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Development of dihydrazide-activated silica supports for high-performance affinity chromatography

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Abstract

A method of preparing dihydrazide-activated silica was developed for use in high-performance affinity chromatography (HPAC). This support was made by oxidizing diol-bonded silica and reacting it with oxalic or adipic dihydrazide. The steps involved in this synthesis were studied and confirmed by FTIR. Items considered in optimizing the preparation of the support included the amount of dihydrazide added and the reaction time or pH used. Control of dihydrazide bifunctional attachment was obtained by varying the extent of diol-bonded silica oxidation. This support was successfully used in the immobilization of oxidized antibodies, horse radish peroxidase (a glycoenzyme) and transfer RNA. In each case, data indicated that immobilization was through site specific coupling rather than non-specific adsorption. Dihydrazide-activated silica was found to be stable for 2–6 weeks after preparation when stored at 5 to 25°C. The linkage between oxidized biomolecules and this support was stable for at least one month in the presence of various solvents commonly used in HPAC.

1. Introduction

High-performance affinity chromatography (HPAC) is a technique which combines the specificity of affinity chromatography with the speed of HPLC for fast and efficient biochemical separations [1]. In this method, a biological compound capable of binding to the analyte of interest is immobilized onto a rigid, high-performance support and packed into a column [1]. Separations based on these columns are selective, take only minutes to perform and are easily automatable [2–7]. These characteristics make HPAC potentially valuable for use in clinical chemistry, pharmaceutical testing and biotechnology.

Most current HPAC methods use silica- and glass-based supports which immobilize biomolecules through free amine, carboxyl or sulfhydryl groups [1,8]. However, this creates a problem when the ligand is a large biological molecule since several such groups can be located throughout the biomolecule's structure. This can lead to improper orientation, denaturation, or multipoint attachment of the immobilized ligand [8,9], resulting in a loss of activity and/or an increase in non-specific binding.

One way of minimizing these effects is to immobilize the ligand through localized groups which are distant from its biologically active site. An example would be the immobilization of an

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antibody through its carbohydrate residues [9,10]. This approach is performed by first oxidizing carbohydrate residues on the antibody to form aldehyde groups using mild treatment with periodate or enzymatic reactions. These aldehyde groups are then reacted with a label or support containing free amine or hydrazide groups to produce the desired antibody conjugate [9,10]. This approach has been used not only for immobilizing and labeling antibodies, but also in modifying a wide range of other biomolecules, including glycoenzymes, RNAs, nucleotides and sugars [10].

Work with biomolecules coupled to agarose, other low-performance supports and various labeling agents has demonstrated that the carbohydrate immobilization approach generally produces higher binding activity than traditional coupling methods [9,10]. However, no successful attempt has yet been reported in adapting this method for use with silica- or glass-based materials. The main difficulty in adapting this method has been in the development of an adequate activated support. For example, Lin *et al.* [11] examined the use of hydrazine in activating silica for antibody attachment but concluded that the actual binding of antibodies to this matrix was controlled by physical adsorption rather than site-specific coupling.

This study will examine the use of dihydrazides in activating silica for biomolecule immobilization in HPAC. The approach to be used in making this support is shown in Fig. 1. First, the silica will be reacted with an epoxysilane to form diol-bonded silica (Step 1) and converted to an aldehyde form by oxidizing it with periodic acid (Step 2). This aldehyde silica will then be reacted with a dihydrazide-containing compound, such as oxalic ($n = 0$) or adipic ($n = 4$) dihydrazide, to form a dihydrazide-activated support (Step 3). This support should react with aldehyde groups on a biomolecule containing oxidized carbohydrate residues to immobilize the biomolecule through a stable hydrazone bond (Step 4) [10]. The same method could be used for glass-based materials after proper pretreatment of the support's surface. A similar approach has been reported for the attachment of T-2 toxin to silica, but based on the direct coupling of dihydrazides to an immobilized epoxysilane [12].

