purify in a bioselective form the peanut lectin, a galactose binding anti-T agglutinin.

We previously synthesized a hydrogel obtained by the reaction of a polybutadiene–acrylic acid oligomer (BuAA) with lactose and hydroxyethylmethacrylate (HEMA).[13] This was able to retain low amounts of proteins, probably due to the difficulty of the biological macromolecule to get lactose groups in the reticular structure. Another way followed was with a bisoxirane (DGEBA), which was used as spacer to expose the carbohydrate out of the reticulated structure, without enhancing the results.

In this article, we present the synthesis of new hydrogels obtained from a polybutadienic hydroxylated-*co*-hydroxyethylmetacrylate (PB–HEMA) gel as a matrix. The epoxidation of this hydrogel was performed by using epychlorhydrin (ECH) as the reactive, by putting oxirane functional groups at the end of the spatial chains, and, then, by reaction with galactose. In this way, we were able to synthesize hydrogels having galactose at the end of spaced-armed chains, pending from the cross-linked structure. Their use in affinity chromatography of peanut lectin is also reported.

EXPERIMENTAL

Materials

The following chemicals were commercially acquired and used: epychlorhydrin (Riedel de Haen), D-galactose (97% Aldrich), and peanut lectins (Sigma).

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Methods

For the determination of carbohydrates and proteins, quantification of galactose was carried out with the phenol–sulfuric acid method[14] and proteins were determined measuring absorbance by UV spectrophotometry at $A_{280}^{1\%} = 7.7\ cm^{-1}$.[11]

IR spectra were carried out on a Nicolet 5-SXC spectrophotometer, Fourier transform infrared (FTIR) spectrometer. Scanning electron microscopy was performed on a Phillips SEM 501 B equipment at the Centre for Research of Material and Metrology (CIMM) Laboratories. UV-visible spectra were recorded with a Shimadzu recording spectrophotometer UV 260.

Swelling indexes (Sw) were determined by ratio of the swelled (Vs) and dried (Vd) volumes of the samples, respectively: $Sw = Vs/Vd$. Determinations of hydroxyl and epoxy equivalents were done using the acetyl chloride and pyridinium chloride methods, respectively.[15]

Preparation of the Specific Affinity Adsorbents

Synthesis of the PB–HEMA Matrix

The polybutadienic-*co*-hydroxyethylmethacrylate (PB–HEMA) matrix was obtained through a reaction using a polybutadienic resin (PB) obtained by us by radical polimerization[16] and previously purified by fractionated precipitation using benzene as the solvent and methanol as the nonsolvent and hydroxyethylmethacrylate (HEMA) in a 1 : 2 ratio, respectively. The reaction was carried out at reflux in benzene with stirring for 2.5 h using benzoyl peroxide as initiator. The product PB–HEMA was dried and purified in a Soxhlet with water, ethanol, benzene, and methanol for 8 h with each solvent.

The swelling indexes of the product PB–HEMA were determined in DMF, benzene, water, acetone, toluene, and dioxane. Determination of the hydroxyl equivalents was done and an IR spectrum was carried out.

Reaction of the PB–HEMA Matrix with ECH

To obtain products with a high content of oxirane reactive groups, several reactions under different conditions of temperature, concentration, and ratio of equivalents of reactives, solvent, and time of reaction were carried out. In every case, the PB–HEMA matrix was mixed with ECH and NaOH under the conditions described in Table I. The reactions were carried out with a magnetic stirrer. The products were purified, and their epoxy equivalents were determined and studied by IR on KBr disks.

Reaction of Galactose with the PB–HEMA–ECH Matrix

Different conditions of the reaction were used to obtain products with galactose coupled to the activated matrices. Then, the concentration and ratio of equivalents of reactives, time, temperature, and solvent were changed in order to obtain higher amounts of galactose in the macromolecular structure (see Table II).

