Effective Immobilization of Protein Linked with Polyethylene Glycol on Silica via Hydrogels Using Silica Sol

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Synopsis

An effective attachment of activated polyethylene glycol (PEG), which has dichloro-*s*-triazinyl functional groups, to silica and protein immobilization with the resulting PEG/SiO_2 composite were studied. The reaction of methanol–silica sol with the PEG–silyl reagent resulted in an efficient PEG attachment to SiO_2 as compared with the usual two-step PEG modification of silica gel and glass surface. The activated PEG/SiO_2 was able to immobilize over 600 mg of bovine serum albumin per unit gram of the PEG composite through hydrogel formation. The application of the present binding method of PEG-linked protein on SiO_2 was also discussed.

INTRODUCTION

Incorporation of polyethylene glycol (PEG) into protein has recently become of much interest because it gives possible control of immunogenicity, stability, solubility, and phase partioning.[1-12] In particular, the modification of enzyme with PEG can improve solubility in organic solvents and, therefore, promises to give a new type of enzymatic organic synthesis.[3,6,7] In this respect, an effective and facile immobilization of PEG enzyme on an appropriate support should be necessary and essential for practical applications.

Protein or enzyme immobilization has been widely studied,[13,14] while there are some interesting studies that peptides, hepalin, an organometallic catalyst, and an affinity ligand linked with PEG spacer exhibit their own native properties free of the matrix effects frequently found in immobilization.[15-19] Also, we observed that immobilization of alkaline phosphatase with PEG spacer on glass led to only 5–10% loss of activity as compared with the free enzyme.[4] However, in most cases attachment of PEG spacer on the support matrix is accomplished to a relatively low extent, and the subsequent immobilization is inefficient. In fact, in our work PEG attached on the glass surface (prepared by route I in Scheme 1) and the enzyme coupled were less than 10 μmol/g and 0.5 mg/g, respectively.[4]

Hence, in order to improve the procedures for the PEG spacer attaching and for immobilizing protein, we have developed a convenient one-step binding (route II in Scheme 1) of the cyanuric chloride-activated PEG to silica via the

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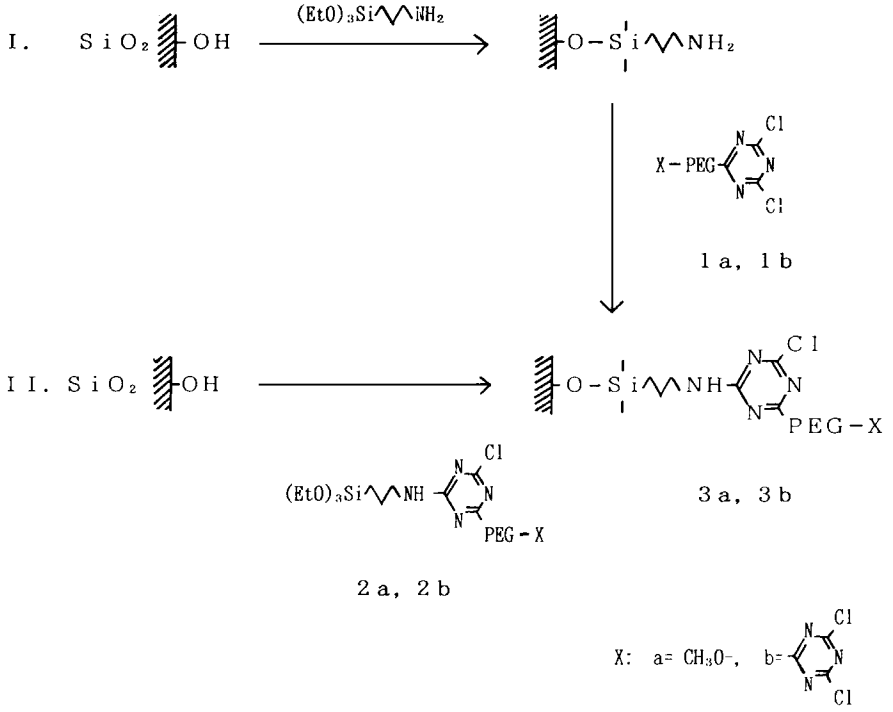
Journal of Applied Polymer Science, Vol. 41, 1443–1450 (1990)
 CCC 0021-8995/90/7-81443-08$04.00