Conditions to be considered in optimizing the

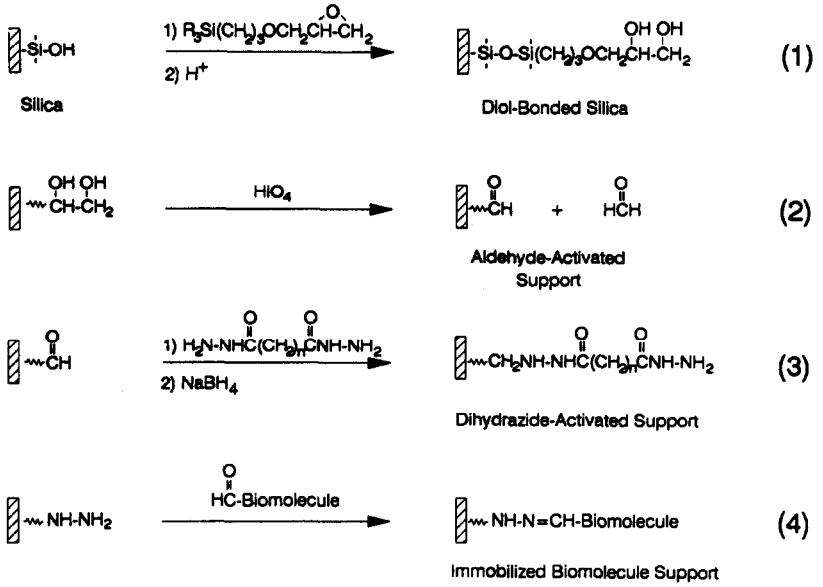

Fig. 1. Preparation of dihydrazide-activated silica. The value of n in Step 3 refers to the number of methylene groups ($-CH_2-$) in the dihydrazide used in the synthesis.

preparation of dihydrazide-activated silica will include the reaction times and pH, the amounts of dihydrazide used and the extent of previous oxidation of the diol-bonded silica support. A comparison between the use of oxalic and adipic dihydrazide in silica activation will be made. The long term stability of the support will also be studied. Finally, the use of dihydrazide-activated silica in the immobilization of antibodies, glycoenzymes and RNA will be examined.

2. Experimental

2.1. Reagents

The glyceraldehyde, horse radish peroxidase (HRP), wheat germ transfer-ribonucleic acid (*t*RNA), goat immunoglobulin G (IgG), periodic acid, iodic acid and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma (St. Louis, MO, USA). The Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) and Nucleosil Si-1000 (7 μm diameter, 1000 Å pore size) were from Alltech (Deerfield, IL, USA). The oxalic dihydrazide, adipic dihydrazide and 3-glycidoxypropyltrimethoxy silane were from Aldrich (Milwaukee, WI, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All other chemicals and biochemicals were of the purest grades available. All solutions were prepared using water from a NANOpure water system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

Samples for the TNBS, *t*RNA and protein assays were analyzed using a Shimadzu UV160U recording UV–Vis spectrophotometer (Kyoto, Japan). Fourier transform infrared spectroscopy (FTIR) of silica samples was performed using an Analect RFX-65 FTIR spectrometer (Laser Precision Corporation, Irvine, CA, USA) in the diffuse reflectance mode. The flow injection analysis (FIA) system consisted of one CM3000 isocratic pump, one CM4000 gradient pump and one SM3100 UV–Vis variable wavelength absorbance detector from Milton Roy (Riviera Beach, FL, USA). Samples in this system were applied using a Rheodyne 7012 injection valve (Cotati, CA, USA) equipped with a PhaseSep event marker (Phase Separations, Queensferry, UK).

2.3. Methods

Diol-bonded Nucleosil was prepared using a modification of previous procedures [13,14]. In this method, 1 g of Nucleosil Si-300 or Si-1000 was placed in 8.5 ml of 0.1 *M* sodium acetate buffer (pH 5.5) and degassed by sonication under vacuum for 10 to 15 min. To this was added 0.20 ml of 3-glycidoxypropyltrimethoxy silane and the resulting solution was shaken for 5 h at 90°C. The epoxy silica formed was washed several times with water and a pH 3.0 sulfuric acid solution. The support was next suspended in the pH 3.0 sulfuric acid solution (100 ml of solution per gram of silica) and was refluxed for 90 min. The diol-bonded silica product was washed with several portions of water, methanol and ether and dried overnight under vacuum at room temperature. The final diol coverages obtained for the Nucleosil Si-300 and Si-1000 by this procedure were 220 ± 20 (1 standard deviation of the mean) and 53 ± 2 μmol per gram of silica, respectively, as determined in triplicate by the periodate oxidation method [15,16].

The final method developed for preparation of dihydrazide-activated silica involved suspending one gram of diol-bonded silica in 20 ml of a 90% (v/v) acetic acid–water mixture containing 1 g of periodic acid. This mixture was sonicated under reduced pressure for 10 to 15 min and shaken for 2 h at room temperature [13]. The aldehyde silica produced was washed four times with deionized water or 0.1 *M* phosphate buffer (pH 7.0) to remove any excess periodic acid. To the aldehyde silica was added 20 ml (for Si-1000) or 50 ml (for Si-300) of 0.10 *M* phosphate buffer (pH 5.0) containing a 5-fold mole excess of oxalic dihydrazide or a 100-fold mole excess of adipic dihydrazide *vs.* initial diol groups on the support. The aldehyde silica–dihydrazide mixture was shaken for 1 to 2 h, centrifuged and

washed four times with 0.1 *M* phosphate buffer (pH 7.0). After activation, any remaining aldehyde groups were reduced by adding a 25 mole excess of $NaBH_4$ (*vs.* initial diol groups) in 20 ml of 0.1 *M* phosphate buffer (pH 8.0) per gram of silica. This mixture was shaken for 90 min and washed four times with water or 0.1 *M* phosphate buffer (pH 7.0). The hydrazide silica was stored under vacuum at room temperature or in the pH 7.0 phosphate buffer at 4°C.