Table I Different Reaction Conditions Among PB–HEMA, ECH, and NaOH

PB–HEMA–ECH Product	PB–HEMA (Equiv)	ECH (Equiv)	NaOH (Equiv)	Temp (°C)	Time of Reaction (h)	Mequiv Epoxy/g of gel	Observed
I	1[a]	8	3.5[b]	40	8	0	[c]
				65	15		
II	1[a]	10	1[d]	60	2.5	0.49	[e,f]
III	1	20	2[d]	60	5.5	0.96	[e]
IV	1[g]	8	3.5[e]	Room temp	26	0.79	[e]
V	1[a]	8	3.5[b]	Room temp	26	0.70	[e]
VI	1	16	1[d]	Room temp	28	1.50	[e]

[a] Prior to reaction, the PB–HEMA matrix was swollen in the least volume of water for 48 h.
[b] The NaOH used was 1*N*.
[c] Product PB–HEMA–ECH (I) was washed in a Soxhlet with water and methanol.
[d] The NaOH used was 10*N*.
[e] Products PB–HEMA–ECH (II)–(VI) were washed in a batch system with water, methanol, and acetone.
[f] After 30 min of reaction, a liquid mixture composed by ECH/water was distilled under reduced pressure. ECH was separated and recycled to the flask in order to continue the reaction for 1 h 30 min. The procedure was repeated once more and the reaction continued for 30 min.
[g] The PB–HEMA matrix was swollen in the ECH used as the reactive.

Table II Different Reaction Conditions Between Epoxidized Matrix and Galactose (Gal)

PB–HEMA–ECH–Gal Product	PB–HEMA–ECH (Equiv)	Gal (Equiv)	NaOH (Equiv)	Temp (°C)	Time	μmol of Gal/g Gel
VIII	1[a]	5[b]	0.02[c]	40	6 h	9.75
IX	1[d]	10[e]	0.1[c]	40	6 h	14
X	1[d]	10[e]	0.1[c]	40	9 h	6
XI	1[d]	10[e]	0.1[c]	40	18 h	8.5
XII	1[d]	10[e]	0.1[c]	40	18 h	
				room temp	6 days	1.74
XIII	1[d]	15[f]	6[g]	45	8.5 h	32.26

[a] PB–HEMA–ECH-activated matrix was swollen in water.
[b] Galactose was dissolved in water : phoshpate buffer, pH 12 (1 : 2).
[c] NaOH 0.5*N* was used.
[d] PB–HEMA–ECH-activated matrix was swollen in DMF for 17 h.
[e] Galactose was dissolved in water : DMF : phosphate buffer, pH 12 (1 : 2 : 2).
[f] Galactose was dissolved in NaOH 0.1*N*.
[g] NaOH 0.1*N* was used.

The epoxy reactive PB–HEMA–ECH (VI) matrix reacted with galactose, NaOH, and BH_4Na (0.2% w/w of galactose). The products obtained were washed extensively in a batch system with water, 0.2*M* sodium acetate buffer (0.5*M* ClNa) at pH 4.06 and, finally, with 0.2*M* borate buffer (0.5*M* ClNa) at pH 8. They were studied by SEM. The covalently binded galactose was quantified after 23 h of submitting the products to hydrolysis with 2 N ClH in dioxane : water (8 : 2) at 100°C.

Specific Retention of Lectin to the Matrix PB–HEMA–ECH–Gal (XIII)

Test of Direct Fluorescence. The test of direct fluorescence with agglutinin peanut labeled with fluorescein isothiocyanate was carried out to confirm visually the adsorption of lectin on the biospecific adsorbent. Observation using a fluorescent microscope was made and showed intense fluorescence.

Affinity Chromatography. The obtained gel XIII was used in a batch system in order to determine the ability to retain peanut lectins. It was previously weighed and put in contact with a solution of peanut lectin in a phosphate saline buffer (pH 6.8) for 16 h at 4°C. The absorbance at 280 nm was measured before and after the experience was carried out and the protein retained was determined.

