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DEVELOPMENT OF SULFHYDRYL-REACTIVE SILICA FOR PROTEIN IMMOBILIZATION IN HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

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Abstract

Two techniques were developed for the immobilization of proteins and other ligands to silica through sulfhydryl groups. These methods made use of maleimide-activated silica (the SMCC method) or iodoacetyl-activated silica (the SIA method). The resulting supports were tested for use in high-performance affinity chromatography by employing human serum albumin (HSA) as a model protein. Studies with normal and iodoacetamide-modified HSA indicated that these methods had a high selectivity for sulfhydryl groups on this protein, which accounted for the coupling of 77–81% of this protein to maleimide- or iodoacetyl-activated silica. These supports were also evaluated in terms of their total protein content, binding capacity, specific activity, non-specific binding, stability and chiral selectivity for several test solutes. HSA columns prepared using maleimide-activated silica gave the best overall results for these properties when compared to HSA that had been immobilized to silica through the Schiff base method (i.e., an amine-based coupling technique). A key advantage of the supports developed in this work is that they offer the potential of giving greater site-selective immobilization and ligand activity than amine-based coupling methods. These features make these supports attractive in the development of protein columns for such applications as the study of biological interactions and chiral separations.

INTRODUCTION

High-performance affinity chromatography (HPAC) is a method of growing interest for the selective separation and analysis of various chemicals.^{1–4} This technique makes use of a support such as HPLC-grade silica that contains a biologically-related molecule as the affinity ligand, or stationary phase. HPAC has been used in such applications as the study of biological interactions, biomolecule purification, chiral separations, and the analysis of clinical, biological and environmental samples.^{5–14} Advantages of HPAC for these applications include its high specificity, speed, ease of automation and ability to use the same ligand for multiple applications^{1–3,15}.

An important factor to consider in the production of a column for HPAC is the way in which the affinity ligand is attached to the chromatographic support.^{16–18} When a protein is utilized as the affinity ligand, this attachment often involves the use of a diol- or glycol-containing support (e.g., diol-bonded silica)¹⁹ and amine-based coupling techniques such as the Schiff base,^{20–26} carbonyldiimidazole,^{27–31} cyanogen bromide,³² succinimidyl ester^{32,33} or epoxy immobilization methods.^{34–39} It is also possible with HPLC supports to attach proteins to amine-activated supports by using the glutaraldehyde^{32,33} or *N*-hydroxysuccinimide (NHS) methods.^{32,33,38,40} In addition, glycoproteins can be immobilized after oxidation of their

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carbohydrate residues by reacting such proteins with hydrazide- or amine-containing supports.^{32,33,36}

Human serum albumin (HSA) is one protein that is often used in HPAC columns. HSA is the most abundant protein in blood and is known to bind a variety of drugs and biological compounds.^{41–43} When immobilized in an HPAC column, this protein can be used under isocratic conditions as a weak-to-moderate ligand for the study of drug-protein interactions⁴⁴ or as a stationary phase for separating a variety of chiral solutes.¹⁹ Most previous work with HSA has involved its immobilization to HPLC supports through its amine groups; however, HSA has a large number of primary amines in its structure (i.e., 58 lysines plus the *N*-terminal amine)⁴² which might lead to such undesirable effects as multipoint attachment, improper orientation or inactivation of this protein during the immobilization process.³²

An alternative route that is appealing for the immobilization of HSA and other proteins is to use free thiol groups in their structures. For instance, normal HSA has only a single free thiol (Cys-34) which could, in theory, be used for the single point attachment of this protein to a support. This study will explore two approaches for such work based on maleimide- and iodoacetyl-activated materials.^{32,33,45–49} Both maleimide and iodoacetyl groups are thought to react selectively with the sulfhydryl group of cysteine.^{32,33,45–49} In addition, these reactions are known to be fast for small thiol-containing compounds,^{45–49} making this a possible route for the rapid immobilization of proteins like HSA to HPLC supports.

Immobilization based on maleimide and iodoacetyl groups has been used in the past for coupling proteins and peptides to quartz disks,⁵⁰ polished silicon wafers,⁵⁰ poly(allylamine) polymers,⁵¹ polystyrene microspheres,⁵² and Sepharose.⁴⁶ However, this approach has not yet been used with any HPLC supports. This study will explore the use of these coupling methods with HSA and HPLC-grade silica. The supports used in this work will be prepared by reacting aminopropyl silica with *N*-(4-carboxycyclohexylmethyl) maleimide (SMCC) or succinimidyl iodoacetate (SIA), as shown in Figure 1. This should give activated materials with good stability towards hydrolysis.^{33,45–49} After these supports have been developed, they will be compared in terms of their specificity for sulfhydryl groups and their ability to immobilize HSA in an active form for use in drug-binding studies and chiral separations. The results will then be compared with those noted for the Schiff base method, a common amine-based coupling technique employed for HSA and other proteins.^{20–26,53}

EXPERIMENTAL SECTION

Reagents

The SMCC (>99% pure; toxic), succinimidyl iodoacetate (>97% pure; toxic) and Slide-A-Lyzer dialysis cassettes (7 kDa MW cutoff, 0.1–0.5 mL or 0.5–3 mL capacity) were from Pierce (Rockford, IL, USA). The D-tryptophan (>99% pure), dimethyl formamide (DMF, 99% pure), acetonitrile (HPLC grade, >99.93% pure; flammable), formic acid (96% pure; corrosive), trifluoroacetic acid (TFA, >99% pure; corrosive), acetic anhydride (>99% pure; flammable) and pyridine (>99% pure; flammable and corrosive) were from Aldrich (Milwaukee, WI, USA). HSA (Cohn fraction V, essentially fatty acid free), 3-(α -acetonylbenzyl)-4-hydroxycoumarin (racemic warfarin, >98% pure; toxic and possible teratogen), racemic tryptophan (>99% pure), L-tryptophan (>98% pure), ibuprofen (>98% pure), carbamazepine (>98% pure), 4-dimethylaminopyridine (DMAP; toxic), trypsin (sequencing grade), guanidine hydrochloride (>99% pure), dithiothreitol (DTT, >99% pure), iodoacetamide (99% pure; light sensitive) and L-cysteine hydrochloride (anhydrous, >98% pure; toxic) were from Sigma (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA, disodium salt and dihydrate, essentially 100% pure) was purchased from J. T. Baker (Phillipsburg, NJ, USA). The separate

forms of *R*- and Swarfarin (>98% pure) were from DuPont Pharmaceuticals (Wilmington, Delaware, USA).

The thiol and sulfide quantitation kit (T-6060) was purchased from Molecular Probes (Eugene, OR, USA); this kit contained the *N*-benzoyl-L-arginine, *p*-nitroanilide (L-BAPNA), papain-S-S-CH₃, and L-cysteine referred to later in this section. The aminopropyl and bare silica Nucleosil Si-300 supports (both 7 μm particle diameter, 300 Å pore size) were from Macherey Nagel (Dren, Germany). All aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and filtered using Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

Apparatus

The system used in the chromatographic studies consisted of a P4000 gradient pump and a UV100 absorbance detector from Thermoseparations (Riviera Beach, FL, USA). Samples were injected using a Rheodyne LabPro valve (Cotati, CA, USA) equipped with a 20 μL sample loop. Chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA). Assays for determining the stability of the activated silica were performed using a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). All HPLC columns were packed using an Alltech column slurry packer (Deerfield, IL, USA). Fourier transform infrared (FTIR) spectroscopy was performed using a Nicolet AVATAR-360 instrument from the Thermo Electron Corporation (Waltham, MA, USA); the FTIR work was conducted using dried silica samples (approximately 10 mg) and 128 scans per analysis in the DRIFT mode. Elemental analysis of the silica samples was performed by Midwest Microlab (Indianapolis, IN, USA).

