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OPTIMIZATION OF PROTEIN IMMOBILIZATION ON 1,1'-CARBONYLDIIMIDAZOLE-ACTIVATED DIOL-BONDED SILICA

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SUMMARY

The activation of diol-bonded silica with 1,1'-carbonyldiimidazole and the coupling of protein ligands was examined as a function of activation conditions, coupling time, buffer, and pH. It was found that extensive sonication and vacuum-degassing during the activation step significantly increased the amount of ligand coupled. In the coupling step, it was found that a subpopulation of active groups was resistant to hydrolysis and resulted in increased coupling yields of ligand over a six-day reaction time. The coupling and hydrolysis reactions were faster in carbonate buffer than in phosphate buffer, but the overall yields were the same. Coupling yields of several proteins were found to be relatively insensitive to pH over the range 4–8.

INTRODUCTION

The use of 1,1'-carbonyldiimidazole (CDI) as an activating agent for affinity chromatographic matrices was first demonstrated by Bethell and co-workers in a series of papers[1–5] and more recently by other workers[6–11]. CDI has also been used for the coupling of proteins to poly(ethylene glycol)[12] and is well-known as a synthetic reagent[13].

The great advantage of CDI for affinity adsorbent preparation is that the alkyl carbamate linkage formed between a hydroxylic support and an amine-containing ligand is neutral[1]. This is in contrast to many other immobilization methods, including the cyanogen bromide[14,15], tresyl chloride[16], epoxide[16], and Schiff's base[16] methods, in which basic groups are introduced when amine-containing ligands are coupled and which can cause non-specific adsorption by ion exchange[15].

Bethell and co-workers primarily examined ligand immobilization on agarose matrices[1–5]. They found high pH to be optimal (typically pH 8.5–10) and that the immobilization of ligands and hydrolysis of reactive groups took place within *ca.* 1 day[2]. Silica and glass matrices, such as are used in high-performance affinity chromatography[16,17], cannot tolerate such alkaline conditions. However, CDI-activated silica and glass matrices have been successfully used at pH 8.5 and below[5,7–11], and CDI-activated glass matrices are commercially available from Pierce. In spite of this, a general study of the CDI-immobilization of ligands on silica or glass matrices has not been published.

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In this paper, the activation reaction in an organic solvent between CDI and diol-bonded silica [prepared by the reaction of silica with (3-glycidoxypropyl)trimethoxysilane[18]] and the coupling reaction between activated silica and an amine-containing ligand in aqueous solution are examined and optimized. It is shown that the optimum conditions for CDI-immobilization are significantly different when diol-bonded silica is used as the matrix rather than agarose.

EXPERIMENTAL

Reagents

Bovine serum albumin (BSA), soybean trypsin inhibitor (STI), rabbit immunoglobulin G (IgG), protein A, eel acetylcholinesterase, bovine trypsin, glucosamine hydrochloride, and acetylthiocholine iodide were from Sigma (St. Louis, MO, U.S.A.). Horse liver alcohol dehydrogenase (HLADH) and β-nicotinamide adenine dinucleotides (NAD and NADH) were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The HLADH was further purified according to a published procedure[19]. Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, U.S.A.). LiChrospher SI500, SI1000, and SI4000 silicas were from Rainin (Woburn, MA).

Methods

Diol-bonded LiChrospher SI500, SI1000, and SI4000 were prepared as described previously[7]. The optimized CDI-activation and ligand-coupling conditions for LiChrospher SI500 diol-bonded silica were as follows: 1.0 g of diol-bonded silica was suspended in 10 ml of acetonitrile (dried over molecular sieves) containing a 10-fold molar excess of CDI relative to the diol groups in the silica. The suspension was degassed by sonication under aspirator vacuum for 15 min and then shaken at room temperature for an additional 30 min. The activated silica was filtered, washed with acetonitrile, and sucked dry on a medium-porosity sintered-glass filter. The activated matrix was suspended in 10 ml of the coupling buffer (0.1 *M* phosphate or carbonate of the desired pH) and degassed for 5 min. The affinity ligand was then added (10–100 mg protein), and the volume was adjusted to 20 ml with deionized water. The suspension was shaken at 4°C in a water bath for 6 days. The support was washed with buffer or water by filtration or centrifugation.

