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Crosslinked glass fiber affinity membrane chromatography and its application to fibronectin separation

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Abstract

Macroporous glass membranes were prepared from glass fiber filters via chemical crosslinking and modification, and used for the membrane affinity chromatography of fibronectin from human blood plasma. The filters were first treated with a piranha solution (a concentrated solution of H_2SO_4+H_2O_2 in water), and then crosslinked with bifunctional organosilanes and modified to introduce amino or aniline moieties. Ligand immobilizations via diazotization and glutaraldehyde pathways were carried out and compared. Characterization of the membranes was performed using bovine serum albumin and trypsin as test ligands. By using a cartridge containing gelatin immobilized affinity membranes followed by another cartridge containing heparin immobilized membranes, fibronectin from human blood plasma could be separated.
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1. Introduction

Membrane-based affinity chromatography has been extensively used for biomolecule purification and immunoadsorption [1–5]. The main advantages of using synthetic membranes compared to conventional bead supports are their incompressibility and the fact that they eliminate internal diffusion limitations. Most investigations have focused on the preparation and modification of the supporting membranes, because, regarding affinity adsorption and elution, abundant information had already accumulated from classical column affinity chromatography [6]. Affinity membranes can be employed as flat sheets, monolithic discs [7] and hollow fibers [8,9].

In membrane affinity chromatography, separation is based on the affinity characteristics of the solute molecules and not their size. For this reason, large pore sizes and high porosities should be used, in order to ensure that even the large impurities pass through. Most of the common commercially available membranes are not suitable for membrane affinity chromatography [10]. In our previous papers [11–14], macroporous cellulose membranes were prepared from high quality filter paper by mercerization followed by chemical crosslinking. The prepared membranes possessed a high porosity (~50%) and large pores (0.4–1.0 μm), had a low cost and were suitable because of their long durability, among other reasons, for affinity chromatography. The prepared membranes were further modified and activated by

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introducing epoxyl, triazine, aldehyde or diazonium groups, which were then coupled with enzymes or other affinity ligands. Using the prepared affinity membranes, the separation and purification of concanavalin A [11], trypsin inhibitors [12], serum proteins, globulins [14], papain inhibitors [13] and peroxidase [14] were carried out.

Besides the cellulose membranes mentioned above, we found that glass fibers are also suitable for the preparation of affinity membranes. In classical column affinity chromatography, the glass beads are the most important inorganic supporting material, because glass and its derivatives have high stability and are not attacked by microorganisms [15]. However, in membrane affinity chromatography, glass, like other inorganic materials, cannot be easily processed as a membrane because it is very brittle. In a previous paper [16], affinity membranes were prepared from commercial glass fiber filters. They were first treated with a piranha solution (a mixture of 70% vol. concentrated sulfuric acid (98%, m/m) and 30% vol. of a hydrogen peroxide solution (30%, m/m)) to enrich the number of silanol groups, followed by silanization to introduce epoxyl or amino moieties as functional groups. Affinity ligands were then immobilized using a glutaraldehyde, diazotization or carbodiimide pathway. The three immobilization methods were investigated in detail by determining and comparing the activities of the immobilized enzymes. The results indicated that the glutaraldehyde method provided the highest performance regarding enzyme immobilization and the highest durability, because of its crosslinking capability.

The objective of this paper was to prepare macroporous glass membranes from glass fiber filters using several chemical crosslinking procedures. The filters were first treated with a piranha solution, to clean the membrane and to increase the surface density of the OH groups. They were then crosslinked using one of the following crosslinkers: bis[3-(trimethoxysilyl)-propyl]amine, 1,2-bis(triethoxysilyl)-ethane or 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane. After crosslinking, the membranes were modified by introducing NH_2 or epoxyl functional groups. The epoxyl groups were thereafter converted to aniline moieties. Ligand immobilization was carried out via diazotization or using glutaraldehyde as a coupling reagent. Characterization of the membranes was carried out using bovine serum albumin and trypsin as test ligands. By using successively two cartridges containing gelatin and heparin immobilized affinity membranes, respectively, fibronectin from human blood plasma could be separated. The separations were monitored and evaluated by electrophoresis.

2. Experimental

2.1. Chemicals

Sigma glass fiber filters (1.5-μm retention), trypsin (type IX), *N*α-benzoyl-L-arginine *p*-nitroanilide (BAPNA), bovine serum albumin (BSA; 99%), human serum albumin (HSA; 99%), gelatin (type A), heparin (140 USP units per mg), fibronectin (FN), phenylmethylsulfonyl fluoride (99%) and human plasma were purchased from Sigma (St. Louis, MO, USA). Bis[3-(trimethoxysilyl)-propyl]amine (90%), 1,2-bis(triethoxysilyl)-ethane (96%), 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane (97%), γ-aminopropyltriethoxysilane (98%), γ-glycidoxypropyltrimethoxysilane (97%), succinic anhydride, 1,4-phenylenediamine, glutaraldehyde (25%), dimethyl sulfoxide (99.9%), sodium dodecyl sulfate (SDS) (99%), β-mercaptoethanol, bromophenol blue, sulfuric acid (98%), nitric acid (70%), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; 99%), sodium nitrite (99.5%) and Coomassie Brilliant Blue G-250 (90%) were purchased from Aldrich (Milwaukee, WI, USA).