Preparation of maleimide-activated silica (SMCC method)

The general scheme used in the SMCC method to prepare maleimide-activated silica is shown in Figure 1(a). This approach was adapted from previous work described for the reaction of amines with NHS-activated compounds.^{33,45,46,54} In this method, 0.8 g of aminopropyl Nucleosil Si-300 was first dried in a vacuum oven for 24 h. A 24 mg portion of DMAP and 50 mg of SMCC were next dissolved in 5 mL of dry DMF. The dried aminopropyl silica was added to this solution and shaken in the dark at room temperature for 24 h (note: similar work in the activation of aminopropyl Nucleosil Si-300 with disuccinimidyl suberate indicated that a reaction time of 8 hr is sufficient for completion of this reaction).⁴⁰ The resulting maleimide-activated silica was collected by centrifugation and washed ten times with DMF. Prior to immobilization, the maleimide-activated silica was washed three times with pH 7.0, 1.3 M potassium phosphate buffer and used immediately. A small portion of the maleimide-activated silica was set aside for later analysis, being washed ten times with DMF, five times with acetonitrile, and five times with diethyl ether; this support was then dried under vacuum at room temperature and stored in the dark at 4°C until use. The maleimide-activated silica was found to be stable for more than 2 months under these storage conditions.

Early work in this report used the maleimide-activated silica directly for protein immobilization. However, later studies used an additional pretreatment step to reduce the effects of unreacted aminopropyl groups on non-specific binding. These aminopropyl groups were capped by reacting them with acetic anhydride to produce acetamide groups. This was performed after support activation by taking 0.5 g of the maleimide-activated silica and adding it to 4 mL of a 1:1 mixture of acetic anhydride and pyridine containing 100 mg of DMAP. This mixture was stirred in the dark for 24 h at room temperature. The capped silica was then washed five times with acetonitrile, three times with diethyl ether, and dried overnight under vacuum. This support was then used for the immobilization of HSA or used directly as a control material.

The conditions used for the immobilization of HSA to maleimide-activated silica were adapted from a previous method reported for the conjugation of antibody F_{ab}' fragments to horseradish peroxidase.⁴⁵ Maleimide groups are known to react preferentially with sulfhydryl groups over the pH range of 6.5–7.5; this forms a stable carbon-sulfur linkage,^{33,45} as shown in Figure 1 (a). Prior to its use in immobilization, the maleimide-activated silica was washed three times with pH 7.0, 1.3 M potassium phosphate buffer. A 5 mL solution of 15 mg/mL HSA in pH 7.0, 1.3 M potassium phosphate buffer was then added to roughly 0.8 g of the activated silica; this solution was degassed by sonication for 5 min and allowed to react with shaking at 4 °C for 36 h. The support was later separated from the rest of the reaction mixture by centrifugation and was washed five times with pH 7.0, 1.3 M potassium phosphate buffer. The final support was stored for up to two days in pH 7.4, 0.067 M potassium phosphate buffer at 4 °C prior to being placed into a column. A control support was prepared in a similar manner by combining 5 mL of 10 mM cysteine (instead of HSA) in pH 7.0, 1.3 M potassium phosphate buffer with approximately 0.4 g of dry maleimide-activated silica.

When using maleimide-activated silica with uncapped aminopropyl groups, there was some HSA that was initially adsorbed non-covalently to the support's surface. The HSA adsorbed on aminopropyl silica was measured using a reducing agent compatible BCA assay from Pierce. It was found that most of this adsorbed HSA could be removed by performing extensive washing of the support after the immobilization step or by capping the aminopropyl groups with acetic anhydride prior to immobilization. The first of these approaches involved the application of 180 or more column volumes of pH 7.4, 0.067 M potassium phosphate buffer to each HSA column prior to its use in chromatographic studies. After this washing it was estimated that less than 7.7 to 7.8 mg of HSA/g of silica was still adsorbed in columns prepared by the SMCC or SIA methods, compared to total protein contents of 61.2 and 20.6 mg HSA/g silica, respectively.

Preparation of iodoacetyl-activated silica (SIA method)

The reaction used in the SIA method for preparing iodoacetyl-activated silica is shown in Figure 1(b). The conditions used in this work with silica were adapted from previous methods described for the reaction of amines with NHS-activated reagents.^{33,45,46,54} In this study, 0.8 g of aminopropyl Nucleosil Si-300 was dried under vacuum for 24 h to remove any residual water. A 25 mg portion of DMAP and 50 mg of SIA were then dissolved in 5 mL of dry DMF. The aminopropyl Nucleosil Si-300 was added to this solution and shaken in the dark at room temperature for 24 h (i.e., a time period over three-fold longer than that estimated to be necessary for completion of the activation reaction).⁴⁰ The resulting activated support was removed from the reaction mixture by centrifugation and was washed ten times with DMF. A portion of this support was washed three times with pH 8.3, 0.050 M borate buffer containing 5 mM EDTA before its use in immobilization. Another portion of the same support was set aside for later analysis after being washed ten times with DMF, five times with acetonitrile, and five times with diethyl ether, followed by drying under vacuum at room temperature and storage in the dark at 4 °C. The iodoacetyl-activated silica was found to be stable for at least 2 months under these storage conditions. Early experiments in this report used the iodoacetyl-activated silica directly for HSA immobilization, but later studies used this support after it had been reacted with acetic anhydride to remove unreacted aminopropyl groups (performed in the same manner as described for the SMCC method).

The conditions used for the immobilization of HSA to iodoacetyl-activated silica were adapted from an earlier reference,³³ which describes the reaction of sulfhydryls with iodoacetyl groups. Iodoacetyl groups are known to react preferentially with sulfhydryl groups under basic conditions (pH > 8) to form a stable carbon-sulfur linkage.^{33,47} Prior to immobilization, the iodoacetyl-activated silica was washed three times with pH 8.3, 0.050 M borate buffer

containing 5 mM EDTA.^{33,55} A 5 mL solution of 15 mg/mL HSA in pH 8.3, 0.050 M borate buffer containing 5 mM EDTA was then added to the iodoacetyl-activated silica and degassed with sonication for 5 min. The immobilization reaction was allowed to occur with stirring at 4°C for 42 h. The final support was separated from the rest of the reaction mixture by centrifugation and washed five times with pH 8.3, 0.050 M borate buffer containing 5 mM EDTA, followed by five additional washings with pH 7.4, 0.067 M potassium phosphate buffer. This immobilized HSA support was stored in pH 7.4, 0.067 M potassium phosphate buffer at 4°C until use. A control support was prepared under similar conditions by immobilizing cysteine in place of HSA to the iodoacetyl-activated silica; in this case, 0.4 g of dry iodoacetyl-activated silica was combined with 5 mL of a 10 mM cysteine solution in pH 8.3, 50 mM borate buffer containing 5 mM EDTA. As stated earlier for the SMCC method, most of the HSA that was non-covalently adsorbed to the support was removed by either performing extensive washing of this material before use or by capping remaining aminopropyl groups on this support with acetic anhydride prior to immobilization.

Schiff base immobilization method

The conditions used for the immobilization of HSA to silica by the Schiff base method were adapted from Ref. 53, with all reactions being carried out at room temperature unless otherwise indicated. In this approach, 1 g of bare silica Nucleosil Si-300 was combined with 0.2 mL of 3-glycidypropyltrimethoxysilane in 8.5 mL of 0.10 M, pH 5.5 sodium acetate buffer. This mixture was heated at 90°C for 5 h with shaking. The resulting epoxy silica was washed three times with water and two times with a pH 3.0 solution of sulfuric acid in water. This silica was refluxed with 100 mL of a pH 3.0 sulfuric acid solution in water for 1 h. The resulting diol-bonded silica was washed three times with water and three times with a 90% solution of acetic acid in water. This silica was then combined with 1 g of sodium periodate (an oxidizing agent) in 20 mL of the 90% acetic acid solution and allowed to react for 2 h with shaking. The resulting aldehyde-activated silica was washed six times with water and twice with 0.10 M, pH 6.0 potassium phosphate buffer.

Immediately after its preparation, the aldehyde-activated silica was combined with 5 mL of 0.10 M, pH 6.0 potassium phosphate buffer, 100 mg HSA and 50 mg sodium cyanoborohydride (a mild reducing agent). This mixture was allowed to react with shaking for four days at 4°C. Any remaining aldehyde groups on the silica were then reduced by slowly adding 0.025 mg sodium borohydride (a strong reducing agent) in 10 mL of pH 8.0, 0.10 potassium phosphate buffer. This support was stored in 0.067 M, pH 7.4 potassium phosphate buffer at 4°C until use. The control support for this material was prepared following the same procedure as described above with no HSA being added during the immobilization reaction.