A spectrophotometric assay was used to determine the degree of CDI activation of the diol-bonded silica. Imidazole standards, CDI-activated silicas and diol-bonded silica blanks (1–3 mg) were suspended in 2 ml of 3 m*M* phosphate buffer (pH 10), sonicated and degassed, then boiled for 30 min to hydrolyze the imidazolyl carbonates. The silica was removed by centrifugation, and the imidazole concentration of the supernatant was determined by absorbance at 210 nm.

Immobilized glucosamine was determined by the alkaline ferricyanide assay[20]. Immobilized protein was determined by either the Lowry protein assay[21] or the Pierce bicinchoninic acid protein assay. In all of these assays, blank values were determined using diol-bonded silica samples. Also, the silica was removed by centrifugation prior to absorbance measurement.

The activities of immobilized HLADH[19] and acetylcholinesterase[22] were determined by enzyme assays. The activities of immobilized STI and protein A were

determined from chromatographic break-through curves[23] of trypsin and IgG, respectively.

RESULTS AND DISCUSSION

Activation step

The CDI-activation of agarose has typically been performed for 15–30 min in dioxane[1–5], acetone[1,5,6], or dimethylformamide[5,12] at room temperature. In our work, acetonitrile was used as the solvent, since better coupling yields were obtained than with dioxane, dimethylformamide, dimethyl sulfoxide, and chloroform[24]. In addition, sonication and vacuum degassing were used to help remove air from the pores of the matrix. Table I indicates the effect of varying the activation parameters on the amount of protein coupled. From Expt. 1, it was apparent that increasing the duration of the sonication–degassing step significantly increased the amount of ligand coupled. Increasing the total activation time, Expt. 2, gave a smaller relative increase in yield. It thus appeared that the sonication–degassing helped to facilitate the reaction between the diol-bonded phase and the CDI.

Since the acetonitrile evaporated as the degassing time increased, Expt. 3 was performed to see whether the change in CDI concentration was important. The results indicated no effect of changing activation volume and CDI concentration.

Further experiments were performed to determine whether extensive sonication fractured the silica. Such fracturing could lead to non-specific adsorption of protein on the bare silica surface. Treatment of several types of diol-bonded silica under conditions identical with the usual activation and coupling conditions, but without CDI, led to the immobilization of 0.15 ± 0.05 mg BSA/g silica. Thus, no significant damage to the silica was observed.

The imidazole assay was used to assess the degree of activation of diol-bonded silica as a function of the surface area of the support. Using diol-bonded silica matrices of surface area 6, 20, and 50 m^2/g and diol coverage of 17, 73, and 190 $\mu mol/g$, respectively, a 10-fold excess of CDI yielded approximately one imidazole per diol group (13, 72, and 156 μmol imidazolyl carbonate/g silica, respectively). In terms of surface coverage, the diol content averaged 3.6 $\mu mol/m^2$ (roughly equivalent to 1.5 monolayers[25]) while the imidazolyl carbonate coverage was 3.2 $\mu mol/m^2$. This activation level can be compared with 1.6 $\mu mol/m^2$ obtained with diol-bonded controlled-pore glass (CPG)[5], 0.4 $\mu mol/m^2$ with the CDI-activated CPG sold by Pierce, and 1.6–3.0 $\mu mol/m^2$ obtained with tresyl chloride-activated diol-bonded silica[19].

To prevent multipoint attachment of macromolecules and a consequent decrease in specific activity[26], it is sometimes necessary to use low activation levels. Fig. 1 shows that low levels of activation were achieved by decreasing the amount of CDI used. In general, the appropriate amount of CDI to use must be determined empirically, since the yield of the activation step was low (*ca.* 11%). The activation levels of cellulose and agarose have also been varied; however, the yields were higher (*ca.* 35–70%)[1,2,5].

Hydrolysis of active groups

Bethell *et al.*[4] found that the imidazolyl carbonate groups were lost by hydrolysis in water at room temperature during reaction times ranging from 1.5 h at

TABLE I

ACTIVATION OF 0.1 g OF LICHROSPHER SI500 DIOL

Expt.	*Sonication–degassing duration (min)*	*Total activation time (min)*	*Activation solution volume (ml)*	*Coupling time (days)*	*BSA coupled (mg/g silica)*
1	1	31	1.0*	2	2.4
	3	33	1.0*	2	2.9
	10	40	1.0*	2	3.8
2	15	15	1.0*	6	7.1
	15	45	1.0*	6	8.5
3	15	45	0.25–paste	6	7.0
	15	45	0.5–0.25	6	7.0
	15	45	1.0–0.75	6	7.3

* Initial volume.

