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The separation of proteins on Cibacron Blue F3GA treated nylon and poly(ethylene) graft copolymers

Clifford G. Beddows[1], Richard A. Morris[1], and James T. Guthrie[2]*

[1]School of Health and Applied Sciences, Leeds Polytechnic, Leeds LS1 3HE, UK

[2]Department of Colour Chemistry, University of Leeds, Leeds LS2 9JT, UK

Summary

Various copolymers have been prepared based on the use of a polyamide support and a polyolefinic support. Various comonomers were used. The resulting copolymers developed into affinity media by treatment with Cibacron Blue F3GA and the total assemblies used to remove protein from human serum. The efficiency of separation is shown to depend on the nature of the copolymer system, though in no case were the protein retention characteristics as good as those obtained with the Sepharose-Cibacron Blue F3GA system.

Introduction

A considerable amount of work has been published on the separation of specific proteins using Cibacron Blue F3GA coupled to polysaccharides, (Thompson et al, 1975, Dean and Watson, 1979). The basis of the purification is accredited to the nucleotide-binding characteristics of the protein. (Stellwagen, 1977, Clonis & Lowe, 1980). A number of polysaccharides have been used including Sepharose and Agarose (Dean and Watson, 1979). Cellulose was reported to be less successful (Angal & Dean, 1977). Advantages associated with the technique relate to the low cost of the dyes compared to materials normally used in biochemical separations and also the fact that the dyes react directly with the support, without prior activation of hydroxyl groups. Gel-based systems have disadvantages. The Cibacron Blue F3GA can leak from the column as the protein is eluted. This introduces the need for further purification. In addition, the gels have poor handling characteristics which does not allow them to be used under pressure. As the effectiveness is dependent upon the diffusion of protein into the gel, the separation can be very time consuming. The gels have the added disadvantage in that other proteins can become occluded and the gels may biodegrade.

Attempts to use other, more stable gels, such as those based on poly(acrylamide) failed to give satisfactory results (Angal & Dean, 1977). Obviously it would be advantageous to utilise other more stable systems provided efficient separation could be achieved. Thresher & Swainsgood (1983) found that glass beads of controlled pore size, linked to Cibacron Blue F3GA through glycerolpropyl groups, had an affinity for bovine lactic dehydrogenase (LDH) similar to that of the free dyestuff although the amount of enzyme that was separated appeared to be small.

Graft copolymers offer many potential advantages as supports assuming that adequate amounts of dye can be attached to sites which are available for access to the protein. The supporting copolymer should ideally have the correct architecture and physical properties. Such properties would allow effective access and elution and inherent stability. The grafted polymer branches can be controlled such that they give a large number of reactive groups as well as providing a suitable microenvironment for any adsorbing species.

* To whom offprint requests should be sent

In this study nylon and polyethylenic substrates were selected in view of their inertness, hydrophobic character and their potential ability to be moulded to a predetermined shape that could be of value for a separation column. Hydroxyethyl methacrylate and vinyl acetate were used as the monomers in grafting. Prior to treatment with Cibacron Blue F3GA, the acetate groups in the vinyl acetate copolymer were hydrolysed to give poly(vinyl alcohol) graft branches.

After coloration, the graft copolymers were assessed in terms of their ability to remove protein from human serum as this has been shown to be a useful measure of the suitability of polysaccharides based systems for protein separation (Angal and Dean, 1978) and also to remove lactic dehydrogenase from a rat liver homogenate (Stockton et al, 1978).

Experimental

Materials Nylon (Grilamid) powder was obtained from Emser Werker Ltd, Switzerland. Low density poly(ethylene) was obtained from Telcon Plastics Ltd.

Hydroxyethylmethacrylate (HEMA) was obtained from Aldrich Chemicals Ltd, Gillingham, UK. Cibacron Blue F3GA, obtained from Ciba-Geigy Ltd, was purified by dissolving in DMF, filtering and precipitating by the addition of acetone dropwise until 1 drop of the supernatant gave a clear ring on filter paper. After filtering, the residue was dried at 298K. Human serum was obtained by centrifuging fresh samples of blood. Lactic dehydrogenase, human serum albumin and other chemicals were obtained from Sigma Chemicals Ltd, St Louis, USA.

Methods - Preparation of Graft Copolymers

In a typical preparation, the purified nylon or poly(ethylene) powder (2g) was dispersed in a solution of monomer (10% w/v) ($30cm^3$) and irradiated at 13 rad/min using Co(60) for 72h at 298K in air, before transferring into methanol ($180cm^3$) and filtering. The solid was extracted with methanol to remove homopolymer and dried to constant weight at 313K. For the grafting of vinyl acetate, 10^{-3}M $CuCl_2$ was included with the monomer to reduce homopolymer formation. The acetate groups of the resulting graft copolymers (1g) were hydrolysed to the corresponding hydroxyl groups with sodium methoxide in methanol (10% w/v) ($50cm^3$) under reflux for 30 min.