After immobilization of a biomolecule to this support, the silica could be used directly in a column or first reacted with glyceraldehyde to remove any remaining activate hydrazide groups. The glyceraldehyde treatment can be performed by placing the silica in 0.10 *M* phosphate buffer (pH 5.0–7.0) and adding a 200-fold excess of glyceraldehyde *vs.* active hydrazide groups on the support. The mixture is shaken at room temperature or 4°C for a minimum of 6 h. The support is then washed several times with 0.10 *M* phosphate buffer (pH 7.0) and stored at 4°C until further use.

The total amount of dihydrazide attached to the silica was determined by carbon/nitrogen combustion analysis (Desert Analytics, Tucson, AZ, USA). The number of active hydrazide sites was determined using a TNBS assay [17]. In this assay, the hydrazide silica was reacted with a five-fold excess of TNBS (*vs.* initial diol groups) suspended in 0.10 *M* phosphate buffer (pH 7.0). A total of 50 ml buffer per gram of silica was used in this reaction. TNBS reacted with free hydrazide groups on the support to form a colored product with an absorption maximum at 425 nm, for oxalic dihydrazide, or 325 nm, for adipic dihydrazide. The amount of colored product was quantitated by suspending 1 to 10 milligrams of the TNBS-reacted silica in 3 ml of a saturated sucrose solution [13], incubating the mixture for 1 h at 60°C and measuring the absorbance of the solution at the appropriate wavelength. A calibration curve was obtained by measuring the absorbance of standards made by adding 100 μl of 0 to 2.5×10^{-3} *M* TNBS in pH 7.0 phosphate buffer to 3 ml of saturated sucrose containing an excess of dihydrazide (*i.e.*, 6.25×10^{-3} *M* adipic dihydrazide or 2.5×10^{-2} *M* oxalic dihydrazide). Assay blanks were prepared by measuring the absorbance of saturated sucrose solutions to which had been added only pH 7.0 phosphate buffer or a known amount of diol-bonded silica. Prior to measurement, all sucrose/sample mixtures were thoroughly vortexed and sonicated to homogenize the suspension and to remove any air bubbles present.

The degree of diol-bonded silica oxidation by periodic acid was determined by FIA. After formation of the aldehyde silica, the silica suspension was centrifuged and 20-μl samples of the supernatant were injected onto the FIA system. The samples were injected into a flow stream containing 90% (v/v) acetic acid applied at a flow-rate of 0.5 ml/min. This stream was combined after the injection port with a solution of 5% (w/v) potassium iodide in deionized water, also applied at a flow-rate of 0.5 ml/min, and passed through a 10 cm × 4.2 mm I.D. reaction chamber containing 75-μm non-porous glass beads (Supelco, Bellefonte, PA, USA). Iodine produced by the reaction of iodide with periodic or iodic acid in the sample was detected by monitoring the absorbance of the eluent at 289 nm. The amount of periodic acid consumed in the production of the aldehyde silica was determined by comparing the response of supernatant samples to the results obtained for standards of periodic acid and iodic acid dissolved in the 90% acetic acid solution.

The HRP, *t*RNA and IgG were oxidized with periodate using previously described methods [18,19]. For the HRP and IgG, 2 ml of 0.10 *M* phosphate buffer (pH 6.0 for HRP, pH 7.0 for IgG) containing 1.3 mg/ml (IgG) or 2.0 mg/ml (HRP) of oxidized or non-oxidized protein was combined with 0.03 g of oxalic dihydrazide-activated Nucleosil Si-300 or diol-bonded Nucleosil Si-300 and reacted at 4°C for one day. A similar procedure was performed with the *t*RNA but instead using 15 ml of a 4.0 mg/ml oxidized or non-oxidized *t*RNA solution in 0.10 *M* sodium acetate buffer (pH 5.0). After the immobilization reaction, the supports were washed several times with 0.10 *M* phosphate buffer (pH 7.0), pH 7.0 phosphate buffer containing 2 *M* sodium chloride, and deionized water. The supports reacted with *t*RNA were also washed several times with 4 *M* sodium chloride and 7 *M* urea.

The final samples were dried overnight under vacuum and analyzed for their biomolecule content. The amount of immobilized protein was determined using the BCA protein assay [20], using HRP or goat IgG as the standard and dihydrazide-activated silica as the blank. Immobilized *t*RNA was determined by suspending silica samples in a saturated sucrose solution and measuring the absorbance at 260 nm, using non-immobilized *t*RNA as the standard and dihydrazide-activated silica as the blank.