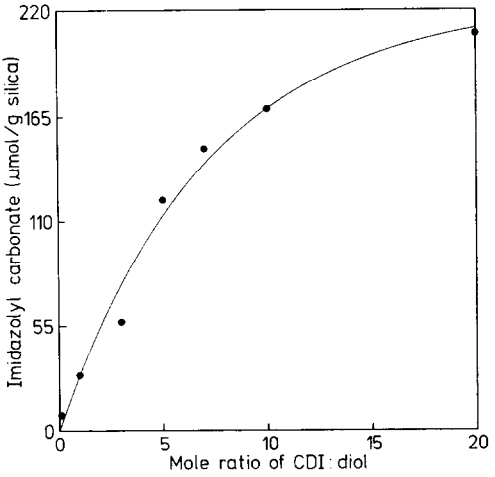

Fig. 1. Imidazolyl carbonate content as a function of the mole ratio of CDI to diol in the activation mixture.

pH 11 to 30 h at pH 8.5 to 20 h at pH 5. Studies of diol-bonded silica were performed in which buffer and pH were varied and the imidazole assay was used to measure the loss of imidazole. The results were imprecise, but the general trend was that hydrolysis was very slow in water and phosphate buffer (more than 6 days at pH 6 and 8) and moderately slow in carbonate buffer (*ca.* 2–4 days at pH 8). It appeared that there might be two subpopulations of active groups present, one of which was hydrolyzed immediately upon contact with water, while the other was more stable and the rate of hydrolysis depended on the buffer salt and the pH.

Coupling of glucosamine

The monovalent ligand glucosamine was used to determine which subpopulation of active groups was primarily responsible for coupling ligands. As shown in Table II, the immobilization rates paralleled the loss of the more stable sites. At pH 8 in carbonate buffer, coupling was complete after 2 days. In phosphate buffer, the reaction was complete after *ca.* 4 days. It is interesting that the same amount of glucosamine was immobilized in both carbonate and phosphate buffers. Apparently, both immobilization and hydrolysis was faster in carbonate buffer.

The yield of the coupling reaction was only *ca.* 11% relative to the initial

TABLE II

IMMOBILIZATION OF GLUCOSAMINE

Buffer	*pH*	*Glucosamine coupled (μmol/g)*				
		0.5 day	*1 day*	*2 days*	*4 days*	*6 days*
Phosphate	6	7	10	17	19	17
Phosphate	8	3	16	12	18	15
Carbonate	8	5	16	18	20	18

number of active sites. The most ligand attached in this laboratory by the CDI method was 1.0 μmol/m^2, or *ca.* 30% relative to the number of diol groups. It thus appears that rapid hydrolysis of many of the active groups ultimately limits the maximum density of immobilized ligands. Using CDI-activated agarose, Hearn *et al.*[2] coupled several monovalent ligands at pH 9–11 with yields of 20–70% relative to the initial number of active groups.

Coupling of albumin and immunoglobulin G

BSA and IgG were chosen to study the immobilization of proteins. Replicate immobilization of BSA yielded a relative standard deviation of 13%. This figure is probably representative of the precision of the immobilized protein data presented below.

The effects of several buffer salts on the immobilization of BSA were examined at pH 8.0 in 0.05 M buffers. The concentrations of BSA attached were: phosphate buffer, 31 mg/g; carbonate, 34 mg/g; borate, 31 mg/g; acetate, 28 mg/g; and trishydroxymethylaminomethane (Tris), 22 mg/g. Thus, all of the buffers, except Tris, yielded similar results. Tris and other amine-containing buffers should not be used for coupling.

Ionic strength effects were briefly examined. When 0.005 *M* phosphate (pH 8) was used, one-half as much BSA was immobilized as when the buffer concentration was 0.05 *M*. Increasing the ionic strength with sodium chloride slightly increased coupling. Similar effects have been observed with other activating agents[27]. However, increasing the ionic strength with phosphate sometimes led to decreased coupling yields.