2.2. Instruments

X-ray photoelectron spectroscopy (XPS) was carried out using a VG-ESCA lab 210 spectrometer operating in the constant analyzer energy mode with a passing energy of 50 eV and Mg Kα radiation as the excitation source. The analysis chamber of the XPS spectrometer had a vacuum of 10^{-10} Torr. The C1s line was taken as an internal reference with a binding energy of 284.8 eV. A Waring LB20G blender (Christison Scientific Equipment, Gateshead, NE8 3AT, UK) was used for sample homogenization. The morphologies of the glass membranes were examined using an Hitachi S-800 scanning electron microscope (Hitachi, Tokyo, Japan). Two peristaltic

pumps were employed for the processing of the mobile phases, a Masterflex 7520-00 (Cole-Parmer Instrument, Vernon Hills, IL, USA) and a TRIS (ISCO, Lincoln, NE, USA). A Sorvall RT600B refrigerated centrifuge (Sorvall Products, L.P., Newtown, CT, USA) was used for the pretreatment of the sample. The concentration of the protein was determined by measuring the absorbance at 595 nm by the Coomassie Brilliant Blue method with a Beckman DU 650 UV–Vis spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The eluant was collected with a Retriever 500 fraction collector (ISCO, Lincoln, NE, USA), and the purity of the protein was determined with an SDS–PAGE (polyacrylamide gel electrophoresis) Phast System electrophoresis system (Pharmacia LKB Biotechnology, Uppsala, Sweden). The electrophoresis results were scanned and analyzed using Metamorph software (Version 4.01, Universal Imaging, Downingtown, PA, USA).

2.3. Pretreatment and purification of the glass fiber filters

A piranha solution (a mixture of 70% vol. concentrated sulfuric acid (98%, m/m) and 30% vol. of a hydrogen peroxide solution (30%, m/m)) [17] was used to remove the organic residues (esters) or other impurities from the glass surface and to generate additional silanol groups. The pretreatment was described previously [16].

2.4. Preparation of membrane cartridges

Home-made membrane cartridges were used in this work. The cartridges have a diameter of 47 mm and an adjustable depth to fit various numbers of membranes [11]. All cartridges used in this paper contained 20 membranes and were 8 mm in total thickness.

2.5. Crosslinking of glass membranes with bifunctional organosilanes

The purified glass membranes were first soaked in ethanol and then packed into cartridges, through which was recirculated, at 15 ml/min, a freshly prepared reacting solution containing 90 ml ethanol and 10–30 ml of one of the following crosslinkers: bis[3-(trimethoxysilyl)-propyl]amine, 1,2-bis(triethoxysilyl)-ethane or 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane. The reaction was carried out at room temperature for 2 h. After reaction, the cartridges were washed first with 200 ml ethanol followed by 500 ml distilled water.

2.6. Silanization of the crosslinked glass membranes to introduce epoxyl or amino groups

The introduction of epoxyl or amino groups into the membranes was carried out through the silanization of the crosslinked glass membranes with γ-glycidoxypropyltrimethoxysilane or γ-aminopropyltriethoxysilane, respectively, using methodologies described previously [16].

2.7. Modification of the glass membrane with 1,4-phenylenediamine for the introduction of aniline moieties

Cartridges containing γ-glycidoxypropyltriethoxysilane-activated membranes were modified using a method similar to that employed on cellulose membranes [11]. The epoxyl containing cartridges were subjected to reacting solutions containing 2 g 1,4-phenylenediamine and 50 g Na_2CO_3 in 100 ml water, which were recirculated at 5 ml/min. The reaction proceeded for 2 h at 50 °C. After reaction, the cartridge was rinsed with distilled water until the latter became colorless.

2.8. Coupling of trypsin, bovine serum albumin, or gelatin to the 1,4-phenylenediamine modified glass membrane

The 1,4-phenylenediamine modified glass membranes were packed into cartridges, activated via diazotization and finally reacted with one of the proteins. The methodologies for membrane activation and protein immobilization were described previously [11].

2.9. Activation of the glass fiber membrane with glutaraldehyde

The γ-aminopropyltriethoxysilane modified membranes were packed into cartridges, which were first equilibrated with a 0.2 *M* NaAc–HAc, pH 7.50 solution for 10 min at room temperature. After the above solution was removed, the cartridges were reacted with a 100-ml 25% (m/m) glutaraldehyde aqueous solution, which was recirculated at 15 ml/min for 3 h at 40 °C. After reaction, the cartridges were washed with 100 ml distilled water, followed by 100 ml of a 2 *M* acetic acid solution and finally 200 ml distilled water, in order to remove the unreacted glutaraldehyde.

2.10. Coupling of heparin, trypsin or bovine serum albumin onto the glutaraldehyde activated glass membrane

Cartridges containing glutaraldehyde activated glass membranes were subjected to a reacting solution containing 200 mg heparin and 0.01 *M* $CaCl_2$ in 100 ml 0.05 *M* Tris–HCl, pH 8.50 buffer (a 0.05 *M* Tris aqueous solution, with its pH adjusted to 8.50 with 1 *M* HCl). The immobilization was carried out by recirculating the heparin solution through the cartridge for 1 h, at 50 °C, at a flow rate of 10 ml/min. The methodologies for the trypsin and bovine serum albumin immobilizations were similar, only the amounts of ligands were different (100 mg).

2.11. Determination of the capacity for BSA immobilization onto the γ-aminopropyltriethoxysilane modified glass membranes via the glutaraldehyde method

A cartridge containing 20 sheets of γ-aminopropyltriethoxysilane modified glass membranes was allowed to react with a solution containing 100–250 mg BSA in 100 ml of 0.05 *M* Tris–HCl, pH 8.50 buffer, using glutaraldehyde as the coupling reagent. The methodology was described previously [16].

