Journal of Chromatography, 299 (1984) 175–183
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,840

PREPARATION AND ANALYSIS OF REACTIVE BLUE 2 BONDED TO SILICA VIA VARIABLE SPACER GROUPS

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(Received February 20th, 1984)

SUMMARY

A variety of aminoalkylated microparticulate porous silicas have been prepared and used to displace the reactive chloride from reactive blue 2 thereby immobilizing the dye in a mild and predictable manner. Methods of analysis of immobilized reactive blue 2 are compared and a rapid new method is recommended which is clean and non-destructive. The biospecific elution of a dehydrogenase from an immobilized dye column is demonstrated.

INTRODUCTION

Textile dyes, particularly reactive blue 2, Color Index 61211, immobilized to Sepharose have found wide application as a vehicle for protein purification. This popularity derives in part from the economy of the dye, its non-biodegradability which facilitates affinity chromatography early in a chromatographic procedure, and its broad specificity which can be made selective through biospecific elution. In order to take advantage of the speed and attendent high resolution of chromatography conducted at high pressure we have begun exploration of protein immobilized dye interactions using silica supports. We decided to take advantage of the inherent reactivity of the triazine chloride moiety of the reactive blue in covalently linking it with aminofunctionalized silica rather than following the more traditional and laborious procedure of Lowe *et al.*[1] of attaching a modified dye to an activated matrix. This communication describes the generation and characterization of silica matrices having reactive blue 2 covalently attached via a variety of spacer arms. In the course of such characterization a convenient analytical procedure for measurement of the amount of immobilized dye was developed and is reported.

EXPERIMENTAL

Materials

Silica and derivatized silica bearing a variety of tradenames and having a range of properties were purchased from several suppliers, LiChrosorb silica 60, LiChrosorb amino silica and LiChrospher Silica 500 were purchased from Merck; Tech-

0021-9673/84/$03.00

sphere silica and Techsphere aminosilica from HPLC Technology; and controlled pore glass carbonyl diimidazole (CPG/CDI) glycophase from Pierce. 3-Chloropropyltrimethoxysilane was purchased from Alfa Products while the other silanes, anhydrous toluene and 1,6-diaminohexane were purchased from Aldrich. Lactate dehydrogenase (EC 1.1.1.27), type XI, and its substrates were purchased from Sigma.

Reactive blue 2 was purchased from Polysciences and from Ciba-Geigy. The Polysciences product exhibited only a trace of hydrolyzed dye, *i.e.* dye having the triazine chloride replaced by a hydroxyl group, when examined by thin-layer chromatography (TLC)[2] while *ca.* 20% of the Ciba-Geigy product contained hydrolyzed dye. The former was used for preparative purposes while the latter was reserved for spectral studies. Neither product exhibited the presence of a colored material other than reactive blue 2 and its hydrolysis product when examined by TLC. The concentration of aqueous solutions of reactive blue 2 was determined spectrophotometrically using 10 m*M* Tris buffer, pH 7.5, as the solvent and an extinction of 13.6 mM^{-1} cm^{-1} at 610 nm[3].

Attachment of spacer group

The following general procedure was used which is an adaptation of the procedure of Vivilecchia *et al.*[4]. All glassware was silanized, washed, dried at 150°C and cooled in a desiccator before use. The silica of choice was heated to 150°C for 24 h and then allowed to cool in a desiccator just prior to use. Up to 3 g of silica were suspended in 50 ml of anhydrous toluene using a magnetic stirrer. Then 27 μl of triethylamine per gram of silica were added followed by 1 ml of silane spacer per gram of silica. This mixture was heated under reflux for 4 h in the absence of moisture, cooled and allowed to settle. The supernatant was decanted, replaced with a small volume of fresh toluene, mixed and centrifuged. The sedimented silica was washed in this manner a further three times with toluene and then twice with ethanol. The final washed suspension was filtered through a fine sintered glass funnel, dried at ambient temperature and then at 150°C. In this manner, 3-aminopropyltrimethoxysilane, N-[3-(trimethoxysilyl)propyl]ethylenediamine, 3-(trimethoxysilyl)propylmethacrylate, 3-chloropropyltrimethoxysilane, and 3-(2,3-epoxypropoxy)propyltrimethoxysilane were treated with silica to generate 3-aminopropyl-[4], 3-(2-aminoethyl)aminopropyl-[5,6], 3-methacryloxypropyl-, 3-chloropropyl-[7] and 3-(2,3-epoxypropoxy)-propyl-[8] functionalized silicas, respectively.