Silica samples for the stability studies on dihydrazide-activated supports were prepared by reacting Nucleosil Si-300 with oxalic dihydrazide, as described earlier. Part of this support was divided into thirty 30-mg portions, which were sealed under vacuum in separate glass tubes stored at 25, 50, 75 or 100°C. Approximately 250 mg of the same support was also stored at 4°C in 0.10 *M* phosphate buffer (pH 7.0). Samples of supports stored under each set of conditions were taken over the course of 0 to 150 days and assayed for active hydrazide group using the TNBS assay.

The stability of the linkage between immobilized biomolecules and the dihydrazide-activated silica was examined using oxidized goat IgG coupled to oxalic dihydrazide-activated Nucleosil Si-300. Portions of a support containing approximately 30 mg of IgG per gram of silica were placed into various test tubes containing 0.10 *M* phosphate buffer (pH 7.0, 2.5, or 8.0), 8 *M* urea or 0.10 *M* sodium chloride. These test tubes were stored at room temperature and silica samples were removed from each tube over the course of 0 to 31 days. Each sample was washed several times with 0.10 *M* phosphate buffer (pH 7.0) and deionized water, dried overnight under vacuum and analyzed for its protein content using the BCA assay.

3. Results and discussion

3.1. FTIR studies of dihydrazide-activated silica synthesis

The reaction scheme in Fig. 1 was initially studied by performing diffuse reflectance FTIR on silica samples obtained at various stages of the synthesis. Representative spectra are given in Fig. 2, using the derivatization of Nucleosil Si-300 with oxalic dihydrazide as an example. This reaction was performed at pH 5.0 using a 25-fold mole excess of periodic acid per diol group in the preparation of the aldehyde matrix and a 5-fold excess of oxalic dihydrazide in the immobilization of dihydrazide to the support. A spectrum obtained for the starting material, underivatized silica, is shown in Fig. 2a. The sharp band at 3740 cm^{-1} is characteristic of vibrations due to the stretching of free or isolated silanol groups on the silica's surface, as reported previously [21].

The first step of the synthesis involved converting the underivatized silica into a diol-bonded form. The resulting product gave the spectrum shown in Fig. 2(b). A comparison of this spectrum to that in Fig. 2a revealed two new bands at 2935 and 2883 cm^{-1}. These bands represent carbon-hydrogen stretching from the organosilane now present on the silica's surface [22]. A third new band that was observed consisted of a broad peak centered at 3400 cm^{-1}. This was assigned to the diol groups produced at the end of the immobilized organosilane [22]. Another difference noted between Figs. 2a and b was that the silanol band at 3740 cm^{-1} was no longer observable with the diol-bonded matrix.

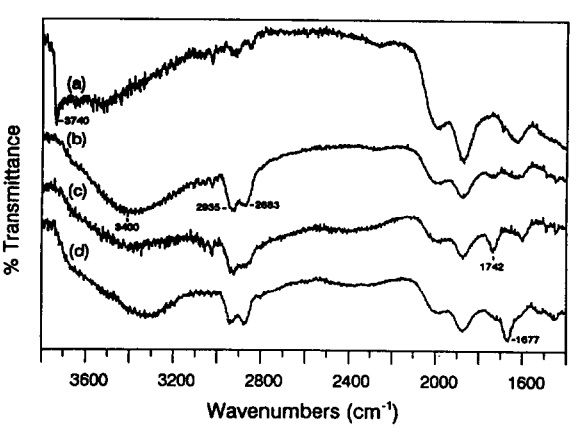

Fig. 2. FTIR spectra of (a) underivatized silica, (b) diol-bonded silica, (c) oxidized silica, and (d) oxidized silica reacted with oxalic dihydrazide. These spectra represent the signal averages of 32 scans obtained using a mixture of the silica in potassium bromide.

This indicated that most of the silanol groups had reacted in the first step of the synthesis. This was confirmed by performing a periodate titration on the diol groups formed. The results indicated that the final coverage of diol groups *vs.* initial silanol groups on this support was 88 ± 3%.

A spectrum of the diol-bonded silica after it was oxidized to an aldehyde form is given in Fig. 2c. An excess of periodate was used in this example to promote the quantitative conversion of diol to aldehyde groups. This is reflected by the large decrease in the diol band at 3400 cm^{-1} and the appearance of a new band at 1742 cm^{-1} characteristic of the aldehyde carbonyl groups produced on the surface of the matrix [22].

The last step in the synthesis involved the reaction of the aldehyde-activated silica with dihydrazide to produce a dihydrazide-activated support. The spectrum obtained for the final product is shown in Fig. 2d. It was found that the aldehyde carbonyl band previously noted at 1742 cm^{-1} had decreased significantly, accompanied by the appearance of a new band at 1677 cm^{-1}. This latter band is characteristic of the amide carbonyl in oxalic dihydrazide [23] and confirmed that oxalic dihydrazide was now present on the silica's surface.

