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Studies of a novel membrane for affinity separations

I. Functionalisation and protein coupling

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ABSTRACT

A study is presented of the potentialities of an electrostatically-spun poly(ether–urethane–urea) as an affinity separation membrane. The spinning process produces a fibrous network with a high internal surface area. A variety of chemical methods has been used for functionalising and activating the membrane.

Assessments of the capacities of the activated membranes for covalent coupling of protein A and human immunoglobulin G have given very encouraging data. Non-specific adsorption of both proteins by the inactivated polymer was negligible.

Geometric considerations suggest that the limiting factor determining protein coupling is the accessible surface area rather than the number of coupling sites.

INTRODUCTION

Membrane-based techniques are playing an increasingly important role in affinity separation and, together with fibre-based systems, seem likely to compete successfully with beads as supports for some purposes. Research in membrane technology in Europe, including aspects related to separation systems, is showing a steady increase. The desirable properties in a membrane designed for affinity separation are well-known and include high macroporosity, large internal and external surface areas, high chemical, biological and mechanical stabilities, a degree of hydrophilicity, low non-specific adsorption of bioactive species and the presence of chemical groups which permit suitable functionalisation. Among membranes which are currently available are those based on "hydrophilic" modified polyvinylidene difluoride (Immobilon AV Affinity Membrane) [1], activated nylon *e.g.* Carboxydyne, Aminodyne, Hydroxydyne (Pall) [2], cellulosic polymers (Genex) [3], and "UltraBind" —a mixture of a polysulphone and polyacrolein (Gelman Sciences) [4].

During recent years we have become familiar with the properties of some electrostatically-spun polymers. The poly(ether–urethane–urea) Biomer (**1**) has been studied in these laboratories by Dr. D. Annis [5] in his work on prosthetic vascular grafts, and it occurred to us that the material has most or all of the desirable properties outlined above. Accordingly, we have investigated its application as an affinity membrane and report the results [6] in this paper. Similar membranes may be made from other poly(ether–urethane)s, *e.g.* Pellethane, Tecoflex, Estane.

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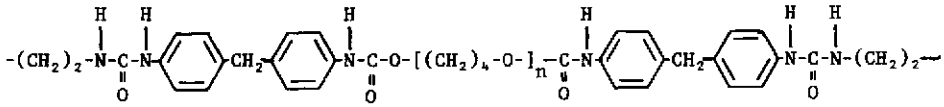


(1) Biomer

EXPERIMENTAL

Materials

The poly(ether–urethane–urea) Biomer was obtained from Ethicon (Somerville, NY, USA) as a 30% solution in N,N-dimethylacetamide (DMAc). The following materials were purchased from Aldrich (Gillingham, UK): poly(ethylene glycol) (PEG), molecular mass 4000, 2-chloroethyl isocyanate and the diisocyanates, Bolton–Hunter reagent, 1,1′-carbonyl diimidazole (CDI), 2-fluoro-1-methylpyridinium toluene-4-sulphonate (FMP), 1-hexanol, 6-aminocaproic acid and rhenium carbonyl. The last was purified by sublimation in vacuum before use. We are indebted to Kodak (Harrow, UK), for a gift of the N-succinimido ester of N-methacrylamido-6-caproate (7); N-acryloxysuccinimide was purchased from Kodak.

2-Isocyanatoethyl methacrylate was bought from Monomer-Polymer and Dajac Labs. (Trevose, PA, USA) and D-glucamine and ethanolamine were obtained from Fluka (Glossop, UK). Protein A (soluble, purified by chromatography) and human immunoglobulin G (IgG) were supplied by Sigma (Poole, UK) and the ^{125}I-labelled proteins by Amersham International (Protein A) (Aylesbury, UK) and NEN Research (human IgG) (DuPont, Stevenage, UK).

Acetonitrile and diethyl ether, dried by refluxing over calcium hydride were distilled before use.

Electrostatic spinning

In this process the spinning solution (polymer solution) is introduced through an appropriate spinneret (*e.g.*, a hypodermic needle) into an electric field to produce filaments which are attracted towards a collecting electrode at a different potential. In our experiments the needle was earthed and the collecting electrode was a stainless-steel rotating mandrel with a diameter in the range 5–10 cm at a potential of −10 kV. The spinning liquid was a solution of Biomer (16%, w/w) in a mixture of N,N-dimethylacetamide and methyl ethyl ketone (1.45:1, w/w). Fibres collected on the mandrel formed a tube which was subsequently removed and opened by cutting to produce a mat or membrane of polymer fibre. Further details are to be found in the paper by Annis *et al.* [5].

Fig. 1 is a typical scanning electron micrograph (SEM) of the spun membrane, which is seen to be essentially a network of very thin fibres of diameter approximately 1 μm. These are melded at many points and enclose irregular holes or pores with a typical dimension in the range 5–10 μm. The pore size and, to a certain extent, the filament diameter, are controllable through the spinning conditions; fibre diameters in the range 0.1–25 μm have been claimed in the patent literature [7,8]. As will appear later, fibres of diameter less than 1 μm and pores smaller than 5 μm could have advantages for present purposes. The overall internal surface area of the membrane is large, 1 g of the material as spun having an area of about 4 m^2; not all of this will be accessible to protein molecules.

