Polymer Journal, Vol. 28, No. 3, pp 284—287 (1996)

NOTES

Interactions between Chondroitin Sulfate Immobilized by Plasma-Polymer and Biochemical Substances

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(Received July 10, 1995)

KEY WORDS Plasma-Polymerized Propargyl Alcohol / Plasma-Polymerized Film / Immobilized Chondroitin Sulfate / Thin-Layer Chromatography of Biochemical Substances / Filtration of Serum Proteins /

Plasma polymerization of propargyl alcohol (2-propyn-1-ol, PA) produces thin film highly hydrophilic on a solid substrate.[1,2] The so-called plasma-polymerized PA (PPPA) was first applied to thin-layer chromatography (TLC) to examine interactions between PPPA and organic compounds.[3,4] The second application dealt with immobilization of enzymes within porous polymer membranes by enclosure with PPPA to prepare sensing elements for electrodes.[5,6] This paper reports further study on the properties of PPPA film deposited on a TLC plate in which chondroitin sulfate (CS) was previously impregnated. Chromatographic behavior of some biochemical substances on the TLC plate was observed to interpret interactions between PPPA or CS and substances tested.

In the field of analytical biochemistry or biofunctional materials, surface electric charge of solid substrates is often pointed out in regard to interactions between the substrates and the biochemical substances.[7–10] At the same time, hydrophilicity is also involved in the discussion to evaluate biocompatibility, especially with regard to antithrombotic activity.[8–13] The PPPA film was therefore thought to be a promising substrate material providing marked hydrophilicity, negative charge, and absorption of water, because of abundant oxygen atoms in the polymer network of PPPA.[14]

CS illustrated in Figure 1 exhibits similar properties to PPPA, while it holds other biochemical functions such as ion-exchange ability, antithrombotic activity, and curing effect against certain damage of connective tissues.[15,16] Immobilization of CS with the PPPA film was therefore supposed to promote these composite functions.

EXPERIMENTAL

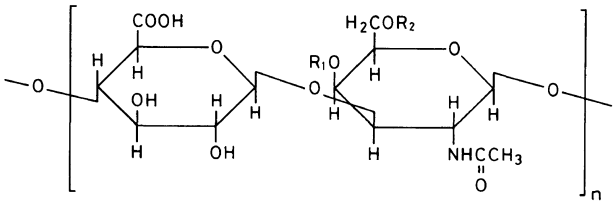
Plasma Coating

PPPA-coating was carried out with a Plasma Deposition System, Model BP-1 (Samco International Lab. Inc., Kyoto) incorporated with a High Vacuum Pump E2M-12 (300 l min^{-1}, Edwards Inc., United Kingdom) and a Thermocouple Vacuum Gauge (Thermocouple 1 and Gauge Head TC1, Edwards Inc.). The procedure for coating was basically the same as that described in the previous papers.[1,5] Radio frequency power (13.56 MHz) applied for sustaining plasma polymerization was 5 W. The pressure before discharge was held at approximately 0.1 Torr under the slow introduction of PA (EP grade, Nacalai Tesque Inc., Kyoto) vapor into the discharge chamber (mass flow rate of PA: 24.0 mg min^{-1}).[1] Under given plasma conditions, the deposition rate of the PPPA film was 450 Å min^{-1}.[1]

Thin-Layer Chromatography

A precoated TLC plate (thickness of silica gel layer, 250 μm, back plate, aluminum; Whatman Inc., United Kingdom) cut into piece of 5×10 cm^2 was dipped into 10% aqueous sodium CS (GR grade, Kanto Chemicals Inc., Tokyo) for impregnation of CS into micropores of the silica gel particles. After air-drying, the plate was processed by plasma-coating for 30 min (film thickness of PPPA: 13500 Å) to immobilize CS, and was finally washed with acetone–water (4 : 1) which was also used as a developing solvent.

Sample solutions were prepared by dissolving biochemical substances (D-[+]-glucose, urea, riboflavin, cyanocobalamin, L-ascorbic acid: GR grade; albumin: crude product from egg; Nacalai Tesque Inc.) in distilled water. The samples were developed on the immobilized CS plate until the solvent front was raised to 8 cm. An untreated TLC plate was used as reference at the same time. Sample spots were detected by exposure to saturated iodine vapor or by spraying 10% sulfuric acid before heating. R_f of each spot was measured and the ratio of R_f for the two plates (sample/reference) was calculated.



$R_1 = SO_3H$, $R_2 = H$
chondroitin sulfate A (chondroitin 4-sulfate).
$R_1 = SO_3H$, $R_2 = H$, C-5′ epimer
chondroitin sulfate B (β-heparin).
$R_1 = H$, $R_2 = SO_3H$
chondroitin sulfate C (chondroitin 6-sulfate).