The latter three functionalized silicas were then treated with 1,6-diaminohexane to generate functionalized silicas each having a terminal amino group. Functionalized silica (1 g)[9] was suspended in 10 ml of dimethylformamide, 1 g of diaminohexane was added and the mixture was stirred gently for 24 h at room temperature. The mixture was then centrifuged and the sediement washed six times with methanol and twice with water. The solid material was then recovered by filtration and dried at 150°C.

Attachment of blue dye

The desired amount of reactive blue 2, normally between 0.54 and 540 μmol, was added to 1 g of the appropriate amino-functionalized silica suspended in 10 ml of diamethylformamide. The mixture was stirred for the desired length of time, normally either 1 or 24 h, centrifuged, washed repeatedly with dimethylformamide until the washings were colorless, washed twice with water, filtered and dried.

Analysis of functionalized silica

The amount of organic amine was determined potentiometically by direct and reverse titration[10] using 10 m*M* sulfuric acid and sodium hydroxide, respectively. Double bond content was determined by bromination followed by back-titration of residual bromine according to the method of Lucas and Pressman[11]. Organic chloride was determined using a chloride specific electrode. About 10 mg of the functionalized silica containing organic chloride was suspended in 5 ml of 1 *M* sodium hydroxide, maintained at 60°C for 30 min, cooled, neutralized with phosphoric acid, diluted to 30 ml with water, and the chloride content measured. The electrode response was calibrated using standard sodium chloride solutions treated in an equivalent fashion. Epoxide content was determined by periodate oxidation subsequent to hydrolysis[12].

The blue dye content of functionalized silica was determined by a modification of the method of Lowe *et al.*[1]. Between 2 and 50 mg of blue silica was placed in 1 ml of 1 *M* sodium hydroxide and maintained at 60°C for 30 min. After cooling, 3 ml of water and 1 ml of 0.241 *M* phosphoric acid were added and the absorbance at 610 nm measured within 1 min of the addition of phosphoric acid. Prior measurements had shown that this addition of acid is required to adjust the final pH of the silica solution to pH 7.0.

The blue dye content was also determined by a refractive index compensation method. Weighed amounts of reactive blue-silica (1–250 mg) were suspended in dimethyl sulphoxide (3 ml) and the suspension transferred to a spectrophotomer cell. The absorbance at 630 nm was recorded within 2 min of mixing and the apparent absorbance of the functionalized silica matrix (Table I) subtracted. An extinction coefficient of 16.3 mM^{-1} cm^{-1} at 630 nm was used to calculate the concentration of immobilized dye.

Chromatography

Chromatography was performed at high pressure and at ambient temperature using an IBM LC/9533 ternary gradient liquid chromatograph at a flow-rate of 1 ml/min at *ca.* 49 bar. Reactive blue 2 coupled to functionalized silica was dry packed in a 20 × 2 mm I.D. stainless-steel column and conditioned with column irrigant, 100 m*M* phosphate buffer, pH 7.0. Reagent injections were made using a 20-μl sample loop. An on-line system for detection of lactate dehydrogenase catalytic activity consisted of an IBM LC 9521 pump, a postcolumn reactor similar in design to that described by Glad *et al.*[8], and an ISCO V4 variable-wavelength absorbance detector set at 340 nm. The assay solution containing 160 μM NADH, 500 μM sodium pyruvate, and 100 m*M* phpsphate buffer, pH 7.0, was pumped at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

As shown in Table I, the procedure employed for the functionalization of silica results in the covalent attachment of between 1.4 and 4.6 μequiv. of organic silane per square meter of silica, values which can be accepted as characteristic for a monomolecular layer[5]. The narrowness of this range suggests that the efficiency of silanization is independent of both the silane reagent and of the silica matrix. As also shown in Table I, the efficiency of attachment of a diaminohexyl group to metha-