2.12. Determination of the activity of trypsin

The activities of the non-immobilized and immobilized trypsin were determined via the hydrolysis rate of a substrate (BAPNA), using a methodology described previously [12]. Under the experimental conditions, 1 U of trypsin activity is defined as a 0.001 increase per minute in the absorbance at 410 nm. The activity of the immobilized enzyme was assayed using a sector-shaped part of a membrane containing enzyme cut into small pieces, suspended through stirring in the substrate solution. Each experiment was repeated at least three times and the experimental uncertainty was below 5%.

2.13. Other determination methods employed

The determinations of (i) the pore size and porosity of the glass fiber membrane, (ii) the protein content by the Coomassie Brilliant Blue method, and (iii) the epoxyl group content of the modified membrane were carried out as described previously [12].

2.14. Gelatin immobilized membrane affinity chromatography

The cartridge containing gelatin affinity membranes was first equilibrated with a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 150 m*M* NaCl, and 1 m*M* EDTA. After the buffer was removed, the cartridge was subjected to 40 ml reconstituted human plasma, which was recirculated for 1 h, at a flow rate of 2 ml/min, at 4 °C. The cartridge was then washed with a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 150 m*M* NaCl, and 1 m*M* EDTA, at a flow rate of 2 ml/min, to remove the non-adsorbed molecules. The bound proteins were eluted with a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 250 m*M* NaCl, 3 *M* urea, and 1 m*M* EDTA, at a flow rate of 2 ml/min. The eluted fractions were collected and dialyzed for 24 h against a 20-fold volume of 50 m*M* Tris–HCl, pH 7.40 buffer, containing 1 m*M* EDTA, and 1 m*M* phenylmethylsulfonyl fluoride. The buffer was changed every 8 h.

The cartridge was regenerated by washing with a 6 *M* urea aqueous solution for 60 min at a flow rate of 0.5 ml/min, followed by equilibration with a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 150 m*M* NaCl, and 1 m*M* EDTA.

2.15. Heparin immobilized membrane affinity chromatography of fibronectin

After dialysis, the affinity fraction of the above gelatin immobilized membrane chromatography, 30 ml in volume, was loaded onto a cartridge containing heparin immobilized affinity membranes and recirculated for 1 h at 4 °C at a flow rate of 1 ml/min. The cartridge was then washed, at a flow rate of 2 ml/min, with a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 1 m*M* EDTA, and 150 m*M* NaCl to remove the weakly adsorbed molecules. Some bound proteins were eluted using the same buffer, at a NaCl concentration of 250 m*M*, at a flow rate of 2 ml/min, and other bound proteins at a NaCl concentration of 500 m*M*.

The cartridge was regenerated by washing with a 6 *M* urea aqueous solution for 60 min at a flow rate of 0.5 ml/min, followed by equilibration with a 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.40 buffer.

2.16. Electrophoresis of fibronectin

The samples were first dialyzed against a 10 m*M* Tris–HCl and 1 m*M* EDTA, pH 8.0 buffer, and then mixed with an equal volume of an aqueous solution containing 5.0% (m/m) SDS, 10% (v/v) β-mercaptoethanol and 0.02% (m/m) bromophenol blue solution. The mixtures were heated at 60 °C for 2 h [18] and then filtered to remove any insoluble material before being used for the SDS–PAGE electrophoresis. A PhastGel Homogeneous 7.5 gel (7.5% separating gel) was used, and the standard procedure [19] for SDS–PAGE PhastSystem was followed.

3. Results and discussion

In our previous work [16], membrane affinity chromatography was carried out on silanized glass membranes, which were prepared from glass fiber filters. Several ligand immobilization pathways have been employed. Among them, the glutaraldehyde method provided the best results, both regarding membrane stability and ligand immobilization capacity. The higher stability occurred because the glutaraldehyde molecules condensed into larger molecules, which crosslinked the glass fibers (Fig. 1).

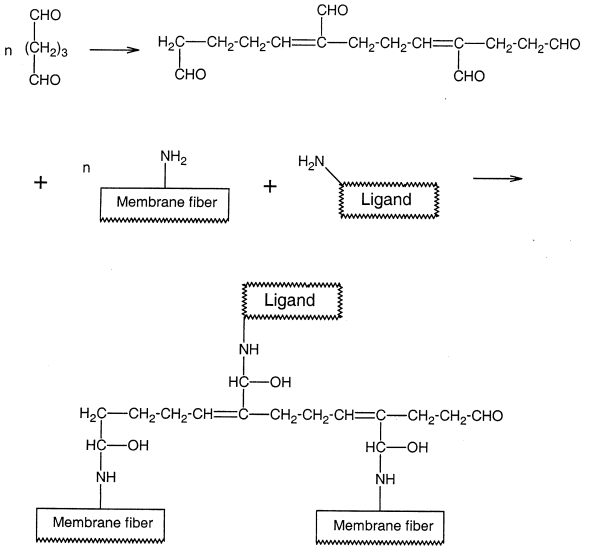

Fig. 1. Crosslinking during the glutaraldehyde immobilization pathway.

In the present paper, the crosslinking was carried out in a more systematic manner. Any bifunctional silane that allows chemical coupling between silanols can be used as a crosslinker. However, the selection of a suitable crosslinker is determined by its solubility, toxicity, and reaction selectivity. In the present paper, the following three crosslinkers were employed: bis[3-(trimethoxysilyl)-propyl]amine, 1,2-bis(triethoxysilyl)-ethane and 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane (Fig. 2).

