Preparation and characterization of a new polymeric support with tannic acid as ligand

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A new polymeric support was synthesized using the co-oligomer of 1,3-butadiene–acrylic acid crosslinked with 2-hydroxyethylmethacrylate. Tannic acid was later attached to this product as a ligand, through a spacer arm of nine carbon atoms. Different assays were carried out in order to characterize this product, and preliminary experiments were performed to determine its potential application in the capture of proteins.

(Keywords: polymer synthesis; polymeric support; crosslinking)

Introduction

Previous work[1–3] has demonstrated that phenolic hydroxyl groups are able to hook an ion bond, a quinone bond and/or a hydrogen bond to a protein or alkaloid. These properties are present in tannic acid, and numerous papers describe the incorporation of tannin in polymeric supports. One method involves the preparation of 'condensation-type' tannins, i.e. with formaldehyde[4,5]; another method is the immobilization of tannin by covalent binding to a support, generally cellulose or other natural supports[6].

In this work, we have synthesized a new support from a co-oligomer of 1,3-butadiene–acrylic acid (BuAA) crosslinked with 2-hydroxyethylmethacrylate (HEMA), with tannic acid covalently bonded through a spacer arm, and investigated its possible application in affinity chromatography.

Experimental

Reagents and materials. Thionyl chloride (Fluka) was distilled prior to use. Hexamethylenediamine (HMDA; 1,6-hexanediamine; Aldrich) was dried *in vacuo* at 30–35°C for 4 days before use. Tannic acid (*m*-digallic acid; Mallinckrodt) was dried *in vacuo* at 80°C for 3 days. Epichlorohydrin (ECH; Fluka) was used without further purification.

All solvents were distilled and dried before use.

Synthesis of activated matrix. Co-oligomer BuAA was obtained by bulk polymerization, using a 2 l stainless steel reactor, with a mechanical stirrer, that had been purged with nitrogen[7].

1,3-Butadiene and acrylic acid at a ratio of 4.5:1 (w/w), were placed into the reactor and 1% benzoyl peroxide was added as initiator. The mixture was then heated to 70°C, at which temperature the system is under 1.1 MPa pressure. After 15.5 h, a colourless resin was obtained, which was collected and dried *in vacuo*.

The resulting co-oligomer was then purified to a more homogeneous product by fractional precipitation, using benzene (solvent) and methanol (non-solvent). Three fractions were collected: F_1, F_2 and F_3.

Crosslinking with HEMA was carried out from F_2 ($M_w = 4500$, determined by vapour pressure osmometry). Thus, 18 g of BuAA were reacted with a 33% (w/v) solution of HEMA in benzene, using 2% benzoyl peroxide as radical initiator, with 1:1 ratio of BuAA double bond equivalents to HEMA double bond equivalents. The mixture was refluxed for 2 h with magnetic stirring. A white product was obtained, which was washed separately with benzene and dimethylformamide (DMF) until no more soluble products were eluted.

The carboxylic groups of the crosslinked polymer were activated by adding 5 ml of $SOCl_2$ (the BuAA/$SOCl_2$ ratio was 1:2 BuAA–COOH equivalents/$SOCl_2$ equivalents) to a 7:3 dry benzene/crosslinked polymer mixture. The reaction was started at 0°C and the mixture was then heated to 40–45°C and held for 60 min with magnetic stirring.

Coupling of a spacer arm. A dark yellowish product was formed by the reaction and was dried *in vacuo*. The introduction of HMDA was carried out as follows: an 18% (w/v) solution of HMDA in dry benzene was added to the previous product in a 1:4 ratio BuAA–COOH equivalents/HMDA–NH_2 equivalents. The mixture was heated to 40°C and held for 3.5 h, with stirring. It was then filtered and washed separately with methanol and water (previously acidified to pH 5 with HCl).

This product was filtered in a vacuum system and then suspended in a 10% (w/v) aqueous solution, adjusted to pH 9 with NaOH. ECH (9 ml) was added at a ratio of 1:3 BuAA equivalents/ECH equivalents, and the suspension was heated to 45–48°C and held for 2 h, with magnetic stirring. The product was then collected, washed with distilled water and again filtered in a vacuum system.