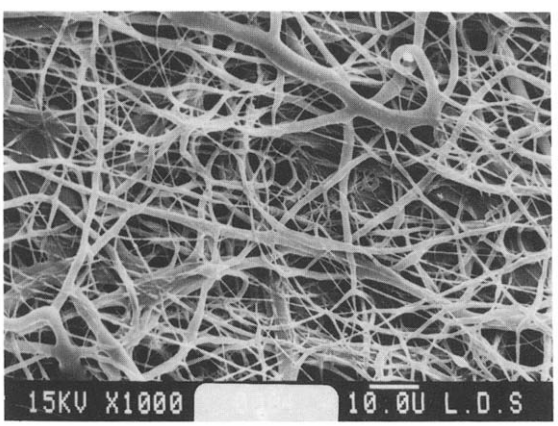


Fig. 1. SEM of electrostatically-spun Biomer membrane.

General procedure

Synthesis of an activated membrane of the type we are considering involves functionalisation of the initial poly(ether–urethane) and activation of the product by attachment of an appropriate active moiety. The general aim is to obtain constructions of the type shown below.

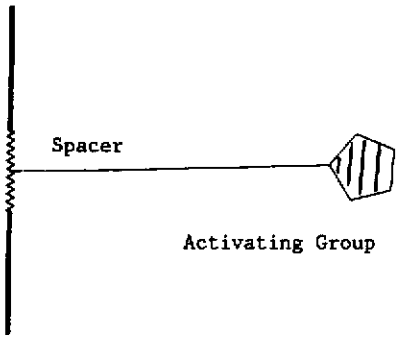


Fig. 2 is a flow-chart for the syntheses of the activated membranes. Functionalisation of Biomer has been carried out both before (a) and after (b) spinning, and satisfactory membranes have been obtained in both cases. Post-spinning activation is preferable and has been employed through-out most of this work; with this technique a larger proportion of the activating groups is accessible and further, the spinning process (best effected at a high relative humidity) can deactivate moisture-sensitive groups.

All processes used for functionalising poly(ether–urethane)s involve isocyanate couplings; subsequent activation may be based on free-radial reactions or direct coupling [9,10]. The following isocyanate derivatives were employed: 2-chloroethyl isocyanate (**2**), 2-isocyanatoethyl methacrylate (**3**), hexamethylene diisocyanate (**4**), 2,4-tolylene diisocyanate (**5**). For activation by free-radial procedures, we used "activated monomers", in which the active group (*e.g.*, succinimidyl) was linked to a polymerisable double bond. These were N-acryloxysuccinimide (**6**) and N-succinimide methylacrylamido-6-caproate (**7**).The activating species used for direct coupling were N-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent) (BH), CDI and FMP. The active moieties so introduced into the polymer react readily with nucleophilic groups (*e.g.*, $-NH_2$, –OH, –SH) in proteins or other biological species so that these become covalently attached to the polymer chains.

Figs. 3–5 set out the reactions employed. Fig. 3 includes the free-radical processes used in activation, leading to the membranes **10**, **11**, **12**; Figs. 4

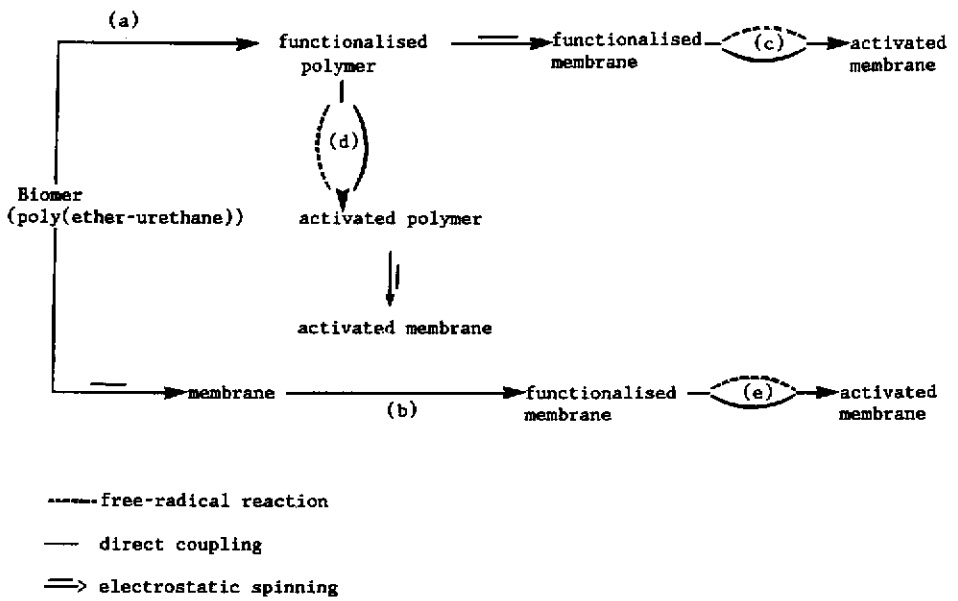
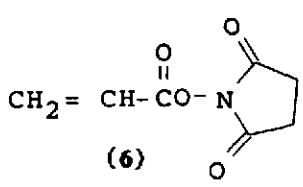


Fig. 2. Flow-chart for preparation of activated membranes.