3.2. Optimization of dihydrazide-activated silica synthesis

Several items were considered in optimizing the preparation of the dihydrazide-activated silica. The first item examined was the relative amount of dihydrazide needed. This was studied by determining the number of active hydrazide sites produced on aldehyde-activated Nucleosil Si-300 as various amounts of oxalic and adipic dihydrazide were added. In this experiment diol-bonded silica was oxidized with a large excess of periodic acid and reacted with dihydrazide for 2 h at pH 5.0. The amount of adipic dihydrazide used varied from a 0 to 450-fold excess *vs.* initial diol groups on the support and the amount of oxalic dihydrazide ranged from a 0 to 50-fold excess.

As the amount of added dihydrazide was increased, there was an initial increase in the number of active hydrazide sites produced. This was followed by a plateau in the number of active sites at higher dihydrazide levels. This plateau began to occur at about a 5-fold excess of oxalic dihydrazide or a 100-fold excess of adipic dihydrazide. These amounts of dihydrazide were used for support preparation in all later work. Above these levels, less than a 5% increase in the number of active sites was noted for oxalic dihydrazide and less than a 20% increase was noted for adipic dihydrazide under the range of conditions studied.

The effect of pH on the production of hydrazide-activated silica was also studied. This was of interest since the reaction of aldehydes with hydrazide compounds is known to be acid-catalyzed, but the non-protonated form of the hydrazide is required for the reaction [9]. The effect of pH in dihydrazide attachment was examined by reacting aldehyde-activated Nucleosil Si-300 for 2 h with a 5-fold excess of oxalic dihydrazide or a 100-fold excess of adipic dihydrazide at six pH values ranging from 2.0 to 7.0. The mean number of active hydrazide groups measured over this pH range was 5.18 ± 0.06(1 S.D.) $\times 10^{-5}$ mol per gram of silica for adipic dihydrazide and $1.55 \pm 0.06 \times 10^{-5}$ mol per gram of silica for oxalic dihydrazide. Neither adipic nor oxalic dihydrazide showed any significant change in the number of active sites produced over the pH range studied (± 1.2% and ± 3.9% variation, respectively). This indicated that any pH between 2 and 7 was suitable for dihydrazide attachment to the aldehyde-activated support. For convenience, an intermediate pH of 5 was used in all further studies.

The effect of reaction time in preparing dihydrazide-activated silica was examined by reacting aldehyde-activated Nucleosil Si-300 with oxalic and adipic dihydrazide under the optimum pH and concentration conditions determined earlier. The reaction for both types of dihydrazides was relatively fast, with 80% of the active hydrazide sites being produced in the first 30 min and 95% being produced within 1 h. In order to obtain quantitative activation (*i.e.*, production of 95% or more of the maximum

active hydrazide sites), a minimum reaction time of 1 h was used for both dihydrazides in all further work.

3.3. Control of bifunctional hydrazide attachment

A potential problem in using dihydrazides for silica activation is that both ends of the dihydrazide may attach to aldehyde groups on the silica's surface. In the extreme case, this would leave few or no free hydrazide groups for the coupling of ligand. This problem was indicated early in our work by the fact that only a small fraction of the immobilized dihydrazide resulted in active sites. This process could be reduced by using an excess of dihydrazide, but even at very high dihydrazide levels a significant fraction of inactive hydrazide groups was noted.

One approach found to be effective in decreasing the bifunctional attachment of dihydrazides was to decrease the degree of oxidation of the diol-bonded silica used in the hydrazide activation step. In theory this should increase the spacing between aldehyde groups on the silica's surface, thereby decreasing the probability that one molecule of dihydrazide will bind to two neighboring aldehyde sites. In this study the degree of diol-bonded silica oxidation was controlled by varying the amount of periodic acid used in Step 2 of the synthesis shown in Fig. 1.

The relationship between the amount of periodic acid added and consumed in the oxidation step of Fig. 1 was examined using flow injection analysis. A linear relationship was noted between added and consumed periodate when less than one equivalent of periodate per diol group was used. The best-fit line over 6 points in the range of 0 to 0.85 equivalents had a correlation coefficient of 0.997. The slope of the best-fit line was 1.04 ± 0.04 (± 1 S.D.) and the intercept was -0.01 ± 0.02. These results demonstrated that the reaction of periodic acid with the diol-bonded silica was quantitative. The linear relationship obtained also indicated that the amount of periodic acid could be used to control the degree of support oxidation. At higher periodate levels (*i.e.*, a 1- to 4-fold excess of periodic acid *vs.* diol groups), approximately one equivalent of periodic acid was consumed regardless of the actual amount of periodic acid added. This suggested that periodic acid was reacting only with the diol groups and did not have any significant side reactions under the conditions used in this study.