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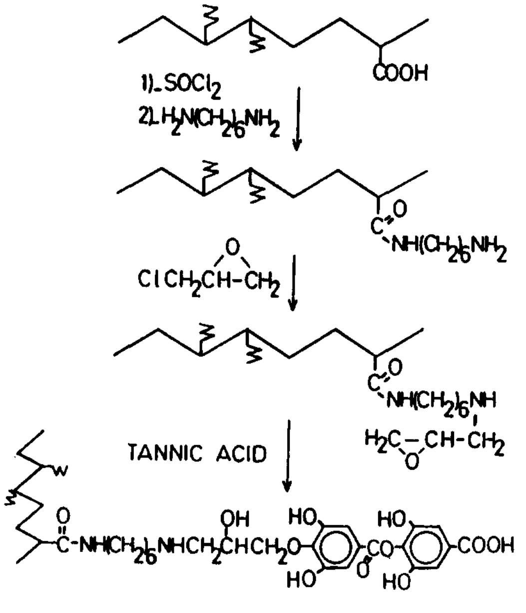

Figure 1 Covalent attachment of spacer arm and ligand

Coupling of tannic acid as ligand. Tannic acid, at a ratio of 1:3 BuAA–COOH equivalents/tannic acid–OH equivalents, was dissolved in distilled water, adjusted to pH 7 with NaOH. The resulting solution (15% w/v) was later added to a swollen gel in water, at the same pH. The reaction was carried out at 45°C for 4.5 h and at room temperature for a further 18 h, with magnetic stirring.

A brown product was obtained, which was collected and purified by washing with buffer (pH 9) and distilled water, followed by several washes with DMF until all remaining tannic acid was removed.

Quantification of functional groups. Determination of carboxyl groups was made by titration using benzene as solvent, with potassium hydroxide in a methanolic solution (0.1 N) and thymol blue as indicator.

Hydroxyl groups were reacted with an excess of acetyl chloride in pyridine. When the reaction was over, the remaining acetyl chloride was hydrolysed. The amount of acids was known from volumetric titration (with NaOH) of the hydrolysis products with *m*-cresol red as indicator. The hydroxyl group quantification was calculated from the difference between the blank titration and the sample[8].

Tannic acid was determined by hydrolysis in HCl–dioxane (1:1), and then quantified by a spectrophotometric method using sulfuric acid (65% v/v) as a colour developer[9].

I.r. spectroscopy. I.r. spectra were obtained on a Nicolet 5-SXC spectrophotometer FT-IR, using KBr discs.

Percentage swelling. The percentage swelling was determined as a function of time, weighing the increase of solvent absorption. When equilibrium was reached, the values of percentage swelling were calculated according to the relation:

$$\text{Swelling}\% = 100(m' - m)/m$$

where m' and m denote the weights of dried gel and swollen gel, respectively. A modified technique of the ASTM Norm D 3616-77 was used.

Scanning electron microscopy (SEM). Studies were performed at CERIDE Laboratories, using a Jeol model 35-C scanning electron microscope. The material was coated with a thin metal layer (gold or gold/platinum mixture) *in vacuo*. Microphotographs were taken using 25 kV high tension values.

Thermogravimetric analysis (t.g.a.). Thermal stability was measured by means a Netzsh STA 429 thermogravimetric analyser at CIMM Labs. The system was purged with nitrogen and heated at a rate of 10°C min^{-1} from room temperature to 650°C. Both t.g.a. and its first derivative (derivative thermogravimetry, d.t.g.) were plotted simultaneously as a function of time and temperature.

Immunological test. A preliminary immunological assay was performed to investigate the gel's capacity as a protein binder. The gel, previously treated with a human gamma globulin solution in buffer PBS (phosphate-buffered saline) with pH 7, was subjected to an immunofluorescent assay, according to the technique described by Coons and Kaplan[10], using suitable antibodies labelled with fluorescein isothiocyanate as fluorochrome.

Results and discussion

Synthesis. The co-oligomer of BuAA was synthesized by bulk polymerization using benzoyl peroxide as initiator, and was later purified by fractional precipitation.

Carboxyl groups of the purified sample were quantified and the obtained result was 0.367 eq. COOH/100 g polymer.

HEMA was incorporated into the BuAA backbone through a radical addition reaction of their double bonds. Both spacer arm and ligand were covalently attached to this product, as shown in *Figure 1*.

The final product contained 0.015 eq. COOH/100 g polymer and 0.415 eq. OH/100 g polymer. The presence of tannic acid as ligand turned the polymer into a brown coloured product with both hydrophilic and hydrophobic characteristics.

Tannic acid covalently bonded to the resin was determined by hydrolysis in acid conditions. The result obtained was 2.64% (w/w).