RESULTS AND DISCUSSION

Synthesis of the PB–HEMA Matrix

The hydroxyl-containing PB–HEMA was a yellowish solid powder product. Its swelling indexes in different solvents were the following: DMF: 8, water: 2.4, dioxane: 2.4, acetone: 2.2, toluene: 1, and benzene: 1. The determination of hydroxyl groups was 3.93 mequiv per gram of dry gel.

The IR spectrum of PB–HEMA showed the appearance of a signal at 3000–3650 cm^{-1} due to the presence of hydroxyl groups and a relative lowering of the signals at 1664 cm^{-1} (C=C bond) and at 910 and 960 cm^{-1} (C—H of alkenes) in relation to the IR spectrum of PB.

Reaction Between PB–HEMA Matrix and ECH

The hydroxyl groups of PB–HEMA were reacted with ECH, obtaining products with different contents of terminal oxirane functional groups in their structures, as can be observed in Table I. During the reaction, the ECH was used in excess to avoid side reactions leading to the formation of cross-linking products.

The first reaction to obtain the product PB–HEMA–ECH (I) was carried out for 8 and 15 h at 40 and 65°C, respectively. Those products contained no free epoxy groups due to possible cross-linking between polymeric chains, according to the temperature and time of reaction used. In later reactions, at temperatures of 60°C, the time of the reaction was decreased or it was increased at room temperature, yielding products with a high content of free epoxy reactive groups (PB–HEMA–ECH II–VI). On the other hand, other reactions were performed using higher concentrations of NaOH to minimize the amount of water in the medium, which could produce side reactions and promote poor efficiency in epoxidation.

To obtain the product PB–HEMA–ECH (II), the mixture of ECH/water was distilled out during the reaction and the ECH was separated and recycled to the flask to remove the excess water. The content of oxirane reactive groups in this product was low (0.49 mequiv g^{-1} of dry gel). For this reason, the concentration of ECH and the amount of NaOH were increased in the next reaction, and we obtained higher values of the oxirane content. In the product PB–HEMA–ECH (III), it rose to 0.96 mequiv g^{-1} of dry gel.

Other reactions, carried out to obtain the products PB–HEMA–ECH (IV) and (V), were performed by maintaining the matrix in a semidry state, using ECH as the reactive and solvent, and swollen in the minimum amount of water, respectively. The determination of epoxide groups showed no significant changes, indicating that previous swelling of the matrix in water in the conditions of time and temperature described do not vary the results.

The product PB–HEMA–ECH (VI) was obtained after submitting the epoxidized product PB–HEMA–ECH (V) to the reaction with an excess of reactives, using a high concentration of NaOH, obtaining in a shorter time of reaction an increase in the amount of oxirane reactive groups in the macromolecule.

The IR spectra of the epoxidized products showed a relative diminution of the signal at 3300–3600 cm^{-1} of the hydroxyl groups in comparison with the IR spectrum of the PB–HEMA matrix and the presence of oxirane reactive groups at 807 and 876 cm^{-1}.[17] The spectrum of product I without free oxirane reactive groups showed the band at 1070 cm^{-1} of the ether bond formed in the cross-linkage.

Reaction of Galactose with the PB–HEMA–ECH Matrix

Galactose was covalently coupled to the PB–HEMA–ECH matrix, as a ligand able to retain peanut lectins. The reaction involved PB–HEMA–ECH as an activated matrix, galactose, NaOH, and $NaBH_4$. This last reactive was added in catalytic amounts in order to avoid decomposition, uncoloring, and oxidation reactions. Table II summarizes the results obtained and gives the amount of coupled galactose under optimal conditions.

The coupling of galactose by the epoxidized matrix was favored by using DMF as solvent, since it is highly swellable in that solvent. By increasing the time of the reaction to obtain products PB–HEMA–ECH–Gal (IX–XII), under the same conditions of concentration, ratio of equivalents, and temperature, the amount of galactose decreased significantly, due probably to hydrolytic reactions. Finally, the best result was obtained when the amount of NaOH and the galactose used were raised and at a temperature of about 45°C for 8.5 h of reaction, yielding the product PB–HEMA–ECH–Gal (XIII).