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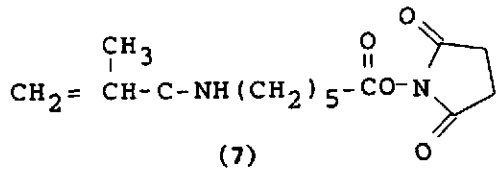


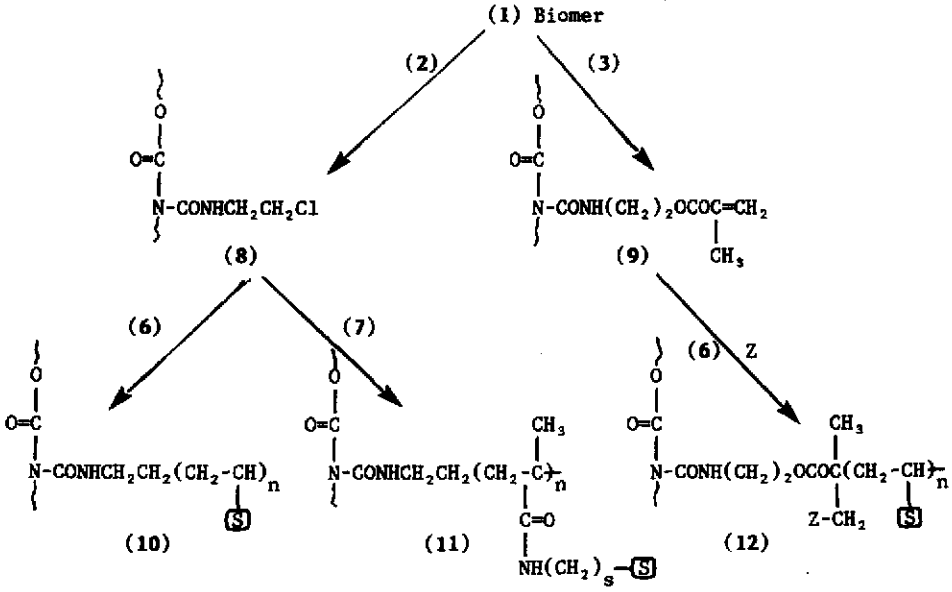
(7)

N-acryloxysuccinimide N-succinimide methacrylamido caproate

and 5 refer to activation by direct coupling forming the membranes **14–19** and **21–27**.

Functionalisation

Pre-spinning functionalisation of Biomer [(a) in Fig. 2] was carried out by reaction with **2** (Fig. 3). A 10-g amount of Biomer and 8 g of **2** were dissolved in 100 ml DMAc and allowed to react at 25°C for three days. The resulting adduct (**8**) was precipitated into water, reprecipitated from DMAc and dried in vacuum.



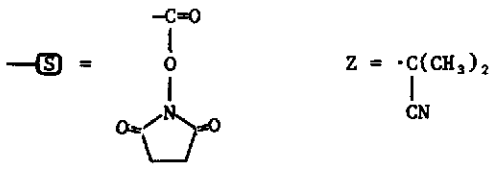
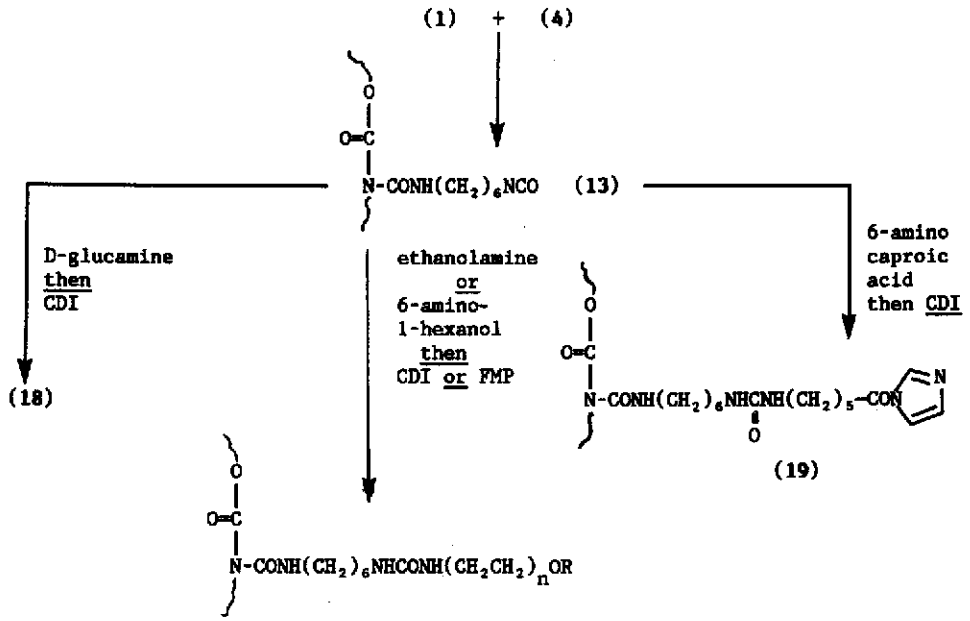


Fig. 3. Activated polymers by free-radical techniques.



Identification of activated polymers

	Activating Group R		
n	-CON		Tos⁻
1	(14)		
3	(15)		
1		(16)	
3		(17)	
D-glucamine	(18)		

Fig. 4. Activated polymers by direct coupling.