Percentage swelling. The percentage swelling shown in *Table 1* reveals that this final product is easily swollen in DMF, ethanol and dioxane, and less easily swollen in water. In solvents such as benzene, it shows an intermediate behaviour. These results indicate that the contribution of backbone fragments is more important than the hydrophilic (tannic acid) contribution.

Table 1 Percentage swelling

Solvent	Swelling (%)
Water	1.78
Benzene	9.15
Dioxane	53.47
Ethanol	57.79
DMF	119.05

I.r. spectroscopy. The difference between the base polymer and the new product was observed by i.r. spectroscopy; the new bands were at 1109–1335 cm^{-1}, R–C–O–C–R (R = aromatic and/or aliphatic); 1660–1680 cm^{-1}, C=O (amide); 1705–1710 cm^{-1}, C=O (acid); 1800–2150 cm^{-1}, C–H (aromatic); and 3500–3600 cm^{-1}, OH (phenolic).

Scanning electron microscopy. SEM studies were performed on 70/100 mesh samples.

From photographs it may be inferred that all particles are irregularly shaped and their size varies in the ranges 250–350 μm in length and 150–250 μm in breadth. Pore sizes of about 1 μm were observed.

Figure 2 shows the inner surface structure of a dry particle of the polymer. It can be seen that the particle has both porous and smooth zones, the latter being the most predominant.

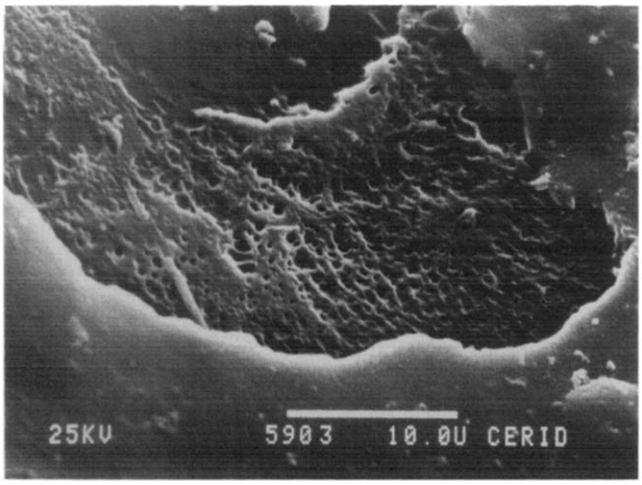

Figure 2 Scanning electron microphotograph of the inner surface of a dry particle. Scale bar = 10 μm

Thermogravimetric analysis. Figure 3 compares the t.g.a. curves of the support BuAA-*g*-HEMA (gel I) and the new activated product (gel II) recorded at a heating rate of 10°C min^{-1}. Up to 375°C, both products showed similar weight loss curves, but above that temperature, gel I showed a greater weight loss than gel II. The initial decomposition temperature was 210°C for gel I and 190°C for gel II. Differences in traces between gel I and gel II at temperatures above 450°C can be attributed to the presence of an aromatic chemical structure in gel II.

Figure 4 compares the degradation rate of the two products, and a typical two-step degradation process is observed for both . The melting temperature, T_m, for gel I was 364°C for the first process, with a weight loss of 50%, and 407°C for the second process, with a weight loss of 46%. For gel II, the T_m was 351°C with a weight loss of 49%, and 439°C with a weight loss of 24% for the two processes, respectively.

Although the maximum degradation rate temperatures are not the same for gel I and gel II, such processes occur at the same range of temperatures. Incorporating a spacer arm and a ligand seems to accelerate the degradation process at low temperatures.

Immunological test. Further qualitative experiments will be carried out in order to find possible applications of this product in affinity chromatography. The immunofluorescence method described above, with a sensitivity range between 10^{-3} and 10^{-9} g, showed that our polymer has a binding capacity for proteins such as gamma globulin.

Conclusions

This newly synthesized product is a brown gel which is easily swollen in DMF, ethanol and dioxane. Characterization studies demonstrate that tannic acid is efficiently bonded to the support through the spacer arm (2.64% w/w).