Figure 1. Chemical structure of chondroitin sulfate (CS).

Permeation through Immobilized CS Filter Paper

A filter paper (No. 2 for quantitative analysis, Toyo Roshi Co., Ltd., Tokyo) impregnated with 10% CS solution was air-dried on a glass plate. The filter impregnated with CS and an untreated filter were coated with PPPA on both sides for 15 min (film thickness of PPPA: 6750 Å). Solutions of bovine serum proteins (albumin: F-V, γ-globulin: 99%; Nacalai Tesque Inc.) were prepared by dissolving the proteins in isotonic sodium chloride solution to make a concentration of 500 mg l^{-1}. Permeation rates of the proteins through those filter papers were comparatively studied.

Filter paper was cut out into circle shape so as to just fit the plate of sintered glass frit at the bottom of the glass funnel (i.d.: *ca.* 30 mm). One piece of filter paper was placed on the sintered glass plate and washed with NaCl solution. Weak tapping was done by a glass stick to fix the filter paper closely on the plate. Multilayers of the filter paper were made by five repeats of the same process and washed with NaCl solution of more than 200 ml.

Exactly 5 ml of the serum protein solution was poured into the glass funnel placed at the upper end of a titration buret and NaCl solution was continuously added in the manner of column chromatography. Every fraction of the filtrate collected in the titration buret was transferred to a photocell and subjected to UV spectrometry at 280 nm (Shimadzu UV-spectrophotometer UV-210A, Shimadzu Inc., Kyoto). The amounts of proteins eluted were calculated from individual calibration curves previously established by known concentrations of the proteins.

The behavior of competitive transportation of the two proteins was studied by use of weight-equivalent mixture solution of them. Albumin in each fraction was detected by the BCG method (formation of bromocresolgreen-albumin complex),[17] a conventional method in clinical analysis.

RESULTS AND DISCUSSION

Interactions of CS with Serum Components

Interactions between CS immobilized by PPPA and six organic components selected from the common biological substances were surveyed by comparing R_f from the TLC tests. Activity of CS was assumed to remain unchanged after the plasma process as in the case of immobilization of enzymes[5,6] under mild plasma conditions, although minute numbers of CS molecules distributed near the surface of the substrate might suffer certain modification by irradiation with the PA plasma. However, it should be also mentioned that CS was more durable than the delicate enzymes during the plasma process. Figure 2 shows the chromatograms on an uncoated plate and a CS-impregnated and PPPA-coated plate. R_f is listed in Table I with the ratios of R_f calculated as coated/uncoated.

Detection of urea was unsuccessful on the PPPA-coated plate by iodine or sulfuric acid. The PPPA film seemed to interrupt direct reaction between urea and the reagents. Riboflavin showed strong tailing on both plates, while albumin did not migrate from the initial position. Other components such as glucose, cyanocobalamin and L-ascorbic acid showed elliptical spots on the PPPA-coated plate in the direction of solvent flow. It was supposed that the developing solvent moved on the PPPA-coated plate somewhat faster than the proper rate for development. R_f of the three components became smaller on the PPPA-coated plate. It was thus thought that the three components were adsorbed more strongly on the CS-immobilized plate than on the untreated plate.

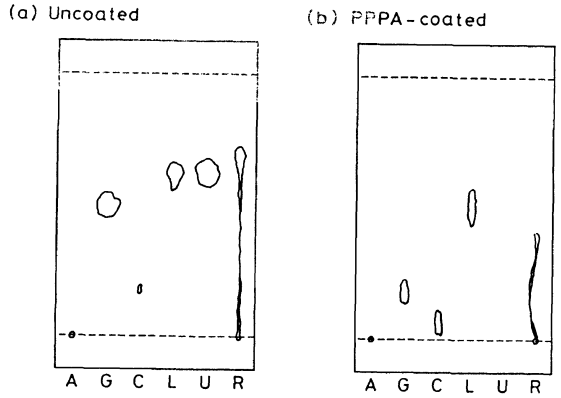


Figure 2. Chromatograms of biochemical substance on PPPA-coated silica gel plate impregnated with CS: A, albumin; G, glucose; C, cyanocobalamin; L, L-ascorbic acid; U, urea; R, riboflavin; developing solvent, acetone/water = 4 : 1.

Table I. R_f of biochemical substances on PPPA-coated silica gel plate impregnated with CS[a]

Compound	R_f-value (SD)		Ratio of R_f-value; coated/uncoated
	Uncoated silica gel	PPPA-coated silica gel	
Glucose	0.50 (0.07)	0.16 (0.05)	0.3
Urea	0.62 (0.01)	—	—
Riboflavin	0—0.74 (0.05)	0≦	—
Cyanocobalamin	0.18 (0.03)	0.09 (0.02)	0.5
L-Ascorbic acid	0.62 (0.02)	0.47 (0.13)	0.8
Albumin	0.00 (0.00)	0.00 (0.00)	—

[a] Developing solvent: acetone/water = 4 : 1. Number of measurements, 7.