For post-spinning functionalisation [(b) in Fig. 2] the membranes were allowed to react heterogeneously with isocyanates **2**–**5** at 25°C to form adducts **8**, **9** (Fig. 3), **13** (Fig. 4), **20** (Fig. 5). Conditions are:

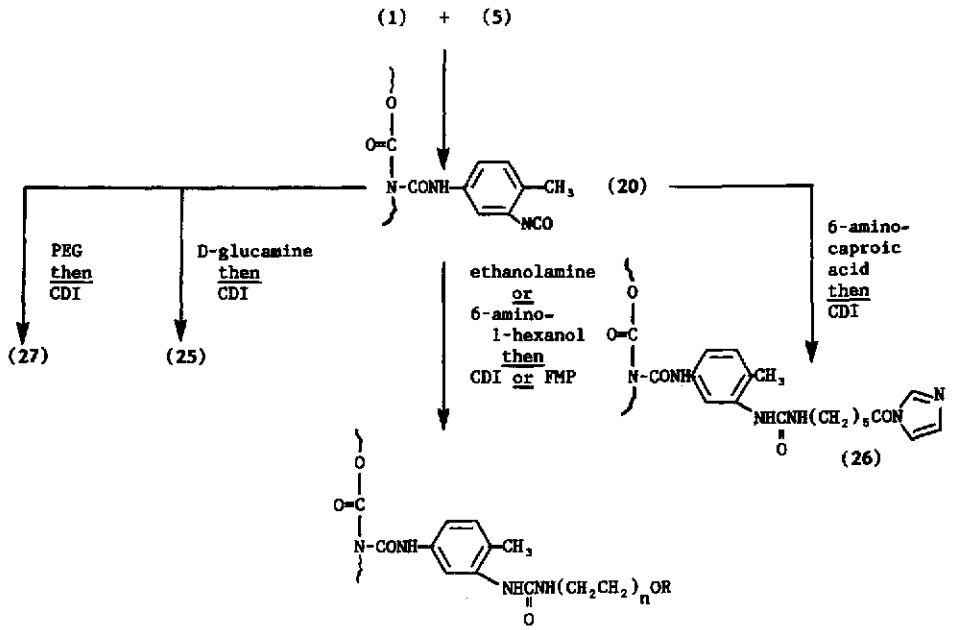
A 4.3-g amount of Biomer membrane was used in each case.
2-Chloroethyl isocyanate: 1 day, hexane solution 8% (w/w), 100 ml.
2-Isocyanatoethyl methacrylate: 5 days, hexane solution 20% (w/w), 100 ml.
Hexamethylene diisocyanate: 4 days, hexane solution 30% (w/w) (excess).
2,4-Toluene diisocyanate: 3 days, bulk (excess).

After reaction the membranes were washed several times with hexane and vacuum-dried.

Activation [(c)–(e) in Fig. 2]

Both free-radical processes and direct coupling were assessed as procedures for attaching active moieties to the polymer.

Free-radical routes. The functionalised polymer **8** (Fig. 3) was used in conjunction with a metal carbonyl to generate "attached" free radicals [11] which propagate through the double bonds in the



Identification of activated polymers

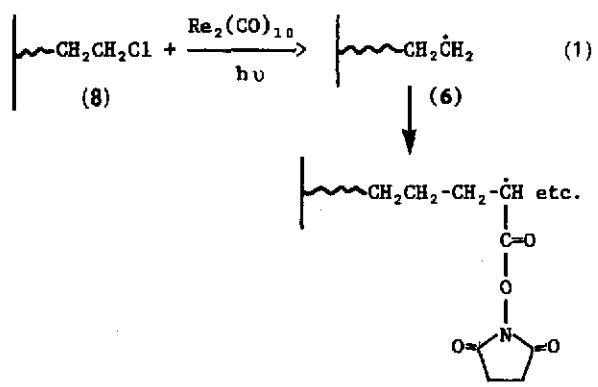
Activating Group R

n	-CON	Tos⁻
1	(21)	
3	(22)	
1		(23)
3		(24)
D-glucamine	(25)	
PEG	(27)	

Fig. 5. Activated polymers by direct coupling.

active monomers **6** and **7**. Eqn. 1 illustrates the reactions between **8** and **6** in these systems. Manganese and rhenium carbonyls ($Mn_2(CO)_{10}$, $Re_2(CO)_{10}$) are very effective for these reactions at wavelengths of 436 and 365 nm, respectively, while molybdenum carbonyl [$Mo(CO)_6$] may be used as a thermal initiator at 60°C or above [11].

A 14.5-g amount of **8** with 3 g of **6** (or **7**) were dissolved in 160 ml of DMAc: a solution of 0.123 g $Re_2(CO)_{10}$ in 15 ml DMAc was then added and the liquid irradiated with light of wavelength 365 nm



from a 100-W high-pressure mercury arc for 5.5 h.

Polymers for subsequent spinning [(d) in Fig. 2] were precipitated in a mixture of diethyl ether and ethyl acetate (9:1, v/v), filtered off and reprecipitated. The products were **10** and **11** (Fig. 3). If activation had been effected after spinning [(c) or (e) in Fig. 2], the membraness were washed with acetonitrile and dried in vacuum.

In a second type of procedure the macromer **9** (Fig. 3) was copolymerised with active monomer **6** with the aid of a conventional free-radical initiator. A 1.9-g amount of **9** was added to a solution of 0.5 g of **6** and 0.2 g azo-bis-isobutyronitrile in 10 ml acetonitrile. After degassing, the polymerisation was carried out thermally at 60°C for 4 h. The membrane was removed, washed with dry acetonitrile and dried in vacuum. The product was composed of units such as **12** (Fig. 3).