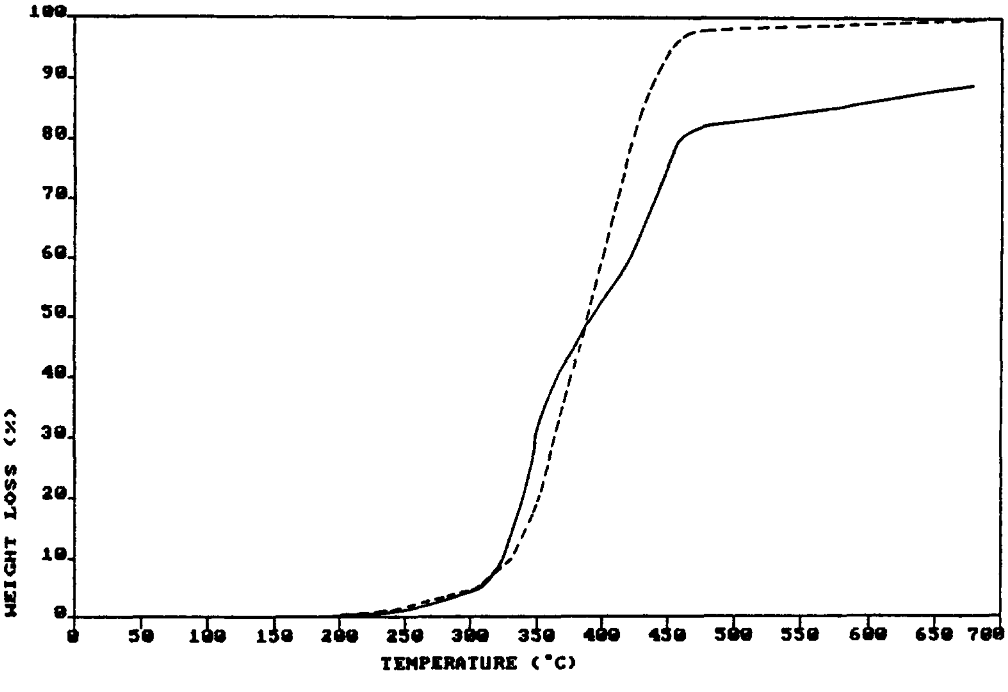

Figure 3 T.g.a. curves of gel I (– – –) and gel II (——)

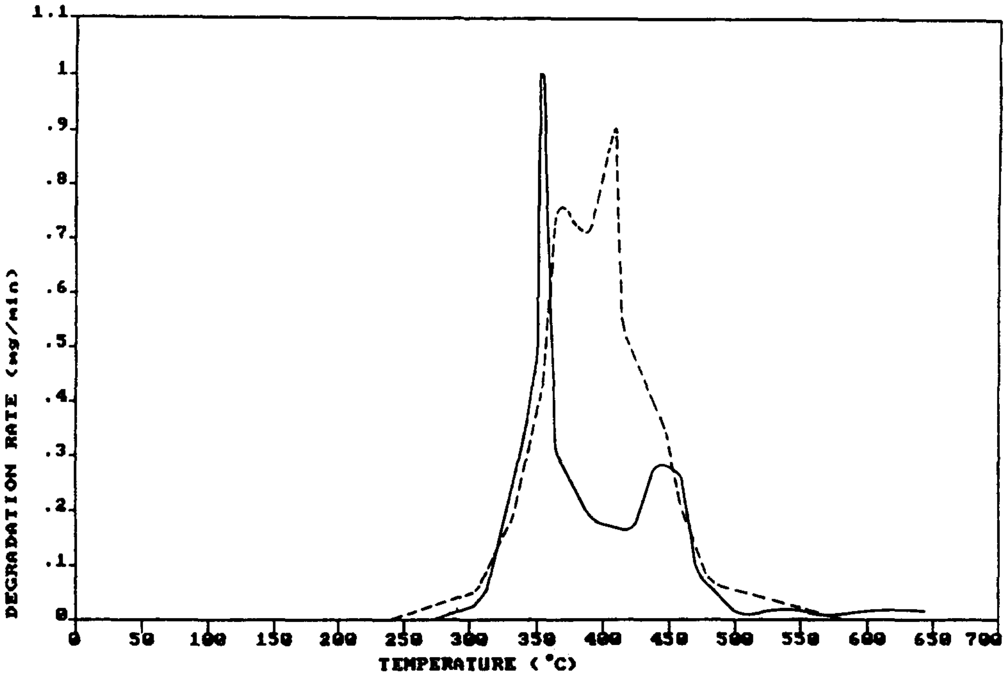

Figure 4 D.t.g. curves of gel I (– – –) and gel II (——)

The capacity of BuAA-*g*-HEMA with tannic acid to capture proteins, such as gamma globulin, was qualitatively demonstrated by means of immunofluorescence assays. Further studies of its application in affinity chromatography should be made by specialist investigators.

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