The effects of the three crosslinkers were compared using scanning electron microscopy (Fig. 3). Fig. 3 shows that after crosslinking, the swelling thicknesses in water were significantly reduced, and the fractions of free fibers were greatly decreased. The latter effect strongly affects the stability of the affinity membranes. In order to examine the effect of the crosslinking, the original membrane and the crosslinked ones were allowed to swell freely in water, the extent of swelling providing a measure of the crosslinking. However, in practical applications, the membranes are packed into cartridges under pressure, and the thicknesses of the non-crosslinked and crosslinked membranes become almost the same. The pore sizes and the porosities of the crosslinked

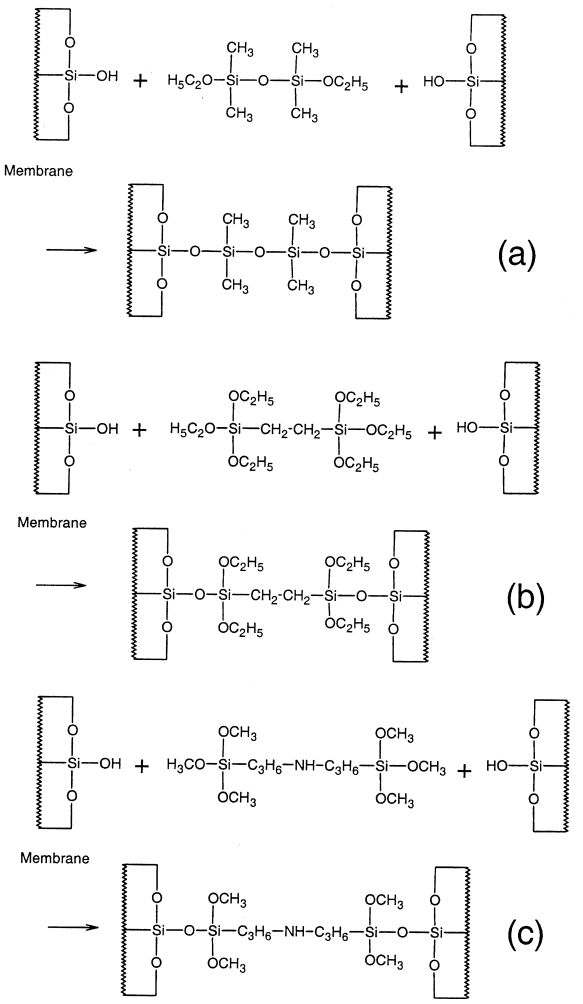

Fig. 2. Crosslinkers employed: (a) 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane; (b) 1,2-bis(triethoxysilyl)-ethane; and (c) bis[3-(trimethoxysilyl)-propyl]amine.

membranes were also determined and the results are listed in Table 1.

As was shown in a previous paper [16], crosslinking increases the stability of the glass membrane. However, crosslinking consumes a fraction of the silanol groups, which can be used for further modification and ligand immobilization. For this reason, we investigated the effects of different crosslinkers on the further modifications of the membranes. The membranes were first crosslinked with various crosslinkers, and then modified with an amino containing silane. The content of N atoms thus introduced into the membranes was thereafter determined by XPS (Table 1). This table shows that bis[3-(trimethoxysilyl)-propyl]amine and 1,2-bis(triethoxysilyl)-ethane consumed fewer silanol groups than 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane. In additional experiments, several membranes were crosslinked with various amounts of 1,2-bis(triethoxysilyl)-ethane, further modified with γ-glycidoxypropyltrimethoxysilane, and compared for their epoxyl content (Fig. 4). Fig. 4 shows that with increasing amount of crosslinker content, the epoxyl content was somewhat reduced. As previously mentioned [16], silanization of glass membranes with γ-glycidoxypropyltrimethoxysilane or γ-aminopropyltriethoxysilane provided almost the same conversion. Consequently, the results presented in Fig. 4 are relevant for both silanization reagents.

The non-specific adsorption of proteins is the main disadvantage of glass. It is caused by the presence of small amounts of metal ions that generate high potential sites [20]. Usually this shortcoming could be overcome by silanization with a hydrophilic group containing silane, such as γ-aminopropyltriethoxysilane [21].

The crosslinking used in the present paper was also found to be effective in generating a uniform layer over the glass surface that capped the high potential sites (Fig. 5). Fig. 5b shows that no metallic ions were present on the surface of glass after crosslinking. This means that the ions present before crosslinking (Fig. 5a) were all capped, and that the surface of the glass was covered by a silane compound.

To introduce the necessary functional groups for further modification and ligand immobilization, the crosslinked membranes were treated with either γ-aminopropyltriethoxysilane or γ-glycidoxypropyltrimethoxysilane, which contain epoxyl and amino functional groups, respectively [16]. The amino groups were used for ligand immobilization via the glutaraldehyde pathway, while the epoxyl groups were transformed into aniline groups, and thereafter used for ligand immobilization via the diazotization pathway.

Compared with the membranes prepared in a previous paper [16], the crosslinked membranes possess a more stable structure because the fibers are linked to each other as demonstrated in Fig. 3. The