Analysis of support activation and stability

A modified version of a papain-based assay^{56–58} was used to determine the degree of modification and stability of the maleimide- and iodoacetyl-activated supports. This assay was performed as shown in Figure 2. In this method, a known excess of cysteine was added to each activated support and allowed to react. Later, the remaining unreacted cysteine was combined with papain-S-S-CH₃, which transformed the inactive form of papain into an active form (papain-SH). The activity of the papain-SH was then measured by using L-BAPNA as a chromogenic substrate.

This assay was performed by placing a known mass (0.7–0.9 mg) of each activated support into a 5 mL test tube. To each test tube was added (1) 7.5 µL of 0.5 mM cysteine in 5 mM sodium acetate buffer containing 50 mM sodium chloride and 0.5 mM EDTA, (2) 7.5 µL of 5 mM sodium acetate buffer containing 50 mM sodium chloride and 0.5 mM EDTA, and (3) 220 µL of pH 7.6, 40 mM sodium phosphate buffer containing 2 mM EDTA. Control samples

containing no cysteine were prepared in a similar manner by adding a known mass of each activated support (0.7–0.9 mg) to (1) 15 μ L of 5 mM sodium acetate buffer containing 50 mM sodium chloride and 0.5 mM EDTA, and (2) 220 μ L of pH 7.6, 40 mM sodium phosphate buffer containing 2 mM EDTA. These solutions were then slowly mixed by a magnetic stirrer and allowed to react at room temperature for 40 min.

A 0.5 mL portion of 0.6 mg/mL papain-S-S-CH₃ in pH 4.0, 5 mM sodium acetate buffer was next added to each test tube. These new mixtures were allowed to react for 1 h at room temperature while being slowly mixed by a magnetic stirrer. After this reaction, 0.5 mL of a 4.9 mM L-BAPNA solution in pH 6.3, 50 mM bis-Tris buffer and 1 mM EDTA was added in 30 s intervals to the test tubes, which were reacted for another 1 h with stirring at room temperature. The final reaction mixtures were filtered in 30 s intervals using a 0.2 μ m nylon syringe filter from Pall (Ann Arbor, MI, USA) and measured for their absorbance at 410 nm. Nanopure water was used as the reference during these measurements. The absorbance of each control sample (i.e., mixtures with no cysteine added) was subtracted from the absorbance of the corresponding unknown sample. This difference in absorbance was used with a calibration curve to determine the amount of maleimide or iodoacetyl groups that was on the activated support.

The calibration curve used in this assay was constructed by using standards that contained known amounts of cysteine. These standards were prepared by adding to a series of test tubes 0, 4, 8, 12 or 15 μ L of a 0.1 mM cysteine solution in 5 mM sodium acetate buffer containing 50 mM sodium chloride and 0.5 mM EDTA. All of these solutions were brought to a total volume of 15 μ L by adding 5 mM sodium acetate buffer containing 50 mM sodium chloride and 0.5 mM EDTA. These solutions were mixed and incubated for 40 min at room temperature. A 0.5 mL aliquot of 0.6 mg/mL papain-S-S-CH₃ in pH 4.0, 5 mM sodium acetate buffer was then added to each standard. This solution was allowed to react for 1 h at room temperature with slow mixing by a magnetic stirrer. Next, 0.5 mL of 4.9 mM L-BAPNA in pH 6.3, 50 mM bis-Tris buffer and 1 mM EDTA was added in 30 s intervals to the test tubes and allowed to react for 1 h with slow mixing by a magnetic stirrer. The absorbance measurements of these solutions were taken in 30 s intervals at 410 nm using water as a reference and were plotted versus the original cysteine concentration to give a calibration curve. This method had a limit of detection of approximately 0.2 μ M cysteine (S/N = 3), with a linear range that extended up to 1.5 μ M cysteine.

Preparation of iodoacetamide-modified HSA

The iodoacetamide-modified HSA (used later in this work to evaluate the immobilization selectivity of the maleimide- or iodoacetyl-activated silica) was prepared by combining 68 mg of iodoacetamide in 337 μ L of 1 M NaOH with 7.5 mL of a 15 mg/mL HSA solution in pH 8.2, 0.1 M ammonium carbonate buffer (i.e., a mole ratio of approximately 200:1 for iodoacetamide versus HSA). This solution was shaken for 30 min in the dark at room temperature; this solution was then divided into one 0.5 mL and two 3 mL portions. The 0.5 mL portion was placed into a 0.1–0.5 mL dialysis cassette and dialyzed twice for 4 h at room temperature against two 1 L portions of water; this was followed by dialysis of the same solution against 1 L of pH 8.2, 100 mM ammonium bicarbonate buffer for 12 h at room temperature and later analysis of this solution by MALDI-TOF mass spectrometry (performed at the Nebraska Center for Mass Spectrometry, Lincoln, NE).

The two 3 mL portions of the iodoacetamide-modified HSA solution were placed into two 0.5–3 mL dialysis cassettes and dialyzed twice for 4 h at room temperature against two 1 L portions of water. One of these dialysis cassettes was then placed in 1 L of pH 7.0, 100 mM potassium phosphate buffer for an additional 12 h of dialysis at room temperature. This solution was later used for the immobilization of iodoacetamide-modified HSA onto maleimide-activated silica.

The other dialysis cassette was placed in 1 L of pH 8.3, 100 mM sodium borate buffer containing 50 mM EDTA for 12 h at room temperature. After dialysis, this solution was used for the immobilization of iodoacetamide-modified HSA with the iodoacetyl-activated silica.

Chromatographic studies

All HSA supports and control supports prepared in this study were placed into separate 4.6 mm I.D. $\times$ 5 cm stainless steel columns. These supports were downward slurry packed at 3500 psi (24 MPa) for 40 min and at room temperature using a column packer from Alltech (Deerfield, IL, USA) and pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. All mobile phases were degassed under vacuum for at least 30 min prior to use. All chromatographic studies were performed at 25 ($\pm$ 0.5) $^{\circ}$ C with the columns being enclosed in water jackets for temperature control.

The frontal analysis studies were performed by applying 2.5–30 μ M carbamazepine in 0.067 M, pH 7.4 potassium phosphate buffer to the HSA columns and control columns. This was typically performed at 0.5 mL/min and 25 $^{\circ}$ C using an approach similar to that described in Ref. 59 (Note: similar results to those seen at 0.5 mL/min were obtained at flow rates ranging from 0.3–1.0 mL/min, with a variation of less than 7% being noted over this range of conditions in the moles of carbamazepine that were required to reach the mean point of the breakthrough curve at any given concentration of this analyte). The elution of carbamazepine was monitored by absorbance detection at 214 nm. The columns were first equilibrated in pH 7.4, 0.067 M potassium phosphate buffer for 30 min, with a switching valve then being used to apply the desired carbamazepine solution. After each frontal analysis study, pH 7.4, 0.067 M potassium phosphate buffer was applied at 0.5 mL/min for 30 min (SIA supports) to 1 h (SMCC supports) to elute any retained carbamazepine prior to the next study. The resulting breakthrough curves were analyzed according to methods described in Ref. 44 to estimate the association equilibrium constants and binding capacities for carbamazepine on the HSA columns. The results obtained in identical experiments on the control columns were used to correct for the void time of the system and for any non-specific binding of carbamazepine to the support. The non-specific binding of carbamazepine to uncapped supports in the SMCC and SIA columns was estimated to be 64–74% and 69–72% of the total binding capacity when HSA was present. After acetic anhydride was used to cap aminopropyl groups on these supports, this non-specific binding was reduced by 20% for carbamazepine and by 80% in studies using warfarin as the analyte.