Scheme 1.

sol–gel process,[20–23] which has lately received much attention as an organic-inorganic composite preparation, by use of silica sol suspended in methanol. In the present work, we wish to describe an effective method for binding the cyanuric chloride-activated PEG to SiO_2 and for immobilizing bovine serum albumin (BSA) (as a model protein) on the PEG/SiO_2 via hydrogel formation. To our best knowledge, protein coupling via PEG spacer on SiO_2 using silica sol is the first example of such immobilization.

RESULTS AND DISCUSSION

PEG Attachment to SiO_2

Since it was observed that gelation takes place in a few minutes after mixing the silica sol with an alkyl silylation reagent such as 3-(triethoxysilyl)propylamine in aqueous and organic solvents, we examined the reaction of the silica sol with MeO–PEG 5000 (monomethoxy PEG of molecular weight of 5000) silyl reagent (2a) (Table I). On mixing the sol with 2a, the gelling occurred in most miscible solvents, e.g., ethanol, chloroform, tetrahydrofuran, benzene.

After gelling, followed by evaporation of solvent and removal of unreacted 2a by centrifugation, the dry gel (3a) was obtained. Formation of chemical bonds between MeO–PEG 5000 and SiO_2 in 3a was confirmed by two characteristic absorption bands at 1458–1440 and 1630–1570 cm^{-1}. These bands are

TABLE I
Attachment of MeO–PEG 5000 to Silica

Method	Form of SiO_2	$NH_2(CH_2)_3$–SiO_2 (g)	1a (g)	MeO–PEG 5000 (μmol/g)
I	Sol	0.8	5.0	4.2
		1.6	5.0	4.0
I	Gel	1.0	4.0	15
		2.0	4.0	16
I	Glass	3.0	2.0	10
		3.0	2.0[a]	5.3[b]
		SiO_2 (g)	2a (g)	
II	Sol	0.9	0.9	42
		0.9	4.5	60
		0.9	6.3	47
		0.9	9.0	38
		0.9	10.8	23
II	Gel	1.0	1.0	16

[a] 2b was used.
[b] The amount of PEG 8000 attached on the glass.

assignable to stretching vibration of the $Si-CH_2$ and $C=N$ (triazine ring) bonds, respectively, on the differential spectrum derived by the subtraction of the absorption signals of SiO_2 and MeO–PEG 5000 from the signals of 3a (Fig. 1). The amount of MeO–PEG 5000 in 3a was dependent on the weight ratio of 1a to SiO_2 in the sol; the maximum attachment of 60 μmol/g was attained at the ratio of 5. Over a five-fold range, the amount of MeO–PEG 5000 attached on SiO_2 decreased with increasing concentration of 2a. The formation of gel from the silica sol is considered to proceed through the following processes: (i) aggregation of silica particles per se induced destruction of the electronic double layer due to the extensive addition of 2a[22] and (ii) crosslinking by 2a among SiO_2 particles. Presumably, in the presence of excess 2a, both processes (i) and (ii) take place, while simultaneous formation of siloxane oligomers, soluble in methanol, from 2a makes the crosslinking among silica particles decrease. However, these composites have small surface area, less than 10 m^2/g, determined by N_2 adsorption at −196°C. This result indicates that the microporous structure did not grow during the gelation, as reported in the porous SiO_2 preparation from $Si(C_2H_5O)_4$ with the sol–gel processing.[23] Thus, the PEG/SiO_2 composite, 3a, is likely to be composed of SiO_2 particles surrounded by the polyoxyethylene chain.