TABLE I

ANALYTICAL VALUES

Identification number	*Matrix*	*Surface area (m^2/g matrix)*	*Concentration (μequiv./m^2 matrix)*		*Residual scattering A_{630} (1%)*	*Conjugated dye* (nequiv./m^2 matrix)*	
			Silane spacer	*Terminal amino*			
	Techsphere	200					
1	$(CH_2)_3NH_2$		2.33	2.33	0.04	3.45	151
	LiChrosphere	50					
2	$(CH_2)_3NH_2$		4.58	4.58	0.17	4.40	
3	$(CH_2)_3NH(CH_2)_2NH_2$		4.16	4.16		71.8	390
	LiChrosorb	550					
4	$(CH_2)_3NH_2$		1.4	1.45	0.02	8.07	60.9
5	$(CH_2)_3NH(CH_2)_2NH_2$		1.83	1.83	0.35		113
6	$(CH_2)_3OCOCH(CH_3)CH_2NH(CH_2)_6NH_2$		1.29	0.29	0.08		5.45
7	$(CH_2)_3NH(CH_2)_6NH_2$		1.95	0.14	0.06	4.15	6.47
8	$(CH_2)_3OCH_2CH(OH)CH_2NH(CH_2)_6NH_2$		1.56	0.63	0.13	4.66	
	Controlled Pore Glass	140					
9	$(CH_2)_3OCH_2CH(OH)CH_2NH(CH_2)_6NH_2$			1.56	0.04		29.4

* Values shown in the left column were obtained using 5.4 μequiv. dye/g matrix and reaction for 1 h. Values shown in the right column were obtained using 540 μequiv. dye/g matrix and reaction for 24 h.

cryloxypropylsilica, chloropropylsilica, and epoxypropoxypropylsilica ranged from 7 to 40%. This variability likely results from the differences in their reaction mechanisms and attendant activation energies. Thus methacryloxypropyl silica will probably undergo a Michael addition reaction in dimethylformamide whereas the chloropropyl and epoxypropoxypropylsilicas will be subject to nucleophilic displacement reactions in this solvent.

The analytical procedure described by Lowe *et al.*[1] was initially employed to measure the number of reactive blue groups covalently attached to the amino-functionalized silica. This procedure involves base hydrolysis, neutralization and measurement of the absorbance of the hydrolyzed dye at 610 nm. However, application of this procedure to silica immobilized dyes presents several difficulties. Firstly, the spectrum of the free dye is significantly perturbed by the hydrolysis and neutralization procedure as shown in Fig. 1A. This change is principally due to the high ionic strength of acid anion required to neutralize the base hydrolyzate. Thus the extinction coefficient for the hydrolyzed dye at neutral pH should be 11.5 and not 13.6 mM^{-1} cm^{-1} as previously assumed. Secondly and much more importantly, the silica in the neutralized hydrolyzate forms colloidal suspensions which increase the observed visible absorbance in an unpredictable fashion dependent in part on sample size, ionic strength, and time. While the colloidal suspension can be removed by centrifugation thus lowering the observed visible absorbance as shown in Fig. 2, the color of the pellet indicates that the collodial suspension either avidly adsorbs hydrolyzed dye or cosediments with a dye–silicic acid thereby removing dye from spectral detection.

Accordingly, we sought an alternative spectral procedure for measurement of the amount of reactive blue 2 immobilized on silica. We have developed a method which seeks to balance the refractive indices of silica with that of a suspending solvent. This method is based on that of Larsson[13] but avoids (i) the practical difficulty of precisely matching the refractive index of the silica and a sucrose solution (*ca.* 70% saturated sucrose in our hands), (ii) the introduction of air bubbles which take a considerable time to dissipate, (iii) the construction of a calibration curve to com-