The sample for the zonal elution studies of D/L-tryptophan was prepared fresh daily in the desired mobile phase and contained 20 μ M of this analyte. Solutions of R/S-ibuprofen and R/S-warfarin (20 μ M) for zonal elution studies were prepared in the desired mobile phase and used over the course of less than a week. Three 20 μ L injections were made of each sample for each experiment. No appreciable changes in retention factors (i.e., random variations of less than 1%) were noted when using up to three-fold higher sample concentrations, indicating that linear elution was present under the zonal elution conditions used in this study. The flow rates used during the separation of D/L-tryptophan, R/S-ibuprofen and R/S-warfarin on the maleimide-activated HSA columns were 1.5, 1.0 and 1.0 mL/min, respectively. The flow rates used during the separation of these same solutes on the iodoacetyl-activated HSA columns were 0.3, 0.5 and 0.3 mL/min. The column backpressures under these conditions ranged from 78 psi (0.54 MPa) at 0.3 mL/min to 500 psi (3.4 MPa) at 1.5 mL/min. No measurable changes (i.e., random variations of less than 2%) were seen in the measured retention factors at either lower or higher flow rates (0.3–1.5 mL/min), as noted previously with other silica-based HSA columns.⁷

The following wavelengths were used for detection in the zonal elution studies: D/L-tryptophan, 280 nm; R/S-ibuprofen, 225 nm; and R/S-warfarin, 310 nm. The following mobile

phases were selected for use in the zonal elution studies based on previous separations that have been reported for the given analytes on HSA columns: D/L-tryptophan, pH 7.4, 0.067 M potassium phosphate buffer;⁷ R/S-ibuprofen, pH 7.0, 0.067 M potassium phosphate buffer containing 8% isopropanol and 5 mM octanoic acid; R/S-warfarin, pH 7.0, 0.067 M potassium phosphate buffer containing 5% isopropanol and 1 mM octanoic acid. In the last two cases, isopropanol and octanoic acid were used as mobile phase modifiers or competing agents to adjust and lower the retention factors for the injected analytes to a reasonable range, as described for other HSA columns.^{60–63}

The system void time was determined by injecting 20 μ L of 5 μ M dimethyl sulfoxide (DMSO) onto the chromatographic system while monitoring the absorbance at 205 nm. The extra-column void time was similarly determined by injecting DMSO onto the chromatographic system after replacing the column with a zero dead volume connector. All the retention times and peak widths were determined by using moment analysis or the B/A_{0.5} method.⁶⁴

RESULTS AND DISCUSSION

Initial evaluation of maleimide- and iodoacetyl-activated supports

The silica supports that were activated by the SMCC and SIA methods were first examined qualitatively by FTIR spectroscopy. Some typical spectra that were obtained are shown in Figure 3. The spectrum for the original aminopropyl silica contained major absorbance bands at 831, 1058, 1309 and 1574 cm^{-1} . These bands are due to the Si-C, Si-O-Si or C-N stretches and the primary amine groups in this material, respectively.⁶⁵ Silica activated by the SMCC method also had these bands (due to remaining aminopropyl groups) as well as the following additional absorbance bands (with tentative assignments given in parentheses): 797 cm^{-1} (due to an alkene group coupled to an amide); 1045 cm^{-1} (from the cyclohexane group in the –CH₂– region); 1504 cm^{-1} (due to a peptide amide bond, NH[δ] and N-CO stretch); 1712 cm^{-1} (due to an amide coupled to an alkene); and 1766 cm^{-1} (due to an amide). Silica activated by the SIA method had similar peaks at 1504 cm^{-1} (due to the peptide amide bond, NH[δ] and N-CO stretch) and 1779 cm^{-1} (due to an amide group).⁶⁵ These results indicated that the SMCC and SIA methods were successful in activating at least part of the aminopropyl groups on the starting material.

The degree of support activation in the SMCC and SIA methods was examined in a more quantitative manner by employing elemental analysis. This was accomplished by measuring the nitrogen and iodine content of the maleimide- and iodoacetyl-activated supports, respectively. It was found that activation of aminopropyl silica by the SMCC method under the conditions used in this work gave 14.3 ($\pm$ 7.1) μ mol maleimide groups per gram of silica for Nucleosil Si-300. Activation of the same original aminopropyl silica by the SIA method gave 13.4 ($\pm$ 0.8) μ mol iodoacetyl groups per gram of silica. Both of these values are comparable to the extent of activation that has been reported for reaction of the same type of aminopropyl silica with *N*-hydroxysuccinimide, which gave 12 μ mol NHS groups per gram of silica.⁴⁰ This similarity is not surprising since the NHS activation process involves a nucleophilic reaction of amine groups with disuccinimidyl suberate,⁴⁰ which is closely related to the types of reactions that were used for support activation in this current study.

The amount of activated sites versus the total aminopropyl sites on the original supports was also determined. In this case, the original support was known to contain 328 ($\pm$ 7) μ mol aminopropyl groups per gram of silica. This meant that the maleimide groups placed on this support in the SMCC method gave rise to activation of only 4.4% of these sites. In the SIA method, 4.1% of these sites were activated with iodoacetyl groups. These values are similar to those reported with the same support when activating it with NHS, where 1.3% activation was reported.⁴⁰ However, up to 150 μ mol activated groups per gram of silica has been reported in

a study using 7 μm , 60 $\text{\AA}$ pore size aminopropyl silica that had been activated using succinic anhydride.⁶⁶ This difference is due, in part, to the higher surface area and larger amount of aminopropyl groups on 60 $\text{\AA}$ versus 300 $\text{\AA}$ pore size silica, which accounts for approximately 50% of this difference in activation. The remaining difference is believed to be the result of 1) hydrolysis of SMCC and SIA during the activation reaction and 2) the use of a lower mole ratio in this work for the activating reagent versus aminopropyl groups (i.e., 0.57–0.67 compared to 0.85 in Ref. 66).

In this study support activation was typically performed using 187 μmol SMCC or 221 μmol SIA per gram of aminopropyl silica, which gave a mole ratio of 0.57–0.67 for these agents versus aminopropyl groups on the support. The fact that this mole ratio was below 1.0 partly explains why not all of the aminopropyl groups were activated. Although a larger excess of activating agent could have been used, the amount of reagent required to do this would have been cost prohibitive. In addition, complete activation of the support was not necessary for the overall goal of this project (i.e., site-selective protein immobilization) since a lower density of activated sites would be favorable for the coupling of a protein through only one or a few residues per molecule. This, in turn, would minimize the occurrence of multipoint attachment and any corresponding deformation of the protein's tertiary structure.³²

The stability of the maleimide- and iodoacetyl-activated supports was examined by using both materials to immobilize cysteine. This was accomplished by monitoring the amount of cysteine that was immobilized on each support before and after storage. No significant change was noted in the amount of cysteine that could be immobilized onto freshly-prepared maleimide-activated silica (after storage in the dark at 4 °C for 2 days) and the same support after it had been stored in the dark for over 65 days at room temperature; the amount of immobilized cysteine on these supports was 4.02 ($\pm$ 0.07) and 4.00 ($\pm$ 0.06) μmol per gram of silica, respectively. No noticeable change was also seen in the amount of cysteine that could be immobilized on the iodoacetyl-activated silica after storage in the dark at 4 °C for 2 days and after storage in the dark at room temperature for 65 days, giving values of 4.25 ($\pm$ 0.03) and 4.35 ($\pm$ 0.05) μmol per gram of silica, respectively.

Use of supports in protein immobilization

It was found through protein assays that both the maleimide- and iodoacetyl-activated supports could be used to couple a model protein (HSA) to silica. This is illustrated in Figure 4, which shows how the overall extent of protein immobilization varied with reaction time when using 1.5 mL of a 15 mg/mL solution of HSA that was combined with approximately 0.15 g of activated support (slurry concentration, approximately 100 mg silica/mL, where the support had not been previously capped to remove aminopropyl groups). The amount of HSA in this mixture was approximately 1.9-fold larger than that needed to obtain monolayer coverage of this protein, based on a surface area for HSA of 40 nm².

Both the SMCC and SIA methods required about one day for the immobilization of HSA under these conditions, with 95% of the maximum protein coverage being obtained in 28–30 h. However, these two methods did differ in the total amount of HSA that was coupled to the support. The SMCC method gave up to 67.8 ($\pm$ 1.3) mg HSA/g silica (before correcting for non-covalent adsorption) after 28 h of immobilization, while the SIA method gave only about half of this amount, or 27.1 ($\pm$ 0.5) mg HSA/g silica. After these supports had been packed and washed with 180 column volumes of the mobile phase (i.e., to remove non-covalently adsorbed HSA), the observed protein contents for the SMCC and SIA methods were 61.2 ($\pm$ 1.3) mg/g and 20.6 ($\pm$ 0.7) mg/g, respectively.