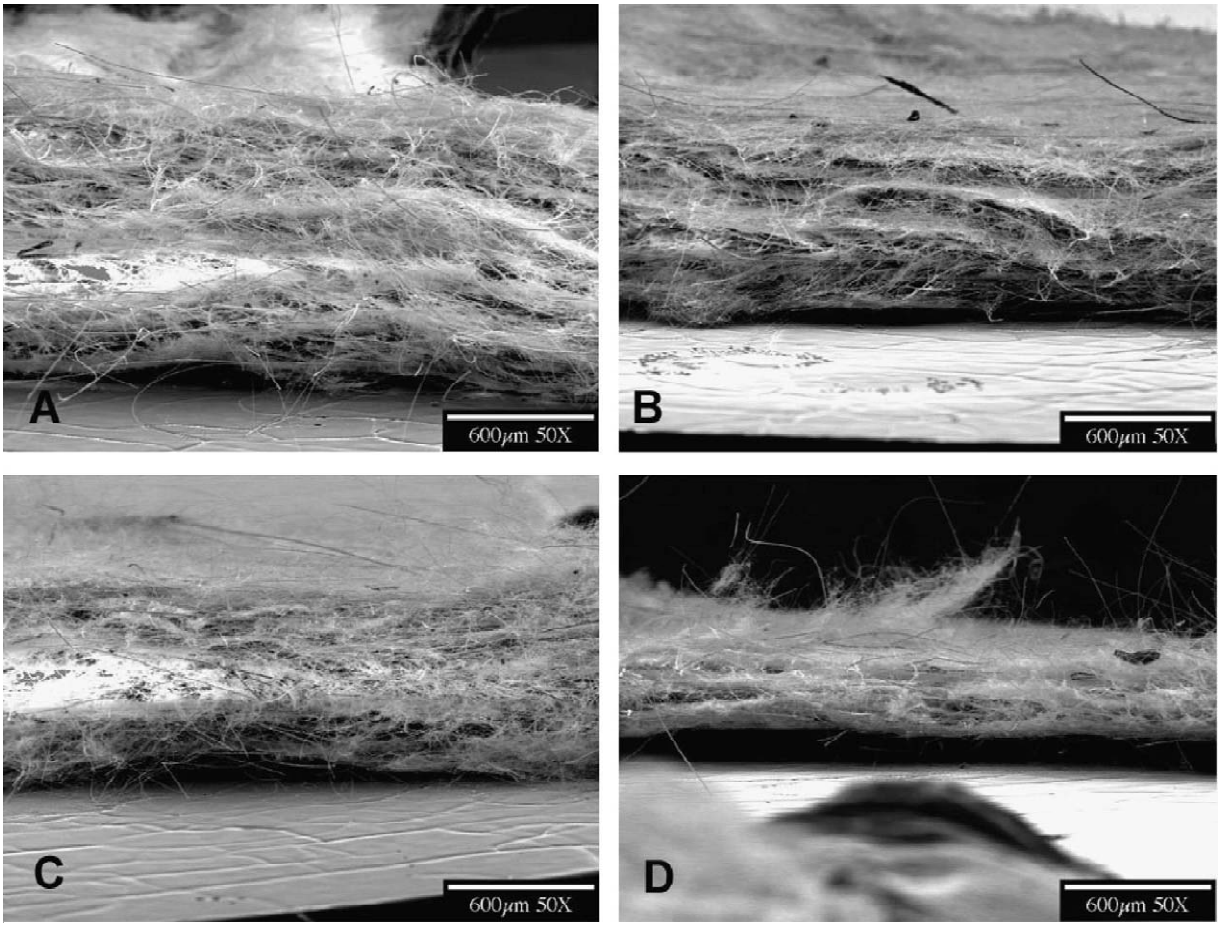

Fig. 3. Scanning electron micrographs of the glass membranes: (A) a non-crosslinked glass membrane; (B) a glass membrane crosslinked with 1,3-diethoxy-1,1,3,3-tetramethyldisiloxane; (C) a glass membrane crosslinked with bis[3-(trimethoxysilyl)-propyl]amine; (D) a glass membrane crosslinked with 1,2-bis(triethoxysilyl)-ethane. A 10 ml amount of crosslinker was used for 20 membranes packed in a cartridge. See Experimental section for details.

Table 1
Effect of three bifunctional silanes on the properties of the crosslinked glass membranes

Crosslinker	Pore size[a] (μm)	Porosity[b] (%)	N content[c] (atom%)
None (original membrane)	3.08	86.9	1.8
1,3-Diethoxy-1,1,3,3-tetramethyldisiloxane	2.76	80.5	0.9
bis[3-(Trimethoxysilyl)-propyl]amine	2.80	78.0	1.6
1,2-bis(Triethoxysilyl)-ethane	2.44	75.4	1.6

A total of 20 membranes packed in a cartridge were crosslinked by reacting them with solutions containing 10 ml of crosslinker.

[a] The pore size was determined using water as the mobile phase, and the equation $J = \frac{\varepsilon r^2}{8\mu\tau} \frac{\Delta P}{L}$, where ΔP is the pressure drop, r the radius of the pore, μ the dynamic viscosity, ε the porosity, τ the tortuosity factor (taken as 2), L the total thickness of the membranes and J the flux through the membrane.

[b] The porosities were obtained by comparing the masses of the soaked and dried membranes.

[c] The N content was determined by XPS, after the non-crosslinked or crosslinked membranes were modified with γ-amino-propyltriethoxysilane.

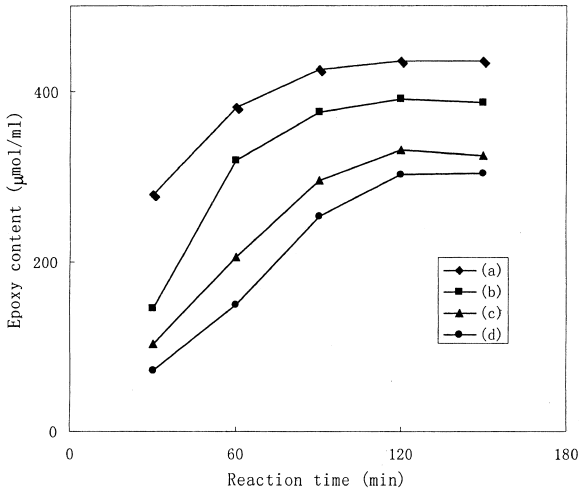

Fig. 4. Effect of crosslinking on the further modification of the glass membranes with γ-glycidoxypropyltriethoxysilane. A total of 20 membranes packed in a cartridge were crosslinked with a solution containing: (a) 0 ml (non-crosslinking), (b) 10 ml, (c) 20 ml and (d) 30 ml 1,2-bis(triethoxysilyl)-ethane, and thereafter modified with a solution containing 10 ml γ-glycidoxypropyltriethoxysilane. See Experimental section for details.