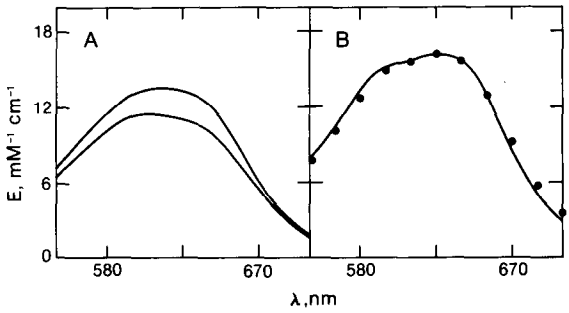

Fig. 1. Absorbance spectra. (A) A portion of the visible absorbance spectrum of reactive blue 2 in aqueous solution. The upper spectrum is that observed for the dye in 10 m*M* Tris–HCl buffer, pH 7.5. The lower spectrum is that observed for the dye subjected to base hydrolysis followed by neutralization. (B) The spectrum observed using dimethyl sulphoxide as the solvent. The continuous line is the spectrum of the unconjugated or free dye in this solvent. The filled circles indicate spectral values obtained from the dye conjugated to Techsphere aminopropylsilica using Techsphere aminopropylsilica as the reference. The conjugated dye was generated using an excess of aminopropylsilica such that all of the reactive blue 2 was covalently attached to the matrix, as judged by the total insolubility of the dye in the solvent.

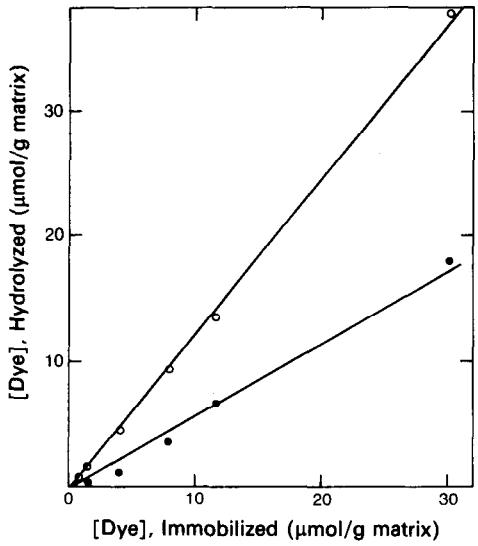

Fig. 2. Comparison of analytical procedures. The ordinate indicates values obtained using the hydrolytic procedure of Lowe *et al.*[1] and the abscissa values obtained for the same dye conjugated silicas using the refractive index procedure developed here. The open and filled circles indicate values obtained prior to and following centrifugation of the neutralized base hydrolyzate, respectively.

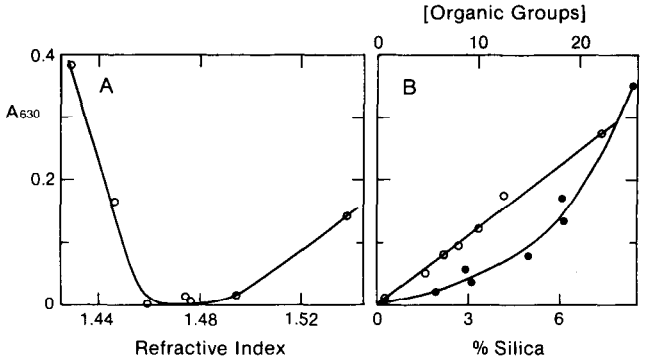

Fig. 3. Apparent absorbance measurements. (A) The effect of the refractive index of the solvent on the apparent absorbance of Techsphere aminopropylsilica. (B) The dependence of apparent absorbance on concentration. The open circles indicate the concentration of Techsphere aminopropylsilica and the filled circles indicate the concentration of the organic groups on all silicas except controlled pore glass. The concentration of organic groups is the product of the concentration of functional groups, μequiv./m^2 matrix (Table I), and the number of non-hydrogen atoms in each functional group. The value for controlled pore glass was not included because of the unique scattering properties of these beads which have a particle size at least an order of magnitude larger than the rest of the functionalized silicas used in this study. All spectral measurements were made at 630 nm and ambient temperature using an optical path of 1 cm.