Carbonate and phosphate buffers were selected for further study. Two concentrations of IgG and BSA were used in these studies. "High" concentration will refer to the use of 5 mg/ml protein concentrations with a maximum immobilization level of 100 mg/g. "Low" will refer to one-tenth of these concentrations.

Fig. 2 shows the immobilization of BSA over a pH range of 2–9. There was

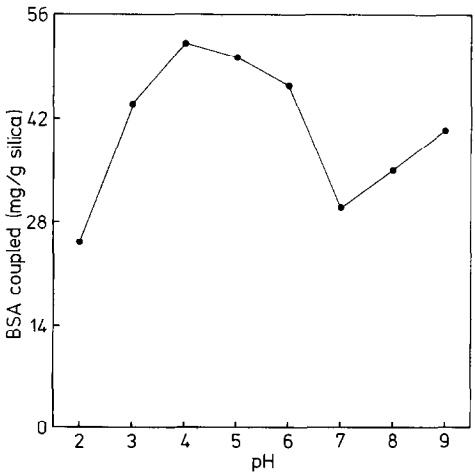

Fig. 2. The pH dependence of BSA coupling using phosphate buffer.

TABLE III

IMMOBILIZATION OF BSA AND IgG

Protein	*Concentration*	*Buffer*	*pH*	*Protein coupled mg/g)*			
				1 day	*2 days*	*4 days*	*6 days*
BSA	Low	Phosphate	6	8	8	8	8
	High	Phosphate	6	31	45	48	57
	Low	Phosphate	8	6	6	6	6
	High	Phosphate	8	28	29	37	42
	Low	Carbonate	6	11	12	12	13
	High	Carbonate	6	43	47	47	52
	Low	Carbonate	8	8	7	7	9
	High	Carbonate	8	26	30	34	34
IgG	High	Phosphate	6	97	100	101	108
	High	Phosphate	8	82	100	103	108
	Low	Carbonate	6	10	10	11	12
	High	Carbonate	6	118	109	111	111
	High	Carbonate	8	61	63	63	63

a broad maximum, centered at pH 4–5. Previous workers utilized high pH for coupling to agarose because monovalent ligands coupled best near the pK_a of the amine group[2,4]. It was thus somewhat surprising to find such a low optimum pH for the BSA. This is very advantageous, since silica matrices are not stable above pH 8.

The time-dependence of immobilization was further examined at pH 6 and 8 (Table III). The data exhibited the same trends as the glucosamine data in Table II. Reaction was more rapid in carbonate than phosphate buffer, but the total amount of protein coupled was similar for both buffers. For BSA, yields were higher at pH 6 than at pH 8, in agreement with Fig. 2. The yields reached *ca.* 90% under "low" conditions and 50% under "high" conditions. IgG appeared to be significantly more reactive than BSA; 100% immobilization was observed under most conditions. It should be noted that 100 mg IgG/g LiChrospher SI500 was close to monolayer coverage. Similar coverages have been obtained for tresyl chloride-activated diol-bonded silica[19].

Immobilization experiments were performed on matrices of variable surface area. Just as in the activation step, the amount of protein attached increased linearly with the surface area of the matrix. Using the same matrices described earlier, "low"

TABLE IV

EFFECT OF THE AMOUNT OF CDI ON PROTEIN COUPLING

Mol CDI/mol diol	*Protein coupled (mg/g)*		
	BSA-low	*BSA-high*	*IgG-low*
10.0	5.3	19.6	7.1
1.0	1.0	1.1	3.1
0.1	0.4	0.7	2.1

TABLE V

REMOVAL OF ACTIVE GROUPS WITH ETHANOLAMINE

Duration of reaction in ethanolamine (days)	*Glucosamine coupled (μmol/g)*
0	18
1	7
2	8
4	2
6	2
Control*	5

* 6 days hydrolysis in buffer.

and "high" BSA concentrations yielded surface coverages of 0.2 and 0.6 mg BSA/m^2, respectively.

BSA was immobilized with decreased amounts of CDI (Table IV). As expected from the activation study (Fig. 1), the coupling yields dropped rapidly as the amount of CDI decreased. Immobilized IgG yields were less affected than BSA yields. In general, the high sensitivity of the yield to the amount of CDI made it difficult to control coverage by this means.