fact that the stability was improved by the crosslinking was also demonstrated by comparing the activities of trypsin immobilized on both non-crosslinked and crosslinked glass membranes (Fig. 6). To examine the structural stability of the membranes, the activity was determined [16] by cutting a sector of one membrane into small pieces, which were suspended in the substrate solutions, and by carrying out the reactions with adequate stirring. Two washing steps were used before the next determination. Fig. 6 shows that the immobilized trypsin prepared via the glutaraldehyde pathway was very stable, for both the crosslinked and non-crosslinked glass membranes. This behavior can be explained through the self-condensing in solution of the glutaraldehyde and the crosslinking of the amino groups of the glass surface by the condensed glutaraldehyde (Fig. 1) [22]. Fig. 6 also shows that the immobilized trypsin on the crosslinked glass membrane prepared via diazotization was much more stable than that on the non-crosslinked glass membrane. This occurred because, in contrast to glutaraldehyde, no crosslinking that can stabilize the structure took place during the diazotization pathway for the uncrosslinked glass membranes. Therefore, the difference in the stabilities of the immobilized trypsin for crosslinked and non-crosslinked glass membranes, is an effect of the crosslinking.

As mentioned above, the crosslinking procedure consumes a fraction of the silanol groups, which, while stabilizing the membrane structure, reduces somewhat the capacity for ligand immobilization. The effect of crosslinking on the ligand immobilization capacity was determined by employing trypsin and BSA as test ligands, and the results are listed in Table 2. The comparisons were made only for the glutaraldehyde pathway, because only in this case is the non-crosslinked membrane sufficiently stable (Fig. 6). For this pathway, the ligand immobilization capacity of the crosslinked glass membrane was only a little lower than that of the non-crosslinked glass membrane. However, the crosslinked membranes should be preferred when an immobilization pathway other than the glutaraldehyde pathway is employed. Indeed, as shown in Fig. 6, the immobilized enzyme on the crosslinked membrane was both more active and more stable than that on the non-crosslinked membrane, when the diazotization pathway was employed. This probably happened because some fibers of the non-crosslinked membrane were easily washed away.

As an application of the prepared crosslinked glass membranes, the separation of fibronectin from human blood was carried out using two kinds of affinity membranes, involving gelatin and heparin as affinity ligands, respectively.

Fibronectin is a large glycoprotein, consisting of two almost identical subunits of ~250 000 each. Fibronectin is believed to play an important role in various physiological activities such as opsonization, phagocytosis, angiogenesis, embryogenesis, tissue organization, and remodeling, as well as in pathological processes such as polyarthritis and oncogenesis [23,24]. Soluble forms of fibronectin are present at high levels in blood plasma and other biological fluids, such as seminal plasma, urine, milk, amniotic fluid and saliva [25,26]. Recently, fibronectin has received great attention in cytobiology, therapy and pharmaceutics [27,28].

Many methods for the separation and purification of fibronectin from various sources have been proposed, because of its affinity to a wide variety of