pensate for light scattering and (iv) the determination of the dye extinction coefficient in sucrose solutions. Similar problems arise in the use of glycerol which has been used with cellulose matrices[14]. As shown in Fig. 3A, suspension of a typical silica in solvents having a refractive index between 1.46 and 1.48 minimizes the scattering of light from the matrix suspension. This results from the matching of the refractive index of the silica employed, 1.472 ± 0.007 with that of the solvent. In this context it should be noted that non-functionalized silica, quartz, has a refractive index of 1.458[15]. Dimethyl sulphoxide having a refractive index of 1.476 was selected as the solvent of choice because unconjugated reactive blue 2 is soluble in this solvent providing for model spectral studies. Further, the residual light scattering of silica as measured by the apparent absorbance at 630 nm, is linearly dependent on silica concentration as shown in Fig. 3B. The residual light scattering observed for the amino-functionalized silicas generated in this study are listed in Table I as the apparent absorbance of a 1% solution observed at 630 nm using a 1-cm light path. As shown in Fig. 3B, the observed residual scattering appears to be a monotonic function of the concentration of organic groups present. Presumably this relationship could be used to determine the concentration of a spacer group of known chemistry conjugated with silica.

While the solvent dimethyl sulphoxide increases the visible absorbance spectrum of unconjugated reactive blue 2, conjugation of the dye to an amino-functionalized silica does not alter the spectrum of the chromophore in this solvent as shown

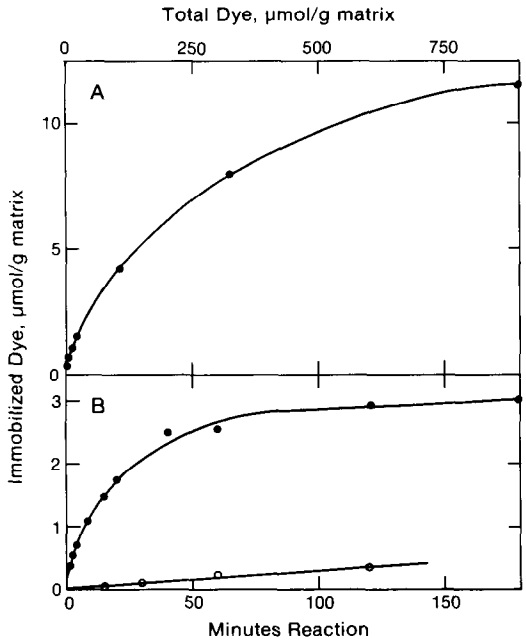

Fig. 4. Progress curves for dye conjugation. (A) Dependence of conjugation on the total dye present in the reaction mixture. Portions of Techsphere aminopropylsilica were separately reacted with increasing concentrations of reactive blue 2 in a total volume of 10 ml for 60 min at ambient temperature. (B) The kinetics of conjugation. The open circles were obtained using aminosilica matrix 2 and the filled circles aminosilica matrix 8. Reactions were conducted using 5 μmole dye per g matrix in a volume of 10 ml at ambient temperature.

in Fig. 1B. Accordingly, measurement of the absorbance at 630 nm of a known weight of washed silica-conjugated dye suspended in dimethyl sulphoxide together with the residual scattering value for the functionalized silica matrix (Table I) and an extinction coefficient of 16.3 mM^{-1} cm^{-1} yields the amount of conjugated dye. As shown in Fig. 2, the hydrolytic and refractive index procedures are quantitatively related. However, in contrast to the hydrolytic procedure, the refractive index procedure is rapid, non-destructive, and free of uncontrolled scattering and adsorption artifacts.

The refractive index compensation procedure was used to observe the features of the conjugation of reactive blue 2 with amino-functionalized silicas. As shown in Fig. 4A, the extent of conjugation exhibits a hyperbolic dependence on the concentration of unconjugated dye added to the reaction mixture. It should be noted that the conjugation reaction asymptotically approaches a level of dye conjugation, *ca.* 12 μequiv./g matrix, considerably less than the total concentration of either the amino-functionalized silica, 466 μequiv./g matrix, or unconjugated dye, 900 μequiv./g matrix. The reaction does not seem to be kinetically limited since reaction with 540 μequiv. dye/g matrix for 24 h generates only 30.2 μequiv. conjugated dye/g matrix, a value representing less than 10% of the total amount of both amino-functionalized silica and dye present. The initial kinetics of dye conjugation exhibit at least two distinct patterns illustrated in Fig. 4B. Conjugation of matrix 2 (Table I) occurs relatively slowly but steadily while conjugation of matrix 8 occurs relatively rapidly, approaching a limiting value after *ca.* 2 h. Inspection of the results shown in Table