Deactivation of excess reactive groups

Active groups remaining after ligand immobilization are frequently removed by reaction with ethanolamine. To test the effectiveness of ethanolamine in removing imidazolyl carbonate active groups, a solution of 0.08 *M* ethanolamine in 0.05 *M* phosphate buffer (pH 8), was allowed to react with CDI-activated diol-bonded silica for various lengths of time. The ethanolamine solution was then removed by filtration, replaced with a glucosamine solution, and allowed to react for an additional 6 days. The amount of glucosamine coupled is listed in Table V. It is apparent that ethanolamine was not much more effective at removing active groups than simple hydrolysis in buffer. This may be due to the high pK_a of ethanolamine (9.5, *vs.* 7.7

TABLE VI

IMMOBILIZATION OF STI AND PROTEIN A

Ligand	*pH*	*Protein coupled (mg/g)*	*Test solute*	*Specific activity (mg test solute/mg ligand)*
STI*	5	19.9	Trypsin	2.7
	6	18.5		2.6
	7	17.3		2.8
	8	17.6		2.1
Protein A**	4	10.2	IgG	0.7
	6	8.6		0.6
	8	9.0		0.5

* STI applied: 200 mg/g silica.
** Protein A applied: 10 mg/g silica.

TABLE VII

IMMOBILIZATION OF ACETYLCHOLINESTERASE AND HLADH

Ligand	*pH*	*Protein coupled (mg/g)*	*Specific activity (immobilized/native)*
Acetylcholinesterase*	4	3.8	0.4
	6	2.7	1.1
	8	2.6	1.0
HLADH**	5.1	15.5	0.0
	6.5	11.7	1.0
	8.0	10.7	1.0

* Acetylcholinesterase applied: 11 mg/g silica.
** HLADH applied: 19.4 mg/g silica.

for glucosamine). A better alternative for ensuring removal of active groups would be to perform the coupling in carbonate buffer or to treat the matrix with carbonate after coupling of ligand is complete.

Immobilization of various other proteins

STI, protein A, acetylcholinesterase, and HLADH were immobilized at several pH levels in phosphate buffer. The matrices were then assayed for protein content and biospecific activity. It should be noted in these experiments that the amount of protein attached could be increased by increasing the concentration and amount of protein used in the coupling step. However, the percentage yields declined. This same trend can be seen in Table III for the BSA data and has been observed for CDI-activated cellulose[6].

Both the yield and activity of immobilized STI (Table VI) were moderately insensitive to pH in the range 5–8. The yield was highest at pH 5 and the specific activity, based on the binding of trypsin, was almost constant over the pH range 5–7. The manufacturer indicated a specific activity of 1–3 mg trypsin/mg STI for the STI; thus almost all of the activity was preserved during immobilization.

Protein A from *Staphylococcus aureus*, which binds to the F_c region of several classes of immunoglobulins, was immobilized at pH 4–6 (Table VI). Both the yield and specific activity for binding IgG increased slightly as the pH decreased from 8 to 4. However, the specific activity was much lower than the manufacturer's value of 13 mg IgG/mg protein A. A comparative study of three coupling methods has shown that CDI is a poor regent for the immobilization of protein A[11].

Acetylcholinesterase appeared to be immobilized in highest yield at pH 4 (Table VII). However, in a second experiment the highest yield was at pH 8. In either case, pH 4 proved unsuitable because the specific activity was significantly less than at pH 6 and 8, where 100% of the initial activity was retained during immobilization.

HLADH, immobilized in the presence of NADH, zinc sulfate and isobutyramide[19], was coupled in highest yield at pH 5.1 but was found to be totally inactive (Table VII). HLADH is known to be denatured at pH values below 5, and this situation occurred briefly during pH adjustment of the protein solution[28]. However, at pH 6.5 and 8.0 the HLADH was immobilized with 100% retention of activity.

Both the yields and activities were comparable with those obtained with tresyl chloride[19].

NOTE ADDED IN PROOF

A recent paper [M. T. W. Hearn, *J. Chromatogr.*, 376 (1986) 245] has also examined the CDI-activation of silica matrices.

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