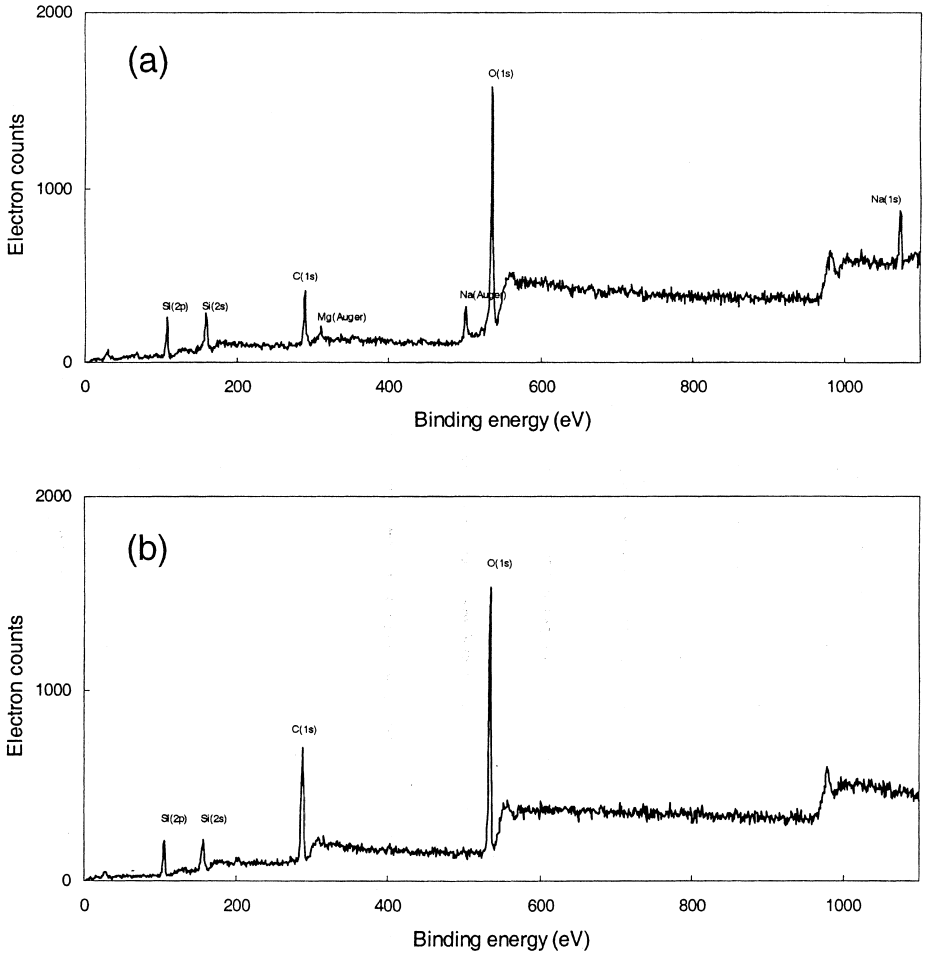

Fig. 5. X-ray photoelectron spectroscopy spectra of the glass membranes: (a) glass membranes after being treated with a ''piranha'' solution; (b) glass membranes crosslinked with 1,2-bis(triethoxysilyl)-ethane. A 10-ml amount of crosslinker was used for a cartridge containing 20 membranes. See Experimental section for details.

ligands, such as collagen, gelatin, heparin, arginine, metal complexes, etc. [29–31]. In the literature, the separation of fibronectin was carried out using either a single step chromatographic procedure [32,33], or by combining two or more successive chromatographic procedures, each one involving a different affinity ligand. The latter method was reported to be more efficient, because it eliminated the 72 000 plasma gelatinase and the fibronectin fragments impurities from the final product [23,34]. Following the suggestion of Poulouin et al. [23], who employed column chromatography, a two-step affinity membrane procedure was used in the present work. The separation of fibronectin was carried out on two kinds of affinity membranes involving gelatin and heparin as affinity ligands, respectively. Gelatin was immobilized via the diazotization method, and heparin via the glutaraldehyde pathway.

The affinity between fibronectin and gelatin, which involves a series of complex interactions [35], has long been investigated and used for the separation and purification of fibronectin. This interaction is so strong that usually the fibronectin containing fractions can be eluted only with a concentrated urea eluant, or a chaotropic reagent such as KSCN. In this paper, the fraction of interest was eluted with 3 *M*

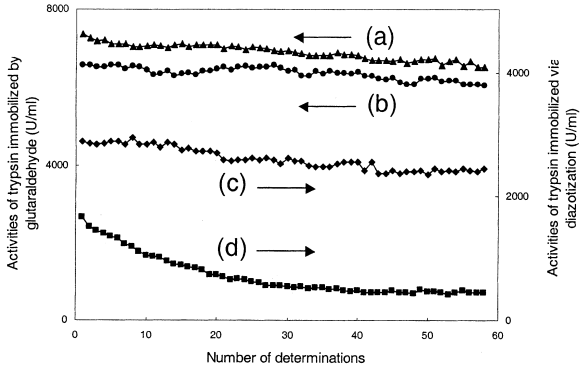

Fig. 6. Dynamic activity of trypsin immobilized on non-crosslinked and crosslinked glass membranes: (a) and (d) refer to non-crosslinked membranes; (b) and (c) to crosslinked membranes. A suitable amount of chopped membrane containing enzyme was assayed in a Buchner funnel. After determination, the membrane pieces were washed twice under suction before the next determination. See Experimental section for details.

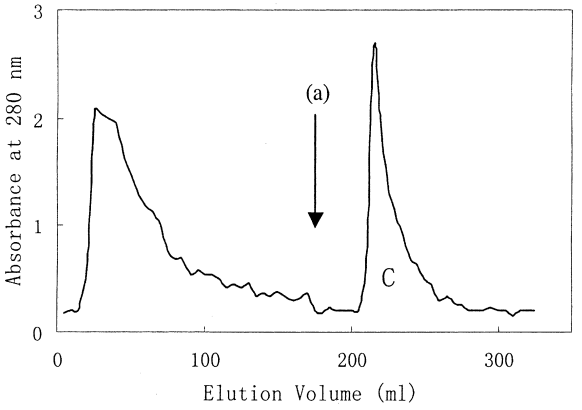

Fig. 7. Affinity chromatography of human plasma on a cartridge containing 20 gelatin immobilized affinity glass membranes. Arrow (a) indicates the beginning of elution with a 50 m*M* Tris–HCl, pH 7.40 buffer containing 250 m*M* NaCl, 1 m*M* EDTA, and 3 *M* urea. Fraction C was collected and used further for separation on a heparin immobilized affinity membrane cartridge (20 membranes) and analyzed by electrophoresis. See Experimental section for details.

urea in a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 250 m*M* NaCl, and 1 m*M* EDTA (Fig. 7). After the removal of urea by dialysis, the product was introduced into a cartridge containing the heparin immobilized affinity membranes.

Owing to its polyanionic character, heparin interacts mainly with cationic molecules. Compared to gelatin, the interactions between heparin and fibronectin are both more simple and weaker. The chromatographic profile is presented in Fig. 8, in which the fractions of interest were eluted successively with a buffer containing increasing concentrations of NaCl.

To monitor the chromatographic procedures and to evaluate the separation, SDS–PAGE electrophoresis was employed (Fig. 9), using commercial human serum albumin and commercial plasma fibronectin (Sigma) as standards. The procedure was carried out under reducing conditions, and the SDS denaturation of the proteins was carried out at the lower temperature of 60 °C, because of the temperature sensitivity of fibronectin [18]. Fig. 9 clearly shows that the major impurity in fibronectin separation is human serum albumin. Fig. 9C indicates that after separation by gelatin affinity membranes, the fibronectin containing fraction was enhanced, compared to that present in Fig. 9B. However, the amounts of impurities remained high, because only one step elution was employed and all the adsorbed proteins were eluted at the same time.