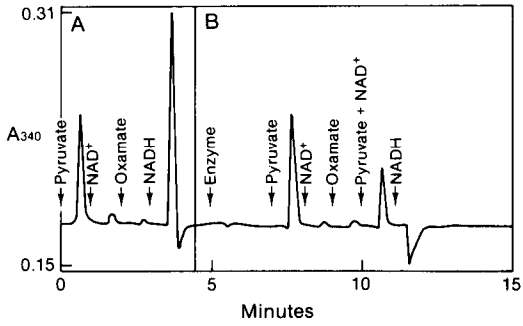

Fig. 5. Chromatography of lactate dehydrogenase on LiChrosphere 3-(2-aminoethyl)aminopropylsilica, matrix 3. The ordinate represents the relative absorbance of the column effluent measured at 340 nm and the abscissa the time in minutes. The baseline having a relative absorbance of *ca.* 0.19 results from the equivolume blending of the chromatography solvent, 100 m*M* phosphate buffer, pH 7, with the enzyme assay reagent, 160 μM NADH and 500 μM sodium pyruvate in phosphate buffer. (A) The absorbance change observed in response to pulsed 20-μl injections of 200 m*M* pyruvate, 8 m*M* NAD, 30 m*M* oxamate or 3.5 m*M* NADH solutions in 100 m*M* phosphate buffer, pH 7.0, to a column containing no lactate dehydrogenase. The arrows indicate the time of the pulsed injection of each chemical. The relatively small absorbance peaks observed after addition of the pulses of pyruvate, NAD and oxamate result from the end absorbance of the high concentration of each of these chemicals applied to the column. The large peak following application of the NADH pulse to the column results from the maximal absorbance of this chemical. (B) The absorbance changes observed in response to pulsed injections of the same components to the same column now preloaded with 10 μg of enzyme. It should be noted that of the pulsed additions illustrated in panel B only that of NADH elutes the enzyme as evidenced by diminution of 340 absorbance. This results from the catalytic transformation of the NADH in the combined column effluent and enzyme assay solutions which are observed by the absorbance detector.

I suggest that matrices 1 and 2 will exhibit slow kinetics and that matricies 3, 4, 7 and 8 will likely exhibit rapid kinetics. However, inspection of their properties does not suggest a rationale for this grouping. Reaction of each matrix with excess dye for 24 h resulted in the conjugation of between 2 and 10% of the amino terminal groups as shown on Table I. This result suggests that the conjugation procedure is to a first approximation independent of the length and polarity of the spacer and the size, shape and porosity of the matrix. It should be noted that although reactive blue 2 is avidly adsorbed from aqueous solution, no conjugation of dye could be observed in aqueous buffered solutions at pH 7, in contrast to the results obtained here using dimethylformamide as the conjugation solvent.

Preliminary measurements using reactive blue 2 conjugated to matrix 3 indicate that proteins will be retained biospecifically by these immobilized dye matrices. As shown in Fig. 5, the enzyme lactate dehydrogenase which binds reactive blue 2 and reactive blue 2 conjugated to Sepharose, is retained by the dye immobilized on silica. The enzyme is selectively eluted by its reduced coenzyme NADH but not by equivalent or excess concentrations of its oxidized coenzyme NAD, by its substrate pyruvate, by neutral salt, or by combinations thereof. This selectivity reflects the relatively high affinity of the dye and of NADH for the coenzyme binding site on the enzyme.

We suggest that the procedures described here can be used to covalently attach reactive dyes of various hues to silica matrices, to analyze the extent of their conjugation and to utilize such matrices for large capacity protein separations at high pressure.

ACKNOWLEDGEMENT

This investigation was supported by United States Public Health Service research grant GM-22109 from the Institute of General Medical Services.

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