The determination of galactose was carried out in concentrated acid medium using dioxane : water as the solvent. In this medium, the product was swollen and the hydrolysis was favored.

Based on the hydrolytic reaction and later quantification by using the method of phenol–sulfuric acid,[14] it was established that, e.g., in product XIII, 32 μmol of sugar (galactose) had been covalently attached per gram of dry gel (6.4 μmol per mL of swelled hydrogel). The modification on the matrix to obtain PB–HEMA–ECH–Gal (XIII) gave a porous-surface hydrogel that was confirmed by SEM, as observed in Figure 1, using a magnification of 640.

Test of Direct Fluorescence

The test of direct fluorescence with agglutinin peanut labeled with fluorescein isothiocyanate on the product PB–HEMA–ECH–Gal (XIII) confirmed the presence of galactose in the gel and the specific adsorption for the lectin of *Arachis hypogaea.*

Affinity Chromatography

The maximum capacity of retention for the adsorbent XIII was 4.2 mg of lectin per mL of the swelled hydrogel. This value was comparable with the results obtained by other authors, using different hydrogels obtained by modification of natural macromolecules.[7,11]

CONCLUSION

The coupling of carbohydrates to activated matrices is an important subject of research in order to obtain adsorbents applicable in affinity chromatography to purify biological macromolecules. The results reported in this work could provide methods to obtain new epoxy-activated matrices with capacities to bind different sugars as ligands for the retention of specific proteins. In this case, the subsequent coupling of galactose led us to obtain products whose incorporation of ligand on activated matrices represent typical examples for the synthesis of affinity adsorbents, since 1–20 μmol ligand per mL of gel are suitable for most of them. The results of the amount of galactose coupled to the activated matrices (32 μmol

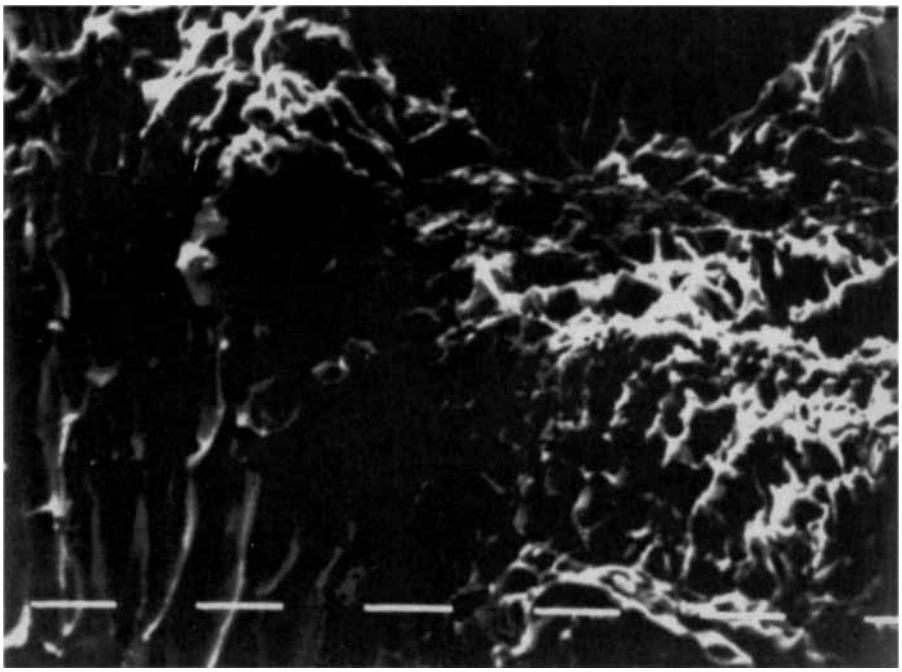

Figure 1 SEM micrograph of PB–HEMA–ECH–Gal (XIII).

per gram of dry gel XIII) and the quantity of lectin retained (4.2 mg per mL of the swelled gel XIII) were comparable with commercial hydrogels.

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