Direct coupling. Figs. 4 and 5 present the reactions by which membranes activated by CDI and FMP were prepared. After functionalisation of the poly(ether–urethane) membrane to give **13** or **20**, the terminal isocyanate residues were converted to hydroxylic forms by reaction with the following series of hydroxyamino compounds or PEG. Reaction conditions are:

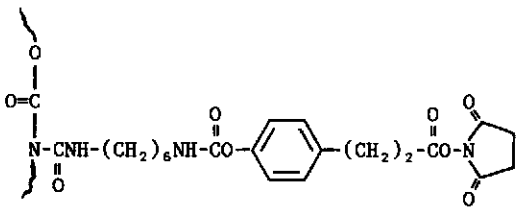
In each experiment the weight of isocyanated membrane was 4.2 g.
Ethanolamine: 25°C, 24 h, bulk, 50 ml.
6-Amino-1-hexanol: 25°C, 24 h, 4% (w/v) in dry ether, 80 ml.
PEG, molecular mass *ca.* 4000: 50°C, 72 h, 50% (w/v) in dry acetonitrile, 50 ml.
D-Glucamine: 25°C, 2 h, 8% (w/v) in formamide, 75 ml.

The hydroxylated membranes were washed with dry acetonitrile and dried in vacuum. Activation of the membranes was carried out with CDI in acetonitrile (15%, w/v). The weight of CDI used was 2–3 times that of the polymer. After 48 h at 25°C the membranes were washed extensively with dry acetonitrile and vacuum-dried.

For activation by coupling with FMP, the hydroxylated membranes (4.2 g) were reacted (25°C, 24 h) with FMP in acetonitrile (5%, w/v) in the presence of 1 ml triethylamine, then washed with acetonitrile and dried.

A further series of membranes was prepared by reacting the functionalised polymers **13** and **20** with 6-amino-caproic acid (Figs. 4 and 5). A 5-g amount of the acid dissolved in 50 ml of 0.75 *M* sodium hydroxide was added to 4.2 g of membrane and reacted for 3 h at 25°C. The acidic membranes produced were washed with water, 0.8% HCl and finally water then dried in vacuum. They were activated by reaction with CDI (5 g) in acetonitrile (50 g) for 48 h at 25°C.

The Bolton–Hunter reagent was condensed with the functionalised membranes **13** and **20** to give the activated membranes **28** and **29**, respectively. For this purpose 1.2 g of **13** or **20** were allowed to react with 0.8 g of BH in 40 ml dry acetonitrile for 4 days at 25°C.



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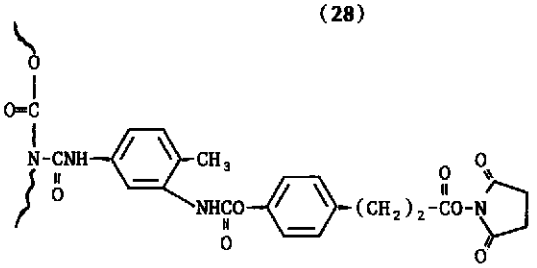

(29)

An aspect of the work (to be discussed later) involved determination of the activating groups in a membrane to establish its influence on the coupling capacity for proteins. This was effected by use of ^{125}I-labelled BH, the coupling experiments being carried out as described above.

Assessments of protein coupling to activated membranes

Discs (diameter 2.54 cm) were cut from sheets of activated membranes (thickness approximately 0.3 mm). For each test one or three such discs were placed in a Millipore Micro-Syringe 25 mm filter holder and a solution of ^{125}I-labelled protein A (1 mg per ml, in 0.1 *M* $NaHCO_3$) was passed through with the aid of a syringe pump at a rate of 1 ml/h. The discs were then washed copiously with 0.1 *M* $NaHCO_3$ followed by deionized water; they were then allowed to stand for 1 h in 2% sodium dodecyl

sulphate solution then washed again with de-ionized water and blotted dry on filter paper.

The protein A contents of the separate discs were determined by counting each in 8 ml of Optiphase scintillant.

All the above procedures were repeated with "unactivated" Biomer (*i.e.*, functionalised but not activated) to determine the non-specific adsorption.

Assessment of the direct coupling of ^{125}I-labelled human IgG to the activated and unactivated polymers was carried out in exactly the same manner as described above.

Assessment of binding of IgG by protein A coupled to activated Biomer membranes

Discs of the activated membranes to which protein A had been coupled were treated with bulk ethanolamine to block unreacted activating residues and washed copiously with deionized water and phosphate-buffered saline (PBS). A 5-ml volume of a solution of ^{125}I-labelled human IgG in 0.15 *M* PBS (2.8 mg per ml) was passed through the discs at a rate of 1 ml/h. The discs were then washed with deionized water and placed in 10 ml of a solution of 0.2% Tween 20 in 0.15 *M* PBS for 1 h to remove non-specifically bound protein. Finally the discs were washed with deionized water, blotted and counted.

RESULTS AND DISCUSSION

Protein A binding

Measurements were made of covalently bound protein A using either one or three discs as described; these are numbered 1, 2 and 3, the order in which they are encountered by the solution. Table I presents total weights of protein A coupled (on all discs); for the purposes of calculation the area is taken as that of one disc.

Table I shows that the non-specific adsorption of protein A by the unactivated membranes is low. As a fraction of the coupling by the corresponding activated membrane it has an average value of 2.0%.