If the gelling were prevented in the reaction system of 2a and the silica sol, it would be expected that 2a efficiently reacted with silanol groups on the sol particles and consequently more effective attachment of MeO–PEG 5000 was attained. Thus, we also tried the reaction of 2a with the silica sol in 1-octanol in the presence (5%) of a surfactant (*N,N,N*-trimethyl-*N*-hexadecylammonium bromide). Indeed, the gelling did not occur during the reaction, but this procedure gave a very small amount (less than 3 μmol/g) of MeO–PEG 5000

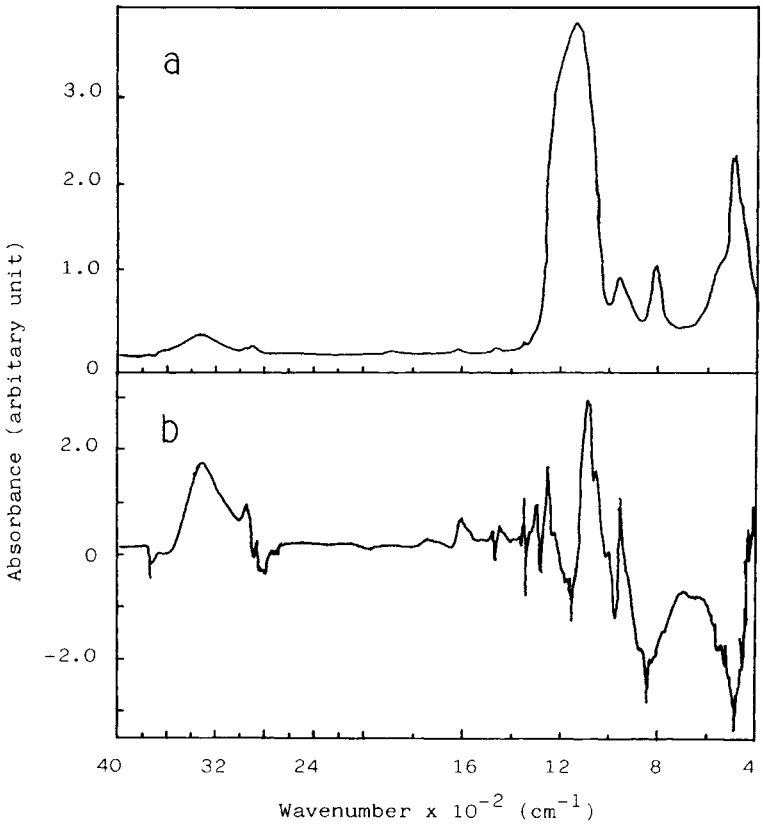


Fig. 1. A FT-IR spectrum (a) of 3a, and the differential spectrum (b) between 3a, MeO-PEG 5000 and SiO_2, obtained by subtraction processing so as to minimize the absorption intensity difference in the range of 3000–2600 cm^{-1}.

attachment. Presumably, 2a is incorporated into the micelle formed at the interface between the silica particles and 1-octanol to a very limited extent.

Meanwhile, attachment of MeO–PEG 5000 to SiO_2, using 1a by the two-step reaction (route I), was also conducted for comparison with the one-step reaction (route II). The amounts of the PEG bound on the surface were 15–16 and 10 μmol/g for silica gel and the glass (specific surface area, 357 and 45 m^2/g), respectively (Table I). The PEG attachment increased with increasing surface area but was not simply proportional to the area. In this case, the use of silica sol led to lower attachment of MeO–PEG 5000 than the silica gel and the glass. Gelling also took place on mixing the sol with 3-(triethoxysilyl)propylamine, and it probably caused propylamino groups to be buried in the crosslinked siloxane network resulting in lower PEG attachment.

At any rate, the one-step reaction of methanol–silica sol with 2a brought about an effective attachment of PEG to SiO_2 to a level four times more than route I and the usual functionalization of SiO_2 surface.

Based on these results, we next examined the preparation of the activated PEG 8000 (molecular weight of 8000)/SiO_2 (3b), which has a dichloro-*s*-triazinyl group at the end of the PEG chain, using the one-step reaction of the

silica sol and the cyanuric chloride-activated PEG 8000–silyl reagent (2b). As shown in Table II, a fivefold use of 2b to SiO_2 in the sol afforded the maximum PEG 8000 as well as in the case of MeO–PEG 5000. However, the PEG 8000 bound to silica was a smaller amount of 20 μmol/g than MeO–PEG 5000, probably due to the greater bulk of the PEG as compared with MeO–PEG 5000.