It was found in further studies with control materials that up to 14.3 mg HSA/g silica in the SMCC and SIA methods was due to non-covalent adsorption of HSA when using activated

supports that still contained aminopropyl groups. However, this process could be eliminated or reduced by first capping these aminopropyl groups with acetic anhydride, as described in the Experimental Section. The presence of non-covalent adsorption plus covalent immobilization explains the biphasic nature of the time profiles in Figure 4. Non-covalent adsorption would be represented by the fast but small levels of immobilization that occurred during the early stages of this procedure (i.e., at times below 1 h), while the slower but larger amount of covalent immobilization is probably represented by the increase in protein content at longer reaction times. The use of capped aminopropyl groups caused a small decrease in amount of covalent immobilized HSA that could be achieved with each support (e.g., 45.7 (± 0.7) mg HSA/g silica in the SMCC method); this effect is probably due to the loss of some maleimide groups on the activated support during the capping process.

The differences found in Figure 4 for the maximum amount of immobilized HSA in the SMCC and SIA methods is interesting since these techniques gave supports with approximately the same number of activated sites and similar stabilities. In both cases the same amount of protein was originally present, but 57% of the added HSA was coupled covalently in the SMCC method while only 14% was coupled in the SIA method. In comparison, the same amount of protein and support in the Schiff base method resulted in 49% of the protein being immobilized, giving a final coverage of 49.4 (± 5.5) mg HSA/g silica. This latter amount is similar to that reported in earlier studies using equivalent support and immobilization conditions (i.e., 54 mg HSA/g support for 300 Å pore size silica).⁶⁷

The different protein coverages obtained by the SMCC and SIA methods was examined in more detail by comparing the specificity of these immobilization reactions. This was studied by comparing the ability of these techniques to immobilize normal HSA and HSA that had been modified to make its sole free sulfhydryl group non-reactive. The modified HSA was prepared by treating HSA with iodoacetamide. The modification of the free cysteine group on this protein was confirmed by comparing MALDI-TOF mass spectra for tryptic digestions of HSA before and after treatment with iodoacetamide.⁶⁸ These two protein preparations were then immobilized under identical conditions to the maleimide- and iodoacetyl-activated supports. In the SMCC method, the amount of covalently immobilized protein was 81% lower for the modified HSA than for normal HSA, with values of 10.4 (± 0.7) mg modified HSA/g silica and 53.5 (± 1.3) mg HSA/g silica, respectively. For the SIA method, the degree of immobilization for the modified HSA was 77% lower than it was for normal HSA, giving 2.9 (± 0.3) mg modified HSA/g silica versus 12.8 (± 0.5) mg HSA/g silica. This indicated that both the SMCC and SIA methods were fairly selective in reacting with sulfhydryl groups on HSA as opposed to other groups on this protein (e.g., amine residues), since the latter would not have been altered by modification with iodoacetamide.

Activity of immobilized HSA in the SMCC and SIA methods

Another item considered in this study was the amount of active HSA that was obtained by the SMCC and SIA methods. One way this was assessed was by comparing their binding for carbamazepine, a probe for the indole-benzodiazepine region of HSA.⁶⁹ Figure 5 shows the results of frontal analysis studies that were obtained with this drug on the HSA columns. As has been noted previously for HSA columns prepared by the Schiff base and NHS methods,^{69,70} linear behavior was seen for double-reciprocal plots obtained with the SMCC and SIA HSA columns (correlation coefficients, 0.996 to 0.999 for $n = 5$) under the experimental conditions that were used in this study.

It was possible from the best-fit lines in Figure 5 to estimate the association equilibrium constant and binding capacity for the major binding site of carbamazepine on HSA.^{2,69} The association equilibrium constant determined in this report at pH 7.4 and 25°C was $1.2 (\pm 0.2) \times 10^4 \text{ M}^{-1}$ for the binding of carbamazepine to HSA immobilized by the SMCC method and

$1.4 (\pm 0.6) \times 10^4 \text{ M}^{-1}$ for carbamazepine and HSA immobilized by the SIA method. These values agreed within one standard deviation with each other and with an association constant of $1.1 (\pm 0.2) \times 10^4 \text{ M}^{-1}$ that has previously been reported for the binding of carbamazepine with HSA immobilized by the Schiff base and NHS methods.⁶⁹

However, these different immobilization approaches did differ in terms of the amount of active HSA that was immobilized. For instance, the total binding capacity estimated for HSA in the SMCC method was $0.80 (\pm 0.11) \mu\text{mol carbamazepine/g silica}$, while the binding capacity for HSA immobilized by the SIA method was $0.25 (\pm 0.05) \mu\text{mol carbamazepine/g silica}$. This information was combined with the total amount of immobilized HSA in each column to give the relative activity of this protein for carbamazepine. This gave a relative activity of $87 (\pm 11)\%$ for HSA immobilized by the SMCC method and $81 (\pm 12)\%$ for HSA immobilized by the SIA method. In comparison, a relative activity of 55–56% has been reported for carbamazepine when using HSA immobilized by the Schiff base method^{69,70} and a relative activity of 37% has been noted for carbamazepine when using HSA immobilized by the NHS method.⁷⁰ These results indicated that both the SIA and SMCC methods gave immobilized proteins with a higher level of activity than common amine-based coupling methods. This is consistent with a model in which more site-selective attachment and fewer problems due to random orientation or multipoint attachment are present for HSA that has been immobilized by the SIA and SMCC methods.

Chromatographic behavior of HSA columns

Additional studies of the SIA and SMCC-prepared HSA columns were performed by using these to separate various chiral solutes: D/L-tryptophan, R/S-warfarin and R/S-ibuprofen. All of these solutes have been examined in previous chiral separations using HSA columns prepared by amine-based coupling methods (e.g., see Refs. 2, 44, 61, 62 and 74); however, these solutes bind to different regions of HSA. For instance, L-tryptophan is a probe for the indole-benzodiazepine region of HSA.^{44,71} The R- and S-enantiomers of warfarin both bind to the warfarin-azapropazone site of HSA.^{2,15,72} HSA is believed to have at least one common site for R- and S-ibuprofen,^{73–79} but S-ibuprofen may have at least one additional binding region on this protein.⁷⁹

Table 1 and Figure 6(a) show the results that were obtained for D/L-tryptophan. When using the SMCC immobilization method, greater than baseline separation was achieved for these analytes on an HSA column (resolution, $R_s = 5.8$; selectivity factor, $\alpha = 14.3$). The corresponding control column (with no HSA present) gave no chiral recognition and a retention factor of 0.49 for D- and L-tryptophan (i.e., 32% and 2%, respectively, of the retention factors noted for these same solutes on the SMCC HSA column). Figure 6(a) also shows some chromatograms for injections of D/L-tryptophan on an HSA column that was prepared by the SIA method. This SIA column gave a baseline separation for D- and L-tryptophan with a resolution of 1.8 and a selectivity factor of 3.83. The SIA control column gave a retention factor of 0.36 for D- and L-tryptophan (i.e., 63% and 16%, respectively, of the retention factors noted on the SIA HSA column). The retention factor due to non-specific binding between D/L-tryptophan and uncapped aminopropyl groups on the support was estimated to be 0.34, which made up roughly 69% and 94% of the retention measured on the SMCC and SIA control supports. However, it was found that this non-specific binding could be significantly reduced (e.g., a ~ 60% reduction for warfarin and 20% for carbamazepine) by capping the aminopropyl groups prior to HSA immobilization.

The results for the separation of R/S-warfarin on the various HSA columns are summarized in Table 2. As shown in Figure 6(b), an HSA column produced by the SMCC method gave a column with a baseline separation for R- and S-warfarin, as represented by a resolution of 1.81 and a selectivity factor of 1.66. The control SMCC column gave a retention factor of 1.55 for

R- and S-warfarin (i.e., 4.4% and 2.7%, respectively, of the total retention noted for R- and S-warfarin on the corresponding HSA column). Near baseline separation was also achieved for R/S-warfarin when using the SIA method for HSA immobilization (resolution, 1.42; selectivity factor, 1.53). In the SIA method, the retention factor for R- and S-warfarin on the control column was 1.42 (i.e., 19% and 13%, respectively, of the retention noted on the SIA HSA column). The retention factor due to non-specific binding between R/S-warfarin and aminopropyl groups on the support was approximately 1.36, making up 88% and 96% of the retention seen for R/S-warfarin on the SMCC and SIA control supports.