Fig. 6 is an SEM of the membrane of the activated polymer **28**. The fibrous structure resembles that of Fig. 1, indicating that the functionalisation process has not greatly altered the porosity. On the other hand the pore structures of polymers **10** and **12** (Figs. 7 and 8) show considerable changes from those of their precursors **8** and **9** (Fig. 3), respectively. Different techniques were used for activating these polymers. For polymer **28**, which gives the best result of the three (Table I), direct coupling with Bolton–Hunter reagent was employed, while **7** and **9** were synthesised by free-radical reactions through the macroinitiator **8** and macromer **9** (Fig. 3), respectively. The activating group was a succinimido

TABLE I

PROTEIN A COUPLING TO ACTIVATED MEMBRANES

Reference number of activated membrane	Number of discs used	Protein A coupled		Non-specific adsorption of protein A	
		mg/g	μg/cm	mg/g	μg/cm
10	1	1.24, 1.66	27.5	0.037	0.79
12	1	0.61	11.8	0.037	0.79
14	1	6.26	93.2	0.014	0.25
15	3	1.46	88.1	0.020	1.35
16	1	0.66	16.9	0.014	0.25
18	3	2.94	183.2	0.055	3.71
19	3	3.64	221.5	0.022	1.51
21	1	0.63	13.6	0.014	0.25
22	3	1.81	114.2	0.020	1.35
23	1	0.29	6.0	0.014	0.25
25	3	3.04	209.7	0.055	3.71
26	3	4.16	269.3	0.023	1.51
27	3	1.43	85.5	0.008	0.77
28	1	3.17	70.3	0.037	0.79

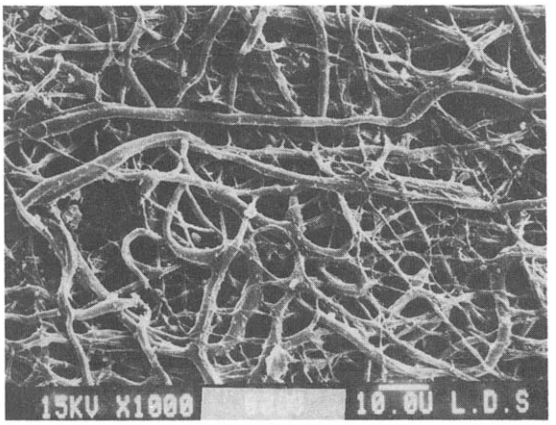

Fig. 6. SEM of membrane **28**, activated with Bolton–Hunter reagent.

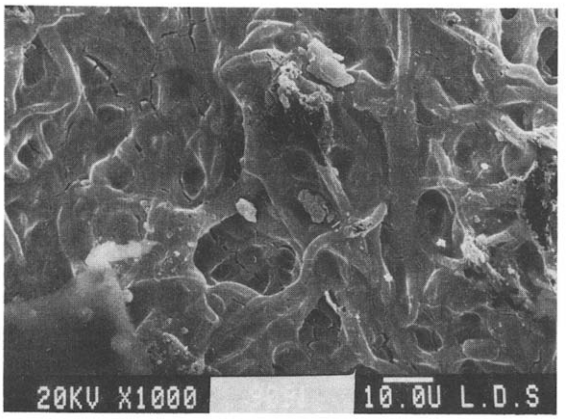

Fig. 8. SEM of membrane **12**, grafted with acryloxysuccinimide by macromer technique.

derivative in each case, and the differences in the capacity for protein coupling are likely to reflect the pore structure. Figs. 7 and 8 show relatively large pores and closely-packed areas; the surface area of the fibres accessible to the proteins is evidently reduced in these structures. Another example of the efficacy of direct coupling is provided by **26** which was activated by CDI (Fig. 5) and has relatively high capacity for protein A. The structure is shown in Fig. 9 and is not very different from those of polymer **29** (Fig. 6) or the original Biomer (Fig. 1). According to these findings direct coupling of the activating group gives more suitable structures than the free-radical methods so later work was focused on the former technique.

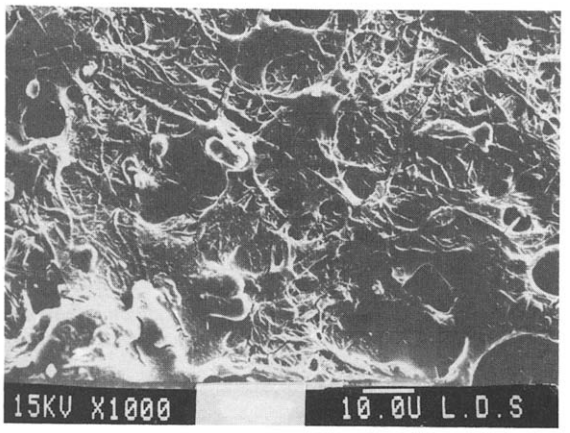

Fig. 7. SEM of membrane **10**, grafted with acryloxy-succinimide by macroinitiator technique.

The choice of activating group is important. It is known that the hydrolysis half-lives at pH 8–9 for N-hydroxysuccinimide ester, imidazole carbamate and 2-fluoro-1-methylpyridinium toluene-4-sulphonate groups attached to a support of cross-linked agarose are of the order: minutes, a few hours and 100 h, respectively. In our experiments (at pH 8) it would seem that for coupling to amino groups (*e.g.* those in protein A) FMP gave relatively low values (polymers **16** and **23**) and CDI high values (polymers **14**, **18**, **19**, **25**, **26**). Although **14** and **18** were both synthesised from **13** the intermediate stages involved the use of ethanolamine and D-glucamine respectively (Fig. 4), while **25** was prepared from **20** with the aid of D-glucamine (Fig. 5). These details may influence the above comparison, which therefore needs further investigation.