The adsorption was considered mainly due to van der Waals' force. Except for albumin, cyanocobalamin has the largest molecular size (ø = 20 Å, MW = 1355)[18,19] in the test components and there is the possibility that it could not pass through the network of PPPA. The others have molecular weights less than 1/3 that of cyanocobalamin, so that these molecular sizes were presumed about 10 Å or less and could pass through the polymer network. This was supported by the previous study for determination of micropore sizes within plasma-polymers derived from pyridine (ø = 10 Å)[19] and tetrafluoroethylene (ø = 20—40 Å).[18] Sensitive response of the glucose or urea electrode[5,6] incorporated with the immobilized enzyme membrane suggested free paths for small molecules through the PPPA film. Therefore, components having relatively small molecular sizes could interact not only with the PPPA film but also with the silica gel surface and CS molecules impregnated within the silica gel particles. Another factor of interactions between the test components and the plasma-processed TLC plate should be that water molecules

have quasi-organized structure within the polymer matrix and surface structure.[8] Since the PPPA film was highly hydrophilic, the polymer film swelled with the developing solvent of acetone–water.[14,20] Even though cyanocobalamin could not directly interact with the CS molecules, the water molecules seemed to draw the component into the polymer network. Interaction with the water molecules coordinated with PPPA and CS would also contribute to partition of the other components toward the stationary phase.

The ratios of R_f for the two TLC plates decreased significantly with glucose, moderately with cyanocobalamin, and slightly with L-ascorbic acid. The affinity of glucose to CS was derived from the close resemblance as sugar compounds and cyanocobalamin was associated with CS by cation-capture abilities. That is, charge-transfer force of the cyanocobalamin contributes to affinity to CS, because cyanocobalamin is a complex involving cobalt as a central cation and CS also involves carboxyl and sulfonic acid groups. Molecular weight and elemental composition of glucose ($C_6H_{12}O_6=180$) are similar to those of L-ascorbic acid ($C_6H_8O_6=176$); however, glucose shows closer resemblance to CS than L-ascorbic acid with respect to molecular structure. Therefore, glucose shows stronger affinity to the CS-immobilized plate than L-ascorbic acid. Such affinity developed by the immobilized CS seems applicable to affinity chromatography of sugars and ion-exchange chromatography of various cations.

Interactions between the test components and PPPA film did not contribute to increase in the affinity on the basis of previous experiments.[3,4] Organic compounds such as azo dyes, amino acids, and cholesterols generally exhibited reduction of adsorption forces on the PPPA-coated plate regardless of the polar or nonpolar developing solvent. This reduction was more significant for compounds having stronger dipole moment.

Interactions with Serum Proteins

Permeable rates of albumin and γ-globulin through multilayers of the CS-immobilized filter paper were examined to determine the interactions between PPPA or CS and serum proteins. Figure 3 shows elution curves of 2.5 mg albumin or globulin through the filter paper prepared in different ways, *i.e.*, (A) uncoated, (B) coated with PPPA on both sides, (C) coated with PPPA on both sides after impregnation of CS.

In the case of (A), both proteins smoothly permeated using 15 ml of the eluate and were hardly detected in further fraction of the eluate. Recovery of proteins in the total eluates was approximately 80%, by which partial adsorption of the proteins on the filter paper was implied. With respect to (B), slow permeation of albumin consuming 20 ml of the eluate was observed while globulin permeated in the same manner as that in (A). Recovery of the proteins exceeded 100%. However, it was assumed that small molecules of PPPA came out of the polymer matrix and increased light absorbance at 280 nm. In the experiment of (C), the filter paper efficiently absorbed water and swelled when washed with NaCl solution. An ordinary elution curve was obtained with globulin, but albumin showed a somewhat complex curve as if temporarily retained within the multilayer.