Table 3 shows the results obtained for R/S-ibuprofen. A baseline separation (resolution, 1.88; selectivity factor, 1.66) was found for R/S-ibuprofen on an HSA column prepared by the SMCC method. A control column for this same method gave a retention factor of 0.42 for these solutes (i.e., 2.4% and 1.4%, respectively, of the retention noted for S- and R-ibuprofen on the HSA column). R/S-Ibuprofen injected onto an HSA column prepared by the SIA method gave baseline separation with a resolution of 1.51 and a separation factor of 1.43; the control column in this method gave a retention factor of 0.38 (i.e., 9.4% and 6.6%, respectively, of the retention noted for S- and R-ibuprofen on the SIA HSA column). Non-specific binding between R/S-ibuprofen and aminopropyl groups on the support gave a retention factor of 0.35, or 83% and 92% of the retention seen for ibuprofen on the SMCC and SIA control supports.

For all three groups of these chiral solutes, HSA immobilized by the SMCC method gave a higher resolution and selectivity factor than HSA that had been immobilized by the SIA method. This greater separating power was mainly due to the higher retention factor that was noted for all solutes in HSA columns prepared by the SMCC method. Although the SMCC supports also gave a slightly higher degree of non-specific binding than the SIA method for all tested solutes, this difference was minor compared to the difference in overall retention seen in HSA columns made by these two techniques. One reason for the greater separating ability of SMCC-prepared columns is the larger amount of HSA that was covalently attached to silica in this method (i.e., 53.5 mg HSA/g silica versus 12.8 mg/g silica in the SIA method). However, this factor alone accounted for only about a four-fold difference in retention. This is short of the 4.9 to 11.5-fold differences in retention seen for D/L-tryptophan in HSA columns made by the SMCC and SIA methods (after correcting for non-specific binding), and the subsequent differences in retention of 4.8- to 5.4-fold for R/S-warfarin and 4.5- to 5.3-fold for R/S-ibuprofen in these same columns. This is believed to reflect differences in the activities of the SMCC and SIA immobilized HSA, as will be examined further in the following section.

Comparison with amine-based immobilization methods

The chiral separation results for both the SMCC and SIA methods were compared to those for an amine-based immobilization technique, as represented here by the Schiff base method, using the same starting support material and preparation of HSA. As shown in Table 1, the retention of D-tryptophan in the SMCC-prepared HSA column was about 1.8-times higher than the retention of this analyte on the HSA column made by the Schiff base method and 2.7-times higher than that of the HSA column made using the SIA method. The retention of L-tryptophan in SMCC-prepared HSA column was 2.5-times higher than that of the Schiff base column and 9.8-times higher than the SIA column.

These observed differences in retention for D- and L-tryptophan on the various HSA supports were much larger than those predicted just based on the total content of HSA in each column. For instance, the amount of protein on the SMCC support (53.5 mg/g silica) was only slightly higher than that of the Schiff base column (49.4 mg/g silica) and 4.1-fold higher than that of the SIA column (12.8 mg HSA/g silica). Thus, these measured differences in retention suggest that there were also differences in the binding capacities and/or relative activities of these immobilized HSA supports for D- and L-tryptophan. This conclusion was further supported

by the differences in selectivity factor for D/L-tryptophan in these HSA columns, where the SMCC-prepared HSA column gave a value that was 1.2-times higher than that for the Schiff base column and 3.7-times higher than that for the SIA column. Similarly, the resolution of D/L-tryptophan in SMCC column was 1.7-times higher than that for the Schiff base column and 3.2-times greater than that for the SIA column, although these latter differences could also reflect differences in the band-broadening properties of these materials.

Some of the same trends were seen for R- and S-warfarin (see Table 2). In this case, the retention for R-warfarin on the SMCC-prepared HSA column was 2.7-times greater than that of the Schiff base column and 4.7-times higher than that of the SIA column, while the retention of S-warfarin on the SMCC-prepared HSA column was 2.1- to 5.4-times higher than that of these other columns. However, the selectivity factor for R/S-warfarin on the SMCC column was now 1.2-fold lower than that for the Schiff base column and only 1.2-times higher than that of the SIA column. The resolution of R/S-warfarin on these columns gave the same type of pattern, with the SMCC and Schiff base columns having similar resolutions and the SIA column having a 1.2-fold lower value. This difference from the D/L-tryptophan results may be a result of the fact that different binding sites on HSA are involved in interacting with these two groups of analytes.^{1,19,44}

When R/S-ibuprofen was used as the analyte, the retention of S-ibuprofen in the SMCC-prepared HSA column was three-times higher than the retention seen for the Schiff base column and four-times higher than the retention measured on the SIA column (see Table 3). The retention of R-ibuprofen on SMCC column was three-times higher than on the Schiff base column and five-times higher than on the SIA column. The selectivity factors and resolutions for R/S-ibuprofen on the Schiff base and SMCC columns were similar and 1.2-times higher than that seen for the SIA column.

The results in Tables 1–3 were examined more closely by normalizing each retention factor for the protein content of the columns. This was accomplished by using the ratio of the retention factor for each solute versus the total amount of HSA in the column, as shown in Tables 1–3 by the term “ $k/(\mu\text{mol HSA})$ ”. This type of ratio has been shown previously to be useful in comparing the relative activity of affinity columns that contain different quantities of the same ligand.^{38,40} As indicated in Table 1, studies performed with D-tryptophan (i.e., an injected probe with weak retention) gave similar values for this ratio for HSA columns prepared by the Schiff base and SMCC methods, with a 1.7-times lower ratio being obtained by the SIA method. This indicated that HSA prepared by the Schiff base and SMCC method were slightly more active for D-tryptophan than HSA that had been immobilized by the SIA method. The SMCC results for L-tryptophan (i.e., an indole-benzodiazepine site probe with moderate retention) gave a ratio that was two- to four-times higher than the ratios for the Schiff base and SIA columns, respectively. This indicated that HSA immobilized by SMCC method was much more active for L-tryptophan than HSA that had been coupled by the other two methods. The different behavior noted here for L- versus D-tryptophan was not surprising since these two enantiomers have been proposed to have different binding regions on HSA,⁴⁴ These different regions, in turn, may have been subject to different immobilization effects in these coupling techniques, as has been suggested previously.⁴⁰

For R/S-warfarin (i.e., warfarin-azapropazone site probes with high retention), the SMCC method gave immobilized HSA with 1.6-times higher relative activity for R-warfarin than the SIA method, and the SIA method gave 1.4-times higher relative activity than the Schiff base method (see Table 2). For S-warfarin, SMCC method gave immobilized HSA with approximately 1.8-times higher activity than both the Schiff base and SIA methods (see Table 2). A similar trend was noted for R/S-ibuprofen (see Table 3). The higher activity of the SMCC versus SIA columns for L-tryptophan, warfarin and ibuprofen could be due, in part, to the

longer spacer arm that was present in the SMCC method (8.67 Å versus 1.50 Å in the SIA method). This latter feature may also explain some of the differences in non-specific binding that were noted in these methods.

The differences between the SMCC and SIA methods versus the Schiff base method are believed to be mainly due to the different types of functional groups on HSA that take part in these immobilization methods. For instance, an amine-based method like the Schiff base technique would involve coupling through the *N*-terminus of HSA or lysine residues, which make up approximately 10% of the amino acids in HSA (59 out of 585 residues in HSA). In contrast to this, there are only 35 cysteines in HSA (5.9% of all residues), with only one of these (Cys-34) normally being present in a free form (i.e., not as part of a disulfide bond).⁴² Due to the much lower abundance of free cysteine groups in HSA as well as in other proteins (e.g., the relative abundance of lysines in proteins is around 7% and for cysteines it is 2.8%),⁸⁰ it would be expected that the use of these sulfhydryl groups would result in an immobilized protein that has a more well-defined orientation and better activity than a protein which has been coupled through a more random or amine-based coupling method.

As noted earlier in this report, both the SMCC and SIA methods were found to react mainly through the free cysteine group of HSA, although some reaction with other side chains may also have been present. In the case of the SMCC method it was estimated that at least 81% of the HSA was immobilized through sulfhydryl groups (i.e., Cys-34), while the remaining 19% of HSA may have been immobilized through other nucleophilic residues. In the SIA method, it was determined that approximately 77% of HSA was immobilized through sulfhydryl groups and less than 23% through other residues. It was also noted that the immobilization of HSA through sulfhydryl groups significantly increased the activity of this protein compared to HSA prepared by the Schiff base method. For example, the relative activity of immobilized HSA for carbamazepine in the SMCC and SIA methods was 81–87%, while the relative activity of HSA for carbamazepine in the Schiff base method was only 55%.