The activated membranes **19**, **26** formed from acid membranes (Figs. 4 and 5) are seen from Table I to be very effective. The content of activating groups is probably high in these materials by virtue of the rapid reaction between carboxyl and CDI and the absence of cross-link (carbonate) formation (which is possible with hydroxylic polymers). However, we shall see later that this factor is unlikely to be responsible for the high coupling capacity of the membranes. The combination of properties possessed by the spacers —which are rather hydrophobic and contain about twenty backbone atoms— could be particularly favourable.

Membrane **27**, (prepared from the same precursor **20** as **26**) (Fig. 5) was also activated by CDI and

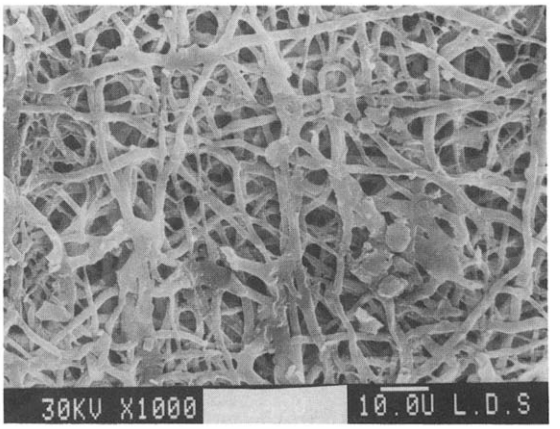

Fig. 9. SEM of acid membrane **26** after direct coupling of CDI.

carried long hydrophilic spacers of PEG having a backbone length of about 270 atoms. These did not confer any marked improvement in capacity (compare Kato and Kito [12] and Phillips *et al.* [13]).

Table II shows the distribution of coupled protein A between the discs.

According to the data in Table II, when three discs are present, the extents of coupling are in the order 1 > 2 > 3. The coupling capacities given in mg/g in Table I for determinations with three discs are therefore underestimates since they are averages based on the total for all three. For example, the maximum capacity for the first disc in polymer **19** calculated from Table II is 8.16 mg/g.

The distribution data lead to interesting conclusions. We assume the three discs of a given polymer are effectively identical in structure. Disc 1 of polymer **26** removes 32.5% of the protein presented to it in the solution so that the average concentration of emerging solution is approximately 67.5% of the initial. Disc 2 might therefore be expected to adsorb 0.675 × 0.976 = 0.659 mg of protein A. Similarly for disc 3 the expected removal is 0.578 mg. These values for discs 2 and 3 are much greater than those observed, 0.247 and 0.141 mg, respectively. The same conclusion may be drawn for polymer **14**. Similar, but smaller effects follow for the polymers **15** and **22**, the expected removals for discs 2 and 3 being 0.184 and 0.175 mg, respectively for **15** and 0.297 and 0.276 mg for **22**. We believe these results reflect the non-uniform nature of the liquid flow through the discs. For instance, they are compatible with a leakage of solution around the edges of the discs, so that some pass through the apparatus virtually unchanged. Inspection of the equipment makes this aspect unlikely. A more probable explanation lies in the range of pore sizes between the

TABLE II

DISTRIBUTION OF PROTEIN A BETWEEN DISCS

(a) = Weight of protein A coupled (mg); (b) = percentage of total coupled protein on each disc; (c) = percentage of total protein initially present in solution removed by disc.

Reference number of activated membrane	Disc 1	Disc 2	Disc 3	Percentage of total protein A in solution which becomes coupled
15 (a)	0.197	0.140	0.112	15.0
(b)	43.9	31.2	24.9	
(c)	6.56	4.66	3.72	
22 (a)	0.335	0.191	0.053	19.3
(b)	57.8	33.0	9.2	
(c)	11.15	6.37	1.77	
19 (a)	0.838	0.198	0.085	37.4
(b)	74.8	17.6	7.6	
(c)	27.94	6.59	2.83	
26 (a)	0.976	0.247	0.141	45.5
(b)	71.5	18.2	10.3	
(c)	32.5	8.24	4.71	

fibres, some pores being sufficiently large to allow solution to pass through them virtually unchanged. Such an effect of large pores is not surprising in view of the small diffusion coefficients of protein molecules. Simple quantification of this model shows that the difference between the expected and actual adsorptions increases with the activity of the membrane in agreement with the above discussion. The above argument assumes a linear relation between weight of protein bound and the solution concentration. If the relation is not linear but has the familiar form with a plateau adsorption the argument is strenghthed.

IgG binding

The coupling of IgG has also been examined. This protein may be bound in two ways: (a) direct to the activated membrane, as described for protein A, and (b) to protein A coupled to the membrane. Data for the two procedures are given in Table III.

Since the coupling values in Table III are given for three discs together, they are unlikely to represent maximum capacities.

As with protein A, the non-specific adsorption of IgG is low, amounting, on average, to approximately 0.64% of the coupling to the activated membrane. The corresponding value given in ref. 14 for "Quenched Immobilon" membrane is about 2%.