Figs. 3 and 4 show how the degree of support oxidation affected the total and relative fraction of active hydrazide sites obtained. From Fig. 3 it was found that all supports gave the largest number of active sites when 100% oxidation of the diol-bonded silica was used. The total number of active hydrazide groups produced with Nucleosil Si-300 was consistently 3–4 times higher than that obtained with Nucleosil Si-1000. This agrees with the fact that Nucleosil Si-300 has a 4-fold larger surface area than Nucleosil Si-1000 (*i.e.*, 100 m^2 per gram silica *vs.* 25 m^2 per gram, respectively).

For both oxalic and adipic dihydrazide, the fraction of active hydrazide groups increased as smaller degrees of oxidation were used (see Fig. 4). As already stated, this would be expected since decreasing support oxidation should increase the average distance between surface aldehyde groups, making bifunctional attachment of dihydrazides less likely to occur.

The data in Figs. 3 and 4 show that oxalic dihydrazide had a greater change than adipic

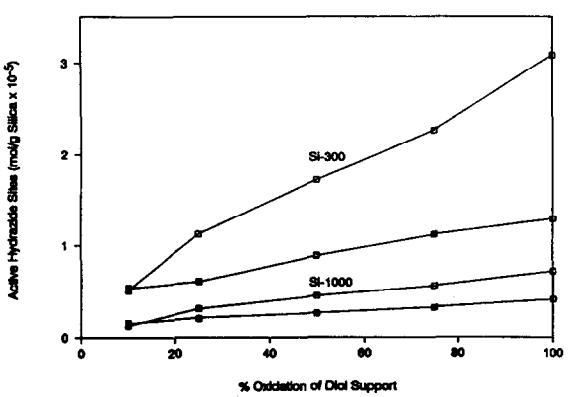

Fig. 3. Effect of diol-bonded silica oxidation on total active hydrazide sites produced for (□) adipic dihydrazide and (■) oxalic dihydrazide on Nucleosil Si-300 and Si-1000. The supports were prepared using the optimized conditions described in the text.

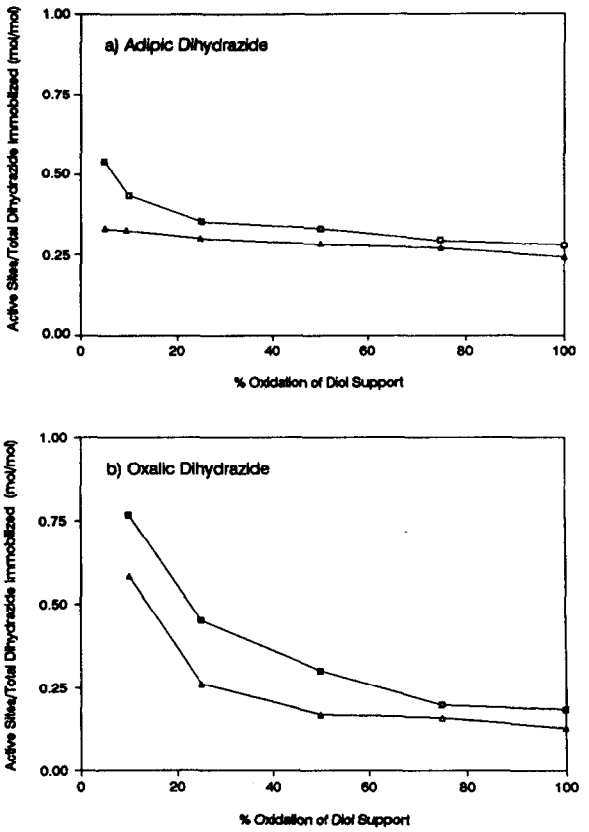

Fig. 4. Effect of diol-bonded silica oxidation on the fraction of active hydrazide sites produced for (a) adipic dihydrazide and (b) oxalic dihydrazide using (△) Nucleosil Si-300 and (□) Si-1000. The experimental conditions used were the same as in Fig. 3.

dihydrazide in the number of active sites produced as the degree of support oxidation was varied. This was anticipated since the oxalic dihydrazide-activated silica was prepared using a much smaller excess of dihydrazide *vs.* diol groups, making this support more susceptible to bifunctional attachment.

At low levels of support oxidation (*i.e.*, 10%), the total number of active sites produced by adipic and oxalic dihydrazide approached the same value (see Fig. 3). This indicates that the spacing of aldehyde groups at this oxidation level was becoming large enough to prevent bifunctional attachment of either the oxalic or adipic dihydrazide. This was confirmed by the data in Fig. 4. At large degrees of oxidation, the adipic and oxalic dihydrazide produced only a small fraction of active hydrazide sites. However, as the degree of oxidation was lowered, the relative fraction of active sites gradually increased to levels as high as 75%.