Immobilization of BSA on the Activated PEG 8000/SiO_2

The PEG/SiO_2 derived from the one-step modification of silica gel using 2b provided BSA binding of 5.4 mg/g, but unexpectedly, one derived from the silica sol by route II resulted in low immobilization of BSA, less than 0.1 mg/g (Table II). From the fact that the reaction of BSA with 3b at pH 8.5 gave almost the same amount of its immobilization as at pH 9.4, it is unlikely that the nucleophilic attack of hydroxyl ions to dichloro-*s*-triazinyl groups should predominate over the attack of amino groups of the protein. The low binding of BSA to 3b seems to be due to slow permeation of the large BSA molecule into the activated PEG/SiO_2 matrices.

However, surprisingly, mixing the buffer (0.05 *M* $Na_2B_4O_7$) solution containing more than 60 mg/mL or less than 400 mg/mL of BSA with over 50 mg of 3b brought about the formation of hydrogel, which exhibited considerable mechanical hardness. This implies that the activated PEG 8000/SiO_2 (3b) can immobilize over 600 mg of BSA per gram of the PEG/SiO_2 composite and is appropriate for binding protein in large quantity. The dried gel was well swollen in water and in organic solvents. The swelling of BSA–PEG 8000/SiO_2 in various solvents was in the following order: H_2O (swelling ratio = 4.77) > $CHCl_3$ (3.28) > $(CH_3)_2NCHO$ (2.55) > C_6H_6 (1.11) > CH_3CN (1.06). These results indicate that this procedure is applicable to the immobilization of enzymes for the purpose of organic synthesis in various organic media. In this regards, the entrapped enzymes in organic polymer gels, prepared by the copolymerization of acrylates or vinyl derivatives in aqueous solution containing the enzymes, are often used.[24] Our present procedure, in contrast, conveniently gave immobilized and/or entrapped protein in the PEG/SiO_2 gels, by only mixing the buffer solutions, without polymerization. Furthermore, the addition of acetone solution of 3b to the aqueous layer of the BSA-containing buffer solution also formed a thin layer of the gel, and successive drying led to successful film processing.

The characterization of the hydrogel as well as the dried gel and investigation of the immobilization effects on the properties of protein are now in progress.

TABLE II
Attachment of the Activated PEG 8000 to Silica by Route II and Immobilization of BSA

Form of SiO_2	2b/SiO_2 (g/g)	PEG 8000 (μmol/g)	Immob. BSA (mg/g)
Sol	3	21	< 0.10
	5	40	0.10 (600)[a]
	7	17	< 0.10
Gel	1	8.2	0.54

[a] Through the hydrogel formation of BSA-PEG 8000/SiO_2 (see the text).

EXPERIMENTAL

General

PEG 8000 and MeO–PEG 5000 were purchased from Aldrich Chem. Methanol–silica sol containing 30% of SiO_2 was offered by Shokubai Kasei Co. Japan, and controlled pore glass (mean pore diameter of 547 Å, surface area of 45 m^2/g) and silica gel (Wakogel C-300; mean pore diameter of 25 Å, surface area of 357 m^2/g) were purchased from Sigma Chem. and Wako Chem., respectively. BSA (Fraction V) was available from Sigma Chem. The cyanuric chloride-activated MeO–PEG 5000 (1a) and PEG 8000 (1b) were synthesized by the method of Harris et al.[25] The ^{13}C-NMR spectra and the FT-IR spectra were recorded on JEOL FX-60 and on JEOL JIR-100 with the diffused reflection method, respectively. The determination of PEG bound on silica was carried out by the literature method.[26]

Synthesis of 2a

A mixture of 1a (2.67 g, 5.3 mmol) and 3-(triethoxysilyl)propylamine (0.14 g, 5.3 mmol) and 20 mL of acetonitrile was stirred with 0.1 mL of *N*-methylmorpholine in a nitrogen atmosphere at room temperature for 2 h. After evaporation of acetonitrile, the resulting solid was dissolved in 30 mL of dichloromethane and precipitated by addition of diethyl ether, and then twice by solution in benzene and addition to two volumes of diethyl ether.