A simple geometric model for estimating the capacity for protein adsorption by electrostatically spun fibres

The present system readily lends itself to idealisation in the form of a model suitable for these estimates. The simplest model consists of a single fibre of which the surface is fully covered by adsorbed protein. Estimation of the maximum weight of protein adsorbed is therefore made in terms of relative areas, on the assumption that there is always an excess of activated groups present. It will appear later that the latter supposition is normally true for our membranes. This model naturally tends to over-estimate the maximum capacity, for, as we have mentioned earlier, the whole of the surface area is not accessible to the protein.

If D and ρ are the diameter and density of the

TABLE III

IgG COUPLING

Reference number of activated membrane	Direct coupling of IgG		Coupling of IgG to protein A on membrane		Non-specific adsorption of IgG	
	mg BH/g	$\mu g/cm^2$	mg/g	$\mu g/cm^2$	mg/g	$\mu g/cm^2$
28	5.65	266.3	2.68	152.0	0.02	1.43
29	5.70	206.2	3.96	290	0.02	1.00

TABLE IV

CONCENTRATIONS OF FREE AND OCCUPIED ACTIVE SITES IN MEMBRANES

S = mol of active sites per g Biomer; S_{occ} = mol of active sites per g Biomer coupled to IgG assuming one bond per protein molecule. Estimated from data in Table III.

Reference number of activated membrane	Active sites present in 1 g Biomer		$10^8\ S_{occ}$ mol/g	S/S_{occ}
	mg BH/g	$10^5\ S$ mol/g		
28'	28.2	10.7	3.77	2838
29'	138	52.4	3.80	13815

fibre, respectively, the length L of fibre weighing 1 g is

$$L = \frac{4}{\pi\rho D^2} \text{cm/g} \quad (2)$$

and the surface area per gram A is

$$A = \frac{4}{\rho D} \text{cm}^2/\text{g} \quad (3)$$

The volume of the constituent chains in a protein molecule of molecular mass M is M/ρ_p cm^3/mol or $M/N\rho_p$ cm^3/molecule, where N is the Avogadro number and ρ_p is the density of the chains. For estimating the packing of the molecules we consider each as a sphere; the volume of this sphere will clearly be greater than $M/N\rho_p$ cm^3 and we may write it was $FM/\text{N}\rho$ cm^3, F being a factor probably in the range 1–10. The radius r of this sphere is given by:

$$r = \left(\frac{3FM}{4\pi N\rho_p}\right)^{1/3} \text{cm} \quad (4)$$

A closely packed monolayer of such spheres would have the centres of neighbours separated by a distance $2r$ so that each sphere in the layer occupies an area $2\sqrt{3}r^2$. From eqns. 3 and 4 we see that the maximum number of protein molecules n which can be accommodated on 1 g of fibre is therefore

$$n = \frac{\text{A}}{2\sqrt{3}r^2} = \frac{2}{\sqrt{3}\rho D}\left(\frac{4\pi\rho_p N}{3FM}\right)^{2/3} \quad (5)$$

Thus the weight of protein bound to 1 g of fibre W is given by

$$W = \frac{nM}{N} = \frac{2}{\sqrt{3}\rho D}\left(\frac{M}{N}\right)^{1/3}\left(\frac{4\pi\rho_p}{3F}\right)^{2/3} \quad (6)$$

The following extreme values of the coupling capacities for the two proteins studied may be deduced from eqn. 6. They are based on $F = 1$ and $F = 10$, with $\rho = 1.0$, $\rho_p = 1.3$ g/cm and $D = 10^{-4}$ cm in each case.

$$\begin{aligned} &\text{Protein A, } M = 42\,000; && W_{max} = 14.7 \text{ mg/g} \\ & && W_{min} = 3.16 \text{ mg/g} \\ &\text{IgG, } M = 150\,000; && W_{max} = 22.4 \text{ mg/g} \\ & && W_{min} = 4.85 \text{ mg/g} \end{aligned} \quad (7)$$

The coupling capacities mentioned earlier lie within the limits in both cases, suggesting that the maximum covering corresponds to a monomolecular layer.

Further evidence supporting this conclusion may be adduced from considerations of the number of activated groups introduced into the sample. We determined the content of such groups as described for two samples of polymer: these, like **28** and **29**, were synthesised from the functionalised polymers **13** and **20** by direct coupling of ^{125}I-labelled Bolton–Hunter reagent. The activated products are designated **28′** and **29′**. We assume that as far as protein coupling is concerned **28′** and **29′** behave similarly to **28** and **29**, respectively.

Data are presented in Table IV.

Two considerations follow: (i) the considerable difference in the site number per gram in the two polymers is not reflected in the IgG capacities (Table III) and (ii) the number of sites greatly exceeds the number of protein molecules bound. Clearly in these samples the initial site density is not a limiting factor determining the protein adsorption, in agreement with our hypothesis.

The data presented allow estimation of the mean area per active site a_s, assuming all sites lie on the fibre surface. This is given by

$$a_s = \frac{4}{\rho DSN} \quad (8)$$

From Table IV, $a_s = 6.2$ Å^2 for **28′** and 1.3 Å^2 for **29′**. Both areas are unacceptably small, indicating that all the active groups cannot be accommodated on the fibre surface. We therefore believe that in the activating process, fibre swelling allows the reaction to proceed to a limited extent beneath the surface. Groups introduced in this way, unlike those lying on the surface, would not be accessible to a protein.

We are currently concerned with optimisation and further exploration of this promising system.

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