3.4. Use of dihydrazide-activated silica in biomolecule immobilization

Table 1 shows the degrees of coverage obtained when oxalic dihydrazide-activated silica was used in the immobilization of oxidized antibodies, HRP and *t*RNA. The non-specific binding of the support was examined by incubating the dihydrazide-activated silica with non-oxidized samples of each compound. For all cases studied, the apparent amount of non-oxidized biomolecule bound to the support was within two standard deviations of the level measured for the blank (*i.e.*, oxalic dihydrazide-activated silica with no biomolecule added). The total amount of each oxidized biomolecule immobilized was five to seven times larger than the

Table 1
Binding of oxidized and non-oxidized biomolecules to oxalic dihydrazide-activated silica

Compound	Amount bound to support (μmol/g silica)[a]	
	Oxidized compound	Non-oxidized compound
Goat immunoglobulin G	0.46 ± 0.04	0.09 ± 0.08
Horse radish peroxidase	0.83 ± 0.18	0.12 ± 0.20
Wheat germ *t*RNA	1.33 ± 0.31	0.26 ± 0.13

[a] Numbers in parentheses represent ± 1 S.D. The molecular masses used in calculating the amounts bound were 144 000 g/mol for goat IgG [26]; 44 050 g/mol for HRP [27]; and 25 000 g/mol for wheat germ *t*RNA [28].

apparent amount of non-oxidized compound coupled. This data indicated the oxidized biomolecules were bound to the dihydrazide-activated silica mainly through site specific attachment, via their carbohydrate residues, rather than through non-specific adsorption to the surface of the support.

The results in Table 1 agree with earlier studies performed using the carbohydrate immobilization method with agarose and other low-performance supports [9,10]. However, the data in Table 1 differs from that reported by Lin *et al.* [11] for hydrazine-activated silica, in which non-specific adsorption was determined to be the main mechanism for oxidized antibody immobilization. This difference indicates that the choice of activation agent (*i.e.*, hydrazine *vs.* dihydrazide) may be an important consideration in obtaining the successful attachment of biomolecules to silica when using the carbohydrate method.

3.5. Stability of dihydrazide-activated silica

The stability of hydrazide silica prior to its use in biomolecule immobilization was examined using an oxalic dihydrazide-activated support. The stability of this matrix was examined by making plots of $\ln(C/C_0)$ *vs.* time, where C and C_0 are the active hydrazide groups measured at times t and 0. The samples tested were stored at 5°C in pH 7.0 phosphate buffer or stored under vacuum at temperatures ranging from 25 to 100°C. A sharp decrease in $\ln(C/C_0)$ between 0 and 14 days was observed for the samples kept at elevated temperatures (*i.e.*, 50 to 100°C). This was followed by a linear decline in $\ln(C/C_0)$ for all samples between times of 14 and 150 days, with correlation coefficients between -0.8517 and -0.9864 (mean, -0.94 ± 0.06) for the five temperatures and six time points tested.

The size of the initial decrease in $\ln(C/C_0)$ for the high temperature samples corresponded to a 50 to 75% loss of active hydrazide sites during the first 14 days of storage, with the degree of loss increasing with storage temperature. These results showed good correlation with changes measured in the total dihydrazide content of the same samples. This indicated that the short term decrease at high temperatures was probably due to the loss of thermally unstable dihydrazide groups from the support.

The linear decrease in $\ln(C/C_0)$ noted for all samples at storage times longer than 14 days indicated that a second mechanism of deactivation was also present which followed pseudo-first order kinetics. This was the only type of deactivation observed for samples stored at or below room temperature. The best-fit slopes obtained for the plots of $\ln(C/C_0)$ *vs.* time between 14 and 150 days were used to estimate the apparent first-order rate constants (k) for this process. The values determined for k with the samples stored under vacuum are given in Fig. 5. The value measured for k at 5°C for silica stored in buffer was $3 \pm 1 \times 10^{-8}$ s^{-1}.

An Arrhenius plot of $\ln k$ *vs.* $1/T$ is shown in Fig. 5 for the samples stored under vacuum between 25 to 100°C. A linear fit was obtained, with a correlation coefficient of -0.9938 over the four data points shown. No apparent difference was noted between the rate constant predicted by this graph at 5°C ($5 \pm 4 \times 10^{-8}$ s^{-1}) and the value measured for silica stored at 5°C in buffer. This suggested that the buffer or solvent was not a major factor in silica deactivation. Further tests indicated that the long-term loss of active hy-

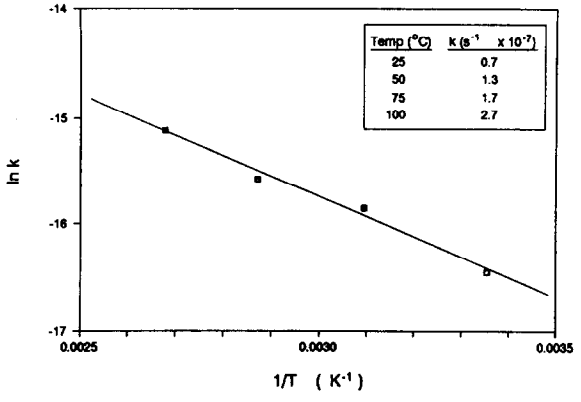

Fig. 5. Arrhenius plot for the loss of active hydrazide groups on oxalic dihydrazide-activated Nucleosil Si-300. The best-fit line shown had a slope of -1910 ± 150 (1 S.D.) and an intercept of -10.0 ± 0.5.

drazide groups was not linked to any significant, corresponding changes in the support's total dihydrazide content. This type of behavior might be produced if the free ends of the immobilized dihydrazides were slowly combining with non-reduced aldehyde groups remaining on the support or with amide carbonyl groups located on neighboring dihydrazide chains.