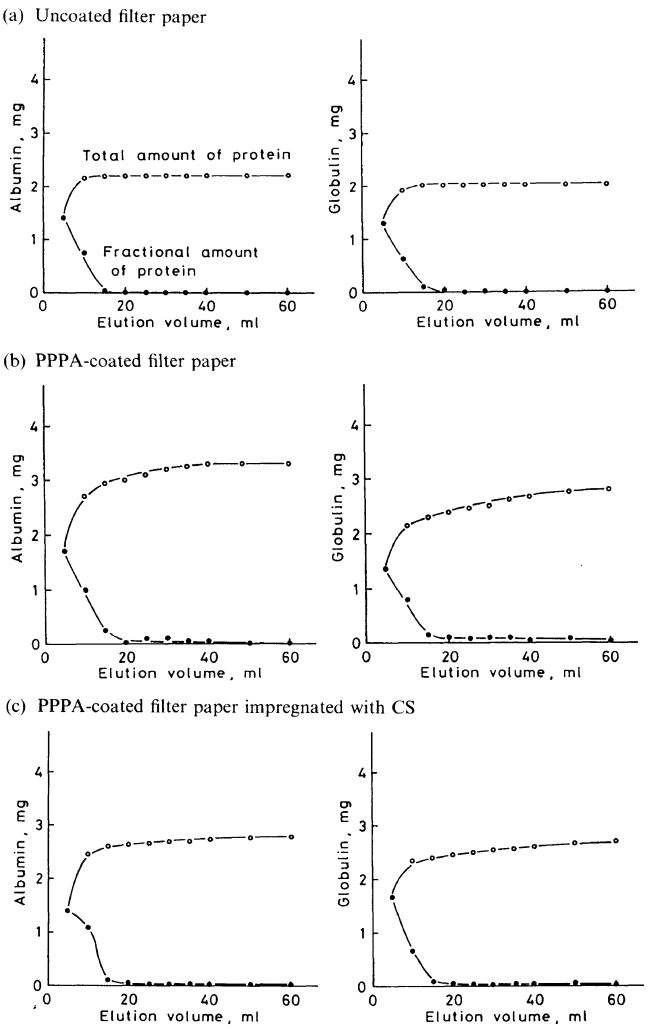


Figure 3. Elution curves of serum proteins through multilayer of filter paper; filter papers, Toyo Roshi No. 2, 5 multilayer sheets.

Recovery of the proteins was nearly 100%.

A similar test was run with a sample solution containing equivalent amounts of albumin and γ-globulin. Figure 4 shows elution curves through the three types of filter paper by plotting absorbance at 280 nm against elution volume. The experiment showed again that PPPA-coating somewhat retarded the permeation and albumin was detected by the BCG method until an elution volume of 30 ml. The CS-impregnated and PPPA-coated filter paper showed significant retention of the proteins and albumin was still detected in the fraction at 50 ml. Since globulin passed much faster than albumin as shown in Figure 3, competitive transportation of the two proteins seemed to enhance the difference of permeation rates. Details of the behavior of the proteins should be further studied by changing the amounts of CS and proteins while using different solute composition or pH of the eluent. Considering the results of these experiments, it may be concluded that stronger intermolecular force appeared in the combination of albumin and CS rather than in the case of globulin.

Although both proteins would primarily not permeate through the PPPA network because of large molecular size, the plasma-coating of the bulky filter paper having complicated fine structure likely left certain areas uncoated. Therefore, interactions of the proteins should

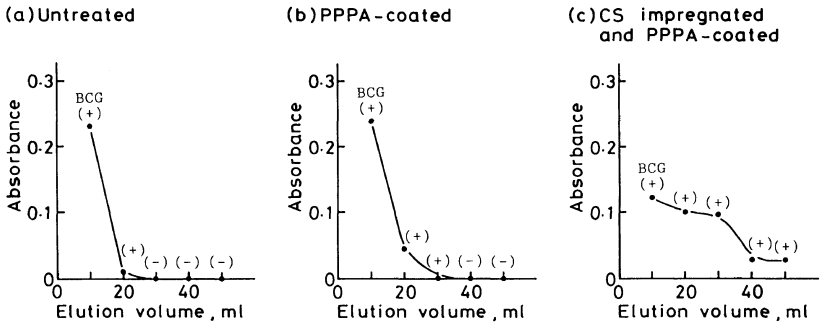


Figure 4. Elution curves of serum protein through multilayers of PPPA-coated filter paper impregnated with CS: serum protein, albumin/globulin = 1 : 1; filter paper, Toyo Roshi No. 2, 5 multilayer sheets.

be considered mainly to PPPA film but also to CS and cellulose. However, many water molecules associated with the latter three materials also played an important role. Interfacial free energy between the proteins and three filter papers would have been reduced by the water molecules resulting in less denaturation of the proteins.[8]

Reduction of the permeable rates by PPPA-coating seemed due to enhanced partition of the proteins into the water-associated PPPA film. This is supported by somewhat slower permeation rate of albumin than globulin, *i.e.*, the former is more hydrated and water soluble than the latter. Comparing the permeation rates for the simply PPPA-coated filter paper with the CS-immobilized filter paper, the latter showed much slower permeation rate. The PPPA film and the immobilized CS showed similar interactions with the proteins, except that CS showed rather characteristic behavior to individual proteins. Further research will be needed to explore the mechanism of interactions, although some analytical data such as contact angle, critical surface tension, and X-ray photoelectron spectra have been reported previously.[1,2,4,14] All information presented here will be helpful to develop other new separation media such as ionically-charged filter membranes or biocompatible materials for medical use.

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