Regarding the second separation on a cartridge containing 20 heparin immobilized affinity membranes, Fig. 9D shows that most of the impurities were washed out with 150 m*M* NaCl in a Tris–HCl, pH 7.40, buffer, and that a small amount of fibronectin was lost at the same time. Two fractions

Table 2
Effect of crosslinking on ligand immobilization onto glass membranes prepared via the glutaraldehyde pathway

Membrane	Activity of immobilized trypsin (U/ml)	Content of immobilized BSA (mg/ml)
Non-crosslinked	7370	8.6
Crosslinked	6590	7.5

A total of 20 membranes packed in a cartridge were crosslinked by reacting them with a solution containing 10 ml 1,2-bis(triethoxysilyl)-ethane. See Experimental section for details.

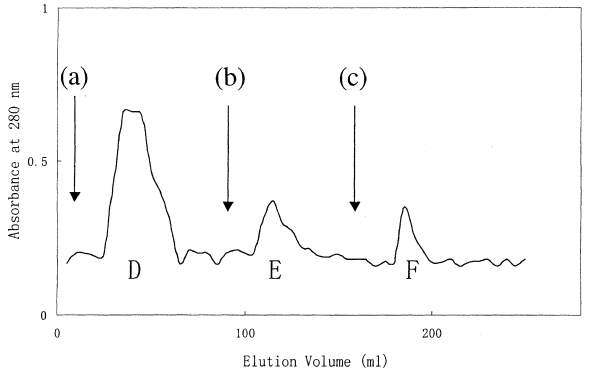

Fig. 8. Separation of plasma fibronectin on a cartridge containing 20 heparin immobilized affinity glass membranes. The arrows indicate elution with (a) 150 m*M* NaCl in a 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.40 buffer, (b) 250 m*M* NaCl in a 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.40 buffer and (c) 500 m*M* NaCl in a 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.40 buffer, respectively. Fractions D–F were collected and analyzed by electrophoresis. See Experimental section for details.

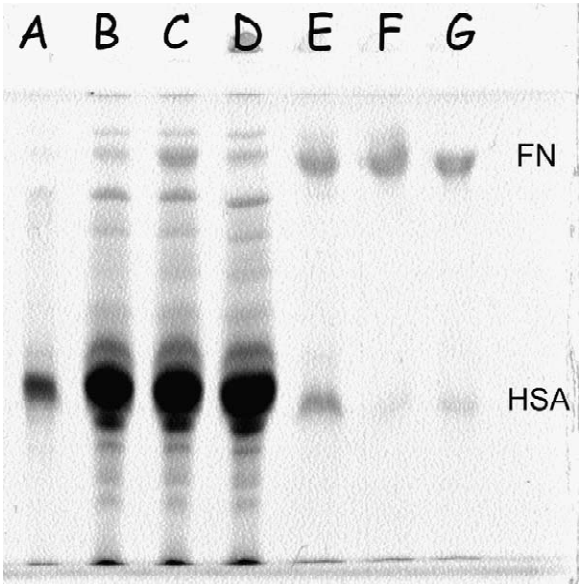

Fig. 9. SDS–PAGE electrophoresis of plasma fibronectin (FN) SDS PhastGel Homogeneous 7.5 gel (7.5% gel content), 60 Vh: (A) commercial human serum albumin; (B) commercial human plasma; (C) fraction obtained using 3 *M* urea in a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 250 m*M* NaCl as eluent from a gelatin immobilized affinity membrane cartridge (Fig. 7); (D) fraction obtained using a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 150 m*M* NaCl and 1 m*M* EDTA as eluent (Fig. 8); (E) fraction obtained using a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 250 m*M* NaCl and 1 m*M* EDTA as eluent (Fig. 8); (F) fraction obtained using a 50 m*M* Tris–HCl, pH 7.40 buffer, containing 500 m*M* NaCl and 1 m*M* EDTA as eluent (Fig. 8); (G) commercial plasma fibronectin (Sigma).

containing purified fibronectin, shown in Fig. 9E,F, were eluted with 250 and 500 m*M* NaCl in the same buffer, respectively. However, the purities of the obtained fibronectin are not the same, because an undesired protein band for serum albumin is present in Fig. 9E. The electrophoresis results were scanned and analyzed using Metamorph software to determine the densities of the fibronectin bands in the products. Using the commercial fibronectin as standard and reference, the yield of this method, on the basis of the fractions E and F, could be calculated to be 0.185 mg fibronectin per ml plasma.

Under the reduced conditions employed, only the major forms (~250 000) of fibronectin were detected by the present electrophoresis procedure [36]. Because of the complex behavior of the fibronectin molecules, their degradability and aggregation, small amounts of minor forms of fibronectin should be also present in both the source sample and purified product [37].

4. Conclusion

Affinity membranes were prepared from glass fiber filters by first treating them with a piranha solution, followed by crosslinking. Of three bifunctional silanes used as crosslinkers, 1,2-bis(triethoxysilyl)-ethane was found to provide the most compact structure. The crosslinked membranes were further modified with either γ-aminopropyltriethoxysilane or γ-glycidoxypropyltrimethoxysilane to introduce amino and epoxyl groups, respectively. Affinity ligands, gelatin and heparin, were immobilized onto the membranes using a diazotization or a glutaraldehyde pathway, respectively. By monitoring the activity of the immobilized enzyme and the content of immobilized bovine serum albumin, the effect of crosslinking on membrane stability was investigated. Separation of fibronectin from blood plasma was carried out by first using a cartridge containing immobilized gelatin affinity membranes, followed by a cartridge containing heparin immobilized affinity

membranes. About 0.18 mg fibronectin was obtained from 1 ml plasma.

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