Anal: Calcd for $C_{241}H_{481}O_{118}N_4ClSi$: C, 53.7%; H, 8.94%; N, 1.04%; Cl, 0.66%. Found: C, 52.2%; H, 8.71%; N, 1.38%; Cl, 0.63%. ^{13}C-NMR (ppm, $CDCl_3$); 7.5 ($Si-CH_2$); 14.0 (CH_3); 58.8 ($SiO-CH_2$); 70.4 (CH_2O); 171.2 (arom. C—Cl); 172.4 (arom. C—O); 181.4 (arom. C—N). IR (cm^{-1}); 1589, 1572 ($\nu_{C=N}$); 1440 (ν_{Si-C}); 1115 (ν_{Si-O}); 962 (ω_{SiO-CH_2}); recorded on the differential spectrum between 1a and MeO—PEG 5000.

Synthesis of 2b

This compound was made by a similar manner to 2a.

Anal. Calcd for $C_{379}H_{748}O_{186}N_7Cl_3Si$: C, 53.5%; H, 8.79%; N, 1.15%; Cl, 1.25%. Found: C, 53.5%; H, 8.91%; N, 1.38%; Cl, 1.19%. ^{13}C-NMR (ppm, $CDCl_3$); 7.5 ($SiCH_2$); 14.0 (CH_3); 58.8 ($SiOCH_2$); 70.4 (OCH_2); 171.2 (arom. C—Cl); 172.4 (arom. C—O); 181.4 (arom. C—N). IR (cm^{-1}); 1600–1570 (broad, $\nu_{C=N}$); 1458–1440 (broad, ν_{Si-CH}); 1115 (ν_{Si-O}); 962 (ω_{SiO-CH_2}); recorded on the differential spectrum between 2b and PEG 8000.

Preparation of 3a by Route I

Four grams of the silica gel or the glass were heated with 4 g of 3-(triethoxysilyl)propylamine in 10 mL of methanol at 60°C for 6 h and thoroughly washed with methanol and then dried at vacuum. The aminopropyl–silica or glass (1.0 g) was put into 40 mL of acetonitrile containing 4.0 g of 1a or 1b and 0.1 mL of *N*-methylmorpholine and heated at 60°C for 12 h. After filtration, the PEG–silica or glass was washed with 100 mL of acetonitrile, and dried at vacuum.

When the silica sol was used, mixing 10 mL of the sol with 3 mL of 3-(triethoxysilyl)propylamine formed the gel. After heating the gel at 60°C for 6 h, followed by adding 100 mL of methanol, the suspension was centrifuged five times at 2500 rpm for 5 min to remove unreacted 3-(triethoxysilyl)propylamine using a total of 300 mL of methanol, and then dried.

Preparation of 3a or 3b by Route II

A mixture of 1 g of the silica gel or 3 mL of the silica sol and the given amount of 2a or 2b was heated in 10 mL of methanol at 60°C for 6 h; the sol was gelled. The unreacted 2a or 2b was removed by the same manner as described previously.

Anal: Calcd: IR (cm^{-1}); 1630–1570 (broad, $\nu_{C=N}$); 1458–1440 (broad, ν_{Si-CH}); 1110–980 (broad, ν_{Si-O-C} and $\nu_{Si-O-Si}$); 962 (ω_{SiO-CH_2}); recorded on the differential spectrum between SiO_2, MeO-PEG 5000 and 3a.

Binding of BSA to 3b

Into 1.0 mL of the buffer solution (0.05 *M* $Na_2B_4O_7$, pH 9.4) was put 50 mg of BSA and 0.5 g of 3b at 0°C. The solution stood in a refrigerator overnight. Nonbound BSA was removed by decantation after centrifugation at 2500 rpm using a total of 200 mL of the buffer, and then lyophilized. Determination of BSA immobilized was carried out by the Biuret method after treating with 0.1 *N* NaOH.[4]

Swelling Ratio

This ratio was estimated by the ratio of the weight of the swelling gel, after immersing in a solvent in a refrigerator for 24 h, to the dry gel.

The authors are very grateful to Shokubai Kasei Co. Japan for offering silica sol and to Dr. K. Tsuji of Mistui Mine Co. Japan for the FT-IR spectrum measurements.

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Received April 10, 1989
Accepted September 26, 1989