Coupling to Cibacron Blue F3GA

The copolymer (1g) was immersed in dye solution ($20cm^3$, $0.1g/100cm^3$). This was made up to $50cm^3$ with H_2O and heated to 100°C for 10 minutes. NaCl (50g) was added followed by heating at 80°C for a further 10 min. $NaHCO_3$ (10g) was then added and the mixture refluxed for two hours or eighteen hours with stirring throughout. The resulting copolymers were filtered, washed with 6M urea ($100cm^3$) and then with 0.5M NaOH until the eluent was clear. The filtrate and washings were combined and the amount of uncoupled dye was determined spectroscopically at 615nm. A portion of the Cibacron Blue F3GA solution was treated in the same manner but without copolymer, to give the control. The amount of dye attached to the copolymer was determined from the difference between the two readings.

The amount of protein retained from human plasma was determined by passing 0.2cm^3 of serum in 0.025M Tris.HCl, (pH 8.5) through a small column (3cm x 1cm diameter) of the copolymer at 80cm^3/h and then eluting with 0.2M KCNS in 0.05M Tris. HCl (pH 8.0). The fractions (0.85cm^3) were collected and assayed for their protein content by the Coomassie Blue method (Sedmark & Grosberg 1977).
In a separate experiment, the serum was dissolved in different buffer solutions of pH 8.5, 7.2 (Tris. HCl), 6.5 (maleate), 6.0, 5.5, 5.0, 3.0 (citrate) and 4.0 (acetate) for the initial passage of the serum.

The protein eluted from one series of experiments was compared to human serum albumin standard and fresh plasma, by electrophoresis at pH 8.6 in 0.06 M veronal. HCl buffer.

Results and Discussion

Graft copolymers were prepared by irradiation of monomers in the presence of nylon and poly(ethylene) powder. Hydroxyethylmethacrylate (HEMA) and vinyl acetate were each grafted onto both substrates. Both low and high levels of graft were selected as these could act as 'extender' arms, with the attached groups keeping them away from the hydrophobic substrate surface. Such 'arms' also provide the opportunity a more hydrophilic environment since it is possible for the hydroxyl groups to extend into the aqueous phase. In addition, a high level of graft provides a large number of groups that could be used in coupling to Cibacron Blue F3GA. The difference in radical yields of the polyamide and the polyolefin would give a statistical difference in the number of grafted chains. In addition the chains themselves would be of different lengths and morphology. Such factors have a bearing on the adsorption characteristics of the derived copolymers.

The levels of maximum add-on obtained with vinylacetate on poly(ethylene) was 21%. The acetate groups in the poly(vinyl acetate) graft copolymers were fully hydrolysed before further treatment.

The graft copolymers were refluxed with Cibacron Blue F3GA for either two hours (which was comparable to that used for polysaccharides (Leatherbarnow and Dean, 1980) or for eighteen hours, to allow for maximum attachment. With the longer period some swelling of the copolymers was observed and some of the dyestuff migrated into the bulk of the substrate. In such a state, the dye would not be available for removing protein.

The amount of ligand immobilised (Table 1) was not affected by the amount of grafted species present. Greater amounts were coupled with the longer reflux period samples as would be expected. The levels of ligand attachment were comparable to those reported for Sephadex-G100 (4-8 M/cm^3) and Sepharose--4B (0.7-2 M/cm^3) (Easterday and Easterday, 1974). This is of interest since unlike the polysaccharides, the Cibacron Blue F3GA must be attached mainly to the surface of the polymeric support.

The Cibacron Blue F3GA-containing graft copolymers were used to remove protein from human serum. The amount of protein retained on small columns of the graft copolymers was not related to the type or amount of graft (Table 2), nor to the amount of ligand. This suggests that the protein -dye interaction must be more complex than suggested from the polysaccharide data, or that other factors, such as the flow-rate, could be important.

Table 1 Concentration of Cibacron Blue F3GA attached to various hydroxy group-containing graft copolymers

Graft copolymer on Nylon	%Add-on	Concentration of ligand ($\mu g/cm^3$)* after refluxing for 2h	18h
Nylon-co-HEMA			
	81	2.4	3.8
	91	0.8	3.8
	146	10.0	2.3
Nylon-co-Vinyl acetate**	72	--	1.7
on Poly(ethylene)			
Poly(ethylene)-co-HEMA	124	0.70	1.5
	117	2.8	10.0
Poly(ethylene)-co-Vinyl acetate**	21	--	4.6

*determined by difference

**the poly(vinyl acetate) was fully hydrolysed before coupling.

Table 2 Retention of protein from human serum ($0.2cm^3$) in 0.025 M Tris.HCl (pH8.5), eluted with 0.2M KCNS

Graft Copolymer	%Add-on	Protein retention ($\mu g/cm^3$) Series A (2h reflux)	Series B (18h reflux)
Nylon			
Nylon-co- HEMA	81	100	150
	91	175	75
	146	150	100
Nylon-co-Vinyl acetate	71	--	155
Poly(ethylene)			
Poly(ethylene)-co-HEMA	124	325	150
	117	225	75
Poly(ethylene)-co-Vinyl acetate	21	--	72