The usable shelf life of dihydrazide-activated silica was determined from the data in Fig. 5. The shelf life was defined in this study as the time required for the support to decay to 90% of its initial activity (*i.e.*, a 10% loss of active hydrazide groups). For oxalic dihydrazide-activated silica, this shelf life was determined to be 17 days when the support was stored under vacuum at 25°C or 40 days when it was stored at 5°C in pH 7.0 phosphate buffer. This stability was considered more than sufficient for the use of dihydrazide-activated silica in biomolecule immobilization; however, it is probably not large enough to warrant long term storage of the activated support. Based on these results, it is recommended that dihydrazide-activated silica be prepared just before use or used for immobilization within 2–6 weeks of its preparation.

The stability of the linkage between immobilized biomolecules and the dihydrazide support was also examined. This was studied by attaching oxidized goat IgG to an oxalic dihydrazide-activated support and placing the resulting matrix into various solvents commonly used for sample application and elution in HPAC. An example of the results obtained in this study are shown in Fig. 6. Linear regression of the entire data set did not reveal any significant decreases in the amount of immobilized IgG over the course of one month. Some random variations in the results were noted, but the mean sample values were always at levels of 80% or more of the initial protein content. From this, it was determined that no appreciable amount of the immobilized IgG was being lost from the support in the presence of these solvents. This work agrees with previous short studies examining the stability of biomolecules immobilized to hydrazine- or hydrazide-activated agarose [24,25].

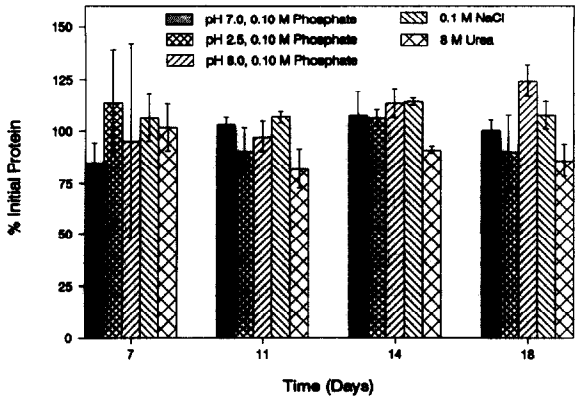

Fig. 6. Stability of goat IgG coupled dihydrazide-activated silica in the presence of various application and elution solvents used in affinity chromatography. The error bars represent a range of ± 2 standard deviations of the mean for two to three analyses of each sample. All other experimental conditions are given in the text.

4. Conclusions

In this work, a method was developed for the preparation of high-performance affinity chromatographic supports based on dihydrazide-activated silica. The steps involved in making this support were studied and confirmed by FTIR. The optimum preparation conditions were determined using both oxalic and adipic dihydrazide as activating agents. Items considered in this optimization included the amount of dihydrazide used, the reaction pH and the reaction time. The effect of bifunctional attachment of dihydrazides was also examined using assays for the active and total dihydrazide on the support. It was found that bifunctional attachment was significant but could be controlled by varying the oxidation of the diol-bonded silica used in the activation step.

Dihydrazide-activated silica was used in the immobilization of oxidized antibodies, horse radish peroxidase and *t*RNA. In each case, it was shown that immobilization was through site specific coupling rather than non-specific adsorption to the support. This observation is important since earlier work found that adsorption was the main mechanism of immobilization for oxid-

ized antibodies on a related support, hydrazine-activated silica [11]. Thus, the dihydrazide-activated silica reported in this work represents an important step forward in using HPAC for the site specific coupling possible with many biomolecules containing carbohydrate residues.

The long term stability of dihydrazide-activated silica was studied both before and after its use in biomolecule immobilization. The activated support was found to be stable (*i.e.*, retain 90% or more of its initial activity) for 2–6 weeks after preparation when stored at 5 to 25°C. Work examining oxidized antibodies coupled to dihydrazide-activated supports determined that the linkage of these biomolecules to the support was stable for at least one month in the presence of various solvents commonly used in HPAC. All of these studies indicate that this support should be a valuable tool in analytical and preparative scale applications of HPAC using antibodies, glycoenzymes, and other carbohydrate-containing molecules.

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