Along with high activity, it is important in many studies to have low non-specific binding for an analyte on an affinity support. As shown in Tables 1–3, all analytes considered in this study gave the lowest degree of non-specific binding in the Schiff base method, with control columns that gave only 0.5–1.2% (for L-tryptophan, R/S-ibuprofen and R/S-warfarin) and 7.3% (for D-tryptophan) of the retention seen on the HSA columns. Non-specific binding by these same analytes was estimated to make up 1.4–4.7% (SMCC method) and 6.6–19% (SIA method) of the total retention noted on HSA columns for L-tryptophan, R/S-ibuprofen and R/S-warfarin and 32% (SMCC method) or 63% (SIA method) of the total retention measured for D-tryptophan.

A final factor considered in comparing these immobilization methods was operational stability of their final supports. This was examined by monitoring any change in the retention factors of L-tryptophan and R/S-warfarin after passing 250 mL of mobile phase (i.e., 385 column volumes) at 0.5 mL/min through columns containing these supports. The results indicated that the SMCC method gave immobilized HSA with the highest stability, with a decrease in retention of only 11–13% for all tested analytes. The second most stable material was SIA-prepared HSA support, which gave a 12–18% decrease in retention factors. The Schiff base method gave HSA supports that had the lowest stability, with a 15–48% decrease in retention being noted. This latter observation could be a result of the use of sodium borohydride (a strong reducing agent) during the Schiff base method, which might cause some reduction of disulfide bonds in HSA and some slow changes in tertiary structure.

CONCLUSIONS

This paper examined the preparation of maleimide- and iodoacetyl-activated silica by the SMCC and SIA methods for the immobilization of proteins, using HSA as a model. The resulting supports were characterized by such methods as frontal analysis, FTIR, elemental analysis and a papain-based assay. The specific activity of the immobilized HSA for carbamazepine was found to be 81–87% for the SMCC and SIA methods, which was much greater than for HSA that had been coupled to silica through an amine-based technique (i.e., 55% activity when using the Schiff base method). Furthermore, the selectivity of the SIA and SMCC methods for free sulfhydryl groups on a protein was estimated to be between 77 and 81%, based on results obtained using normal and iodoacetylated HSA.

HSA supports prepared by the SMCC, SIA and Schiff base methods were also compared in terms of their retention and ability to separate chiral substances such as D/L-tryptophan, R/S-warfarin and R/S-ibuprofen. It was found that the SMCC and SIA methods gave HSA supports with comparable or improved activity and stability versus HSA columns made by the Schiff base method, with the SMCC method giving the best overall behavior. These features should make the SMCC and SIA methods useful for work with other proteins or sulfhydryl-containing ligands and offer the potential of giving greater site-selective immobilization and activity than amine-based coupling methods. This makes these techniques attractive for the development of protein columns for such analytical applications as the study of biological interactions and chiral separations. It should also be possible to adapt these methods for use with alternative proteins and other materials based on silica or glass when it is desired to couple ligands to such supports through sulfhydryl groups.

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References

1. Hage DS, Austin J. *J Chromatogr B* 2000;739:39–54.
2. Hage DS. *J Chromatogr B* 2002;768:3–30.
3. Larive CK, Lunte SM, Zhong M, Perkins MD, Wilson GS, Gokulrangan G, Williams T, Afroz F, Schoeneich C, Derrick TS, Middaugh CR, Bogdanowich-Knipp S. *Anal Chem* 1999;71:389R–423R.
4. Lillehoj EP, Malik VS. *Adv Biochem Engin Biotech* 1989;40:19–71.
5. Chattopadhyay A, Tian T, Kortum L, Hage DS. *J Chromatogr B* 1998;715:183–190.
6. Liu Y, Zhao R, Shangguan D, Zhang H, Liu G. *J Chromatogr B* 2003;792:177–185.
7. Yang J, Hage DS. *J Chromatogr* 1993;645:241–250. [PubMed: 8408417]
8. Clonis YD. *J Chromatogr A* 2006;1101:1–24. [PubMed: 16242704]
9. Sun X, Chiu JF, He QY. *Expert Rev Proteomics* 2005;2:649–657. [PubMed: 16209645]
10. Voit R. *BIOspektrum* 2005;11:337–339.
11. Linhult M, Guelich S, Hober S. *Prot Peptide Lett* 2005;12:305–310.
12. Cuatrecasas P, Wilchek M. *Encyclopedia Biol Chem* 2004;1:51–56.
13. Schriemer DC. *Anal Chem* 2004;76:440A–448A.
14. Jungbauer A, Hahn R. *Curr Opin Drug Discov Develop* 2004;7:248–256.
15. Hage DS, Tweed SA. *J Chromatogr B* 1997;699:499–525.
16. Chaiken, IM., editor. *Analytical Affinity Chromatography*. CRC Press; Boca Raton: 1987.
17. Scouten, WH. *Affinity Chromatography: Bioselective Adsorption on Inert Matrices*. Wiley; New York: 1981.
18. Turkova, J. *Affinity Chromatography*. Elsevier; Amsterdam: 1978.

19. Loun B, Hage DS. *Anal Chem* 1996;68:1218–1225. [PubMed: 8651495]
20. Bjoerklund M, Hearn MTW. *J Chromatogr A* 1996;743:145–162. [PubMed: 8817878]
21. Hornsey VS, Prowse CV, Pepper DS. *J Immunol Methods* 1986;93:83–88. [PubMed: 3021857]
22. Stults NL, Asta LM, Lee YC. *Anal Biochem* 1989;180:114–119. [PubMed: 2554747]
23. Suda Y, Nakamura M, Koshida S, Kusumoto S, Sobel M. *J Bioactive Compatible Polymers* 2000;15:468–477.
24. Suzuki N, Quesenberry MS, Wang JK, Lee RT, Kobayashi K, Lee YC. *Anal Biochem* 1997;247:412–416. [PubMed: 9177706]
25. Thomas DH, Beck-Westermeyer M, Hage DS. *Anal Chem* 1994;66:3823–3829.
26. Frey T, Cosio EG, Ebel J. *Phytochem* 1993;32:543–550.
27. Burton SJ, Stead CV, Lowe CR. *J Chromatogr* 1990;508:109–125. [PubMed: 2380311]
28. Koyama T, Terauchi KI. *J Chromatogr B* 1996;679:31–40.
29. Potempa LA, Motie M, Anderson B, Klein E, Baurmeister U. *Clin Materials* 1992;11:105–117.
30. Sudi P, Dala E, Szajani B. *Appl Biochem Biotech* 1989;22:31–43.
31. Taylor RF. *Anal Chim Acta* 1985;172:241–248.
32. Kim, HS.; Hage, DS. *Handbook of Affinity Chromatography*. Hage, DS., editor. CRC Press; Boca Raton: 2005. p. 35-78.
33. Hermanson, GT. *Bioconjugate Techniques*. Academic Press; San Diego: 1995.
34. Berruex LG, Freitag R, Tennikova TB. *J Pharmaceut Biomed Anal* 2000;24:95–104.
35. Gupalova TV, Lojkina OV, Palagnuk VG, Totolian AA, Tennikova TB. *J Chromatogr A* 2002;949:185–193. [PubMed: 11999734]
36. Jiang T, Mallik R, Hage DS. *Anal Chem* 2005;77:2362–2372. [PubMed: 15828768]
37. Luo Q, Zou H, Zhang Q, Xiao X, Ni J. *Biotechnol Bioengin* 2002;80:481–489.
38. Mallik R, Jiang T, Hage DS. *Anal Chem* 2004;76:7013–7022. [PubMed: 15571354]
39. Ostryanina ND, Vlasov GP, Tennikova TB. *J Chromatogr A* 2002;949:163–171. [PubMed: 11999732]
40. Kim HS, Kye YS, Hage DS. *J Chromatogr A* 2004;1049:51–61. [PubMed: 15499917]
41. Kragh-Hansen U. *Pharmacol Rev* 1981;33:17–53. [PubMed: 7027277]
42. Peters, TJ. *All About Albumin: Biochemistry, Genetics and Medical Applications*. Academic Press; San Diego: 1995.
43. Sengupta A, Hage DS. *Anal Chem* 1999;71:3821–3827. [PubMed: 10489529]
44. Yang J, Hage DS. *J Chromatogr A* 1997;766:15–25. [PubMed: 9134727]
45. Hashida S, Imagawa M, Inoue S, Ruan KH, Ishikawa E. *J Appl Biochem* 1984;6:56–63. [PubMed: 6490581]
46. Partis MD, Griffiths DG, Roberts GC, Beechey RB. *J Protein Chem* 1983;2:263–277.
47. Rector ES, Schwenk RJ, Tse KS, Sehon AH, Chan H. *J Immunol Methods* 1978;24:321–336. [PubMed: 102705]
48. Thorpe PE, Ross WCJ, Brown ANF, Myers CD, Cumber AJ, Foxwell BMJ, Forrester JT. *Eur J Biochem* 1984;140:63–71. [PubMed: 6608447]
49. Yoshitake S, Imagawa M, Ishikawa E, Niitsu Y, Urushizaki I, Nishiura M, Kanazawa R, Kurosaki H, Tachibana S, et al. *J Biochem* 1982;92:1413–1424. [PubMed: 6759501]
50. Rezanian A, Johnson R, Lefkow AR, Healy KE. *Langmuir* 1999;15:6931–6939.
51. Tada T, Mano K, Yoshida E, Tanaka N, Kunugi S. *Bull Chem Soc Japan* 2002;75:2247–2251.
52. Tournier EJM, Wallach J, Blond P. *Anal Chim Acta* 1998;361:33–44.
53. Larsson PO. *Methods Enzymol* 1984;104:212. [PubMed: 6371445]
54. Brinkley M. *Bioconj Chem* 1992;3:2–13.
55. Domen PL, Nevens JR, Mallia AK, Hermanson GT, Klenk DC. *J Chromatogr* 1990;510:293–302. [PubMed: 2401701]
56. Singh R. *Bioconj Chem* 1994;5:348–351.
57. Singh R, Blaettler WA, Collinson AR. *Methods Enzymol* 1995;251:229–237. [PubMed: 7651201]