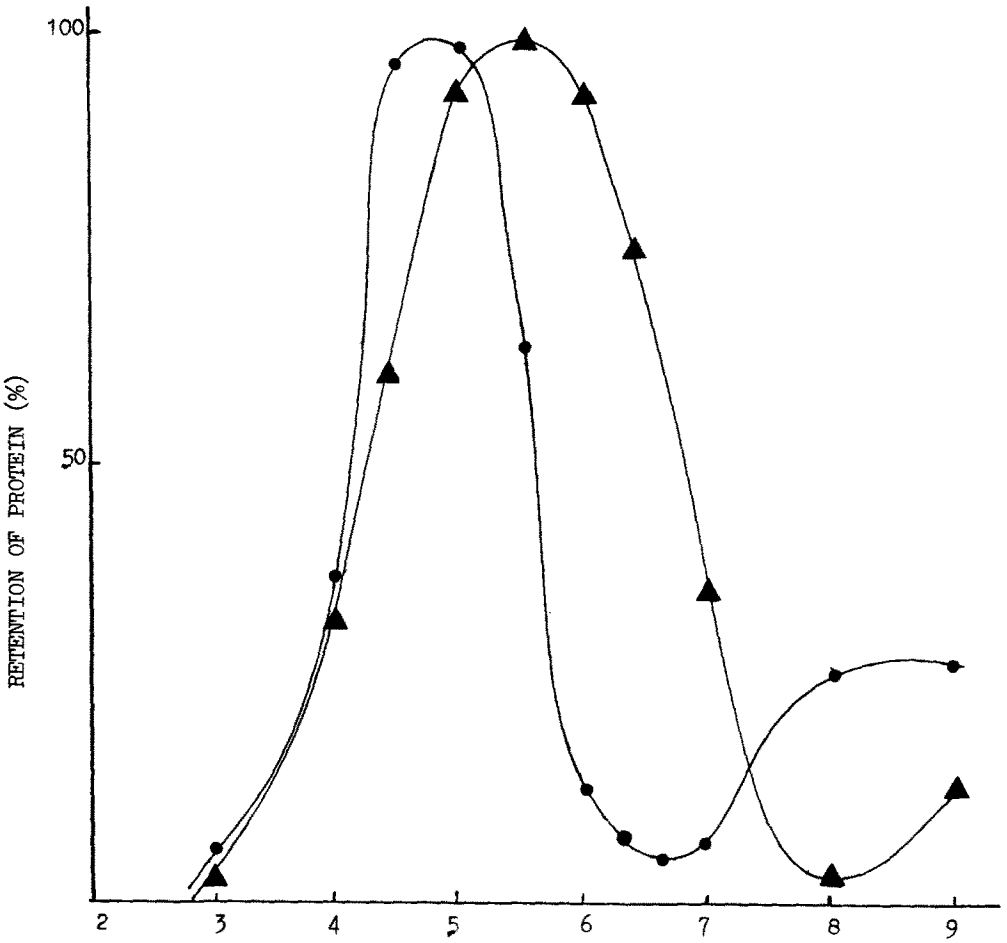

FIGURE 1 The % retention of protein from serum by cibacron blue - nylon and polyethylene graft copolymers vs pH.
▲——▲ Polyethylene - co-HEMA ●——● Nylon - co-HEMA

Consequently, a portion of the serum was left in contact with the copolymer-dye overnight before being washed through at a very slow rate and then eluting. No significant increase in protein retention was observed. Analysis of the protein retained and that washed through showed that 99.8% of the protein was accounted for.

Earlier workers have reported that the amount of protein retained is affected by the pH and that more non-specific protein interaction occurred at pH = 5 (Hey & Dean 1981). Consequently, a poly(ethylene)-co-HEMA and a Nylon-co-HEMA graft copolymer were treated with human serum over a range of pH values. The results (Figure 1) show a difference between the two graft copolymers. The poly(ethylene) had an optimum retention at pH=5.5 with negligible amounts being retained at pH=8. The Nylon copolymer had a narrower optimum at pH=5.0 and a minimum at pH=6 to pH=7.

It is somewhat difficult to relate the results for protein retention for the graft copolymers to those obtained with the hydrophilic gels because the volumes were significantly different. However, it would seem that the amount of protein being retained was significantly less than for Sepharose-Cibacron Blue F3GA (Travis et al, 1976) despite the similar amounts of ligand being present.

The protein retained by the graft copolymer at pH=8.5 and pH =5.0 was examined by electrophoresis. The protein removed at pH=8.5 appeared as a single band and appeared to be pure whereas the protein obtained from the pH=5.0 column, was a mixture of proteins as would be expected, but the major band was the same as that found in the pH 8.5 eluent.

Graft copolymer systems continue to offer a means of investigating the nature of protein adsorption phenomena and we are continuing with this type of investigation.

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