58. Singh R, Blattler WA, Collinson AR. *Anal Biochem* 1993;213:49–56. [PubMed: 8238881]
59. Rollag JG, Beck-Westermeyer M, Hage DS. *Anal Chem* 1996;68:3631–3637.
60. Williams ML, Wainer IW. *Therap Drug Monit* 2002;24:290–296. [PubMed: 11897974]
61. Patel, S.; Wainer, IW.; Lough, WJ. *Handbook of Affinity Chromatography*. Hage, DS., editor. CRC Press; Boca Raton: 2005. p. 571–594.
62. Allenmark, SG. *Chromatographic Enantioseparation Methods and Applications*. Ellis Horwood; Chichester, UK: 1988.
63. Hayball PJ, Holman JW, Nation RL. *J Chromatogr B* 1994;662:128–133.
64. Anderson DJ, Walters RR. *J Chromatogr Sci* 1984;22:353–359.
65. Pretsch, E.; Buehlmann, P.; Affolter, C. *Structure Determination of Organic Compounds: Tables of Spectral Data*. Springer; Berlin: 2003.
66. Jarret HW. *J Chromatogr* 1987;405:179–189.
67. Chen J, Ohnmacht C, Hage DS. *J Chromatogr B* 2004;809:137–145.
68. Wa C, Cerny R, Hage DS. *Anal Biochem* 2006;349:229–241. [PubMed: 16356458]
69. Kim HS, Hage DS. *J Chromatogr B* 2005;816:57–66.
70. Kim HS, Mallik R, Hage DS. *J Chromatogr B* 2006;837:138–146.
71. Muller WE, Wollert U. *Naunyn-Schmiedeberg's Arch Pharmacol* 1975;288:17–27.
72. Fehske KJ, Mueller WE. *Pharmacol* 1986;32:208–213.
73. Rahman MH, Yamasaki K, Shin YH, Lin CC, Otagiri M. *Biol Pharmaceut Bull* 1993;16:1169–1174.
74. Domenici E, Bertucci C, Salvadori P, Motellier S, Wainer IW. *Chirality* 1990;2
75. Watanabe H, Yamasaki K, Kragh-Hansen U, Tanase S, Harada K, Suenaga A, Otagiri M. *Pharmaceut Res* 2001;18:1775–1781.
76. Cheruvallath VK, Riley CM, Narayanan SR, Lindenbaum S, Perrin JH. *Pharmaceut Res* 1996;13:173–178.
77. Chen J, Fitos I, Hage DS. *Chirality* 2005;18:24–36. [PubMed: 16278829]
78. Kim HS, Austin J, Hage DS. *Electrophoresis* 2002;23:956–963. [PubMed: 11920883]
79. Hage DS, Noctor TAG, Wainer IW. *J Chromatogr A* 1995;693:23–32. [PubMed: 7697161]
80. Garrett, R.; Grisham, CM. *Biochemistry*. 2. Saunders; Fort Worth: 1999.
81. Smith, P. K.; (Pierce Chemical Co., USA). Application: US, 1989, 2 pp, Cont of U S Ser No 618,727, abandoned.
82. Walker, JM. *Protein Protocols Handbook*. 2. 2002. p. 11–14.
83. Zaia DAM, Zaia CTBV, Lichtig J. *Quimica Nova* 1998;21:787–793.
84. Walker JM. *Methods Mol Biol* 1994;32:5–8. [PubMed: 7951748]

(a) SMCC Method **(b) SIA Method** **Figure 1.**

Reactions involved in the (a) SMCC method and (b) SIA method, using aminopropyl silica as the starting material. Abbreviations: SMCC, N -(4-carboxycyclohexylmethyl) maleimide; SIA, succinimidyl iodoacetate; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; EDTA, ethylenediamine tetraacetic acid; KPB, potassium phosphate buffer.

(a) Production of Active Papain

(b) Detection of Active Papain

Figure 2.

Determination of the amount of active maleimide and iodoacetyl groups on silica by a papain-based assay. In the first step of (a) excess cysteine is added to the activated support and allowed to react. In the second step of (a), inactive papain is added to the reaction mixture, allowing conversion of papain to its active form as it undergoes a disulfide exchange reaction with residual cysteines. In (b), the active papain is measured by allowing it to convert its substrate L-BAPNA to the product *p*-nitroanilide. Absorbance of the product *p*-nitroanilide is then measured at 410 nm.

(a) Aminopropyl Silica **(b) Maleimide-Activated Silica (SMCC Method)** **(c) Iodoacetyl-Activated Silica (SIA Method)** **Figure 3.**

IR spectra of (a) aminopropyl silica (b) maleimide-activated silica (SMCC method) (c) iodoacetyl-activated silica (SIA method). See the text for assignment of the peaks.

Figure 4.

Amount of HSA immobilized at various reaction times and at 4°C when using silica that had been activated by the SMCC or SIA methods. In this study the original sample contained 0.15 g of activated silica plus 1.5 mL of a 15 mg/mL HSA solution in the immobilization buffer or 1.5 mL of the immobilization buffer with no protein present. After a given reaction time, a 20 μ L aliquot of this mixture was withdrawn and centrifuged, with the protein content of the supernatant then being measured by using a BCA protein assay.^{81–84} The final amount of protein on the maleimide-activated silica (SMCC method) or iodoacetyl-activated silica (SIA method) was determined using a reducing agent compatible BCA assay. These particular studies were performed using activated supports in which the aminopropyl groups had not been capped with acetic anhydride; the presence of these aminopropyl groups gave rise to the adsorption of 14.3 mg HSA/g silica after a reaction time of 52 h.

(a) SMCC-HSA column

(b) SIA-HSA column

Figure 5.

Frontal analysis studies for carbamazepine on HSA columns prepared by the (a) SMCC method or (b) SIA method. In the double-reciprocal plots shown at the bottom of this figure, m_{Lapp} is the apparent moles of solute (carbamazepine) that is required to reach the mean point of the breakthrough curve at a given concentration of the applied solute. The equations for the best-fit lines in these latter plots were as follows: (a) $Y = \{2.42 (\pm 0.01) \times 10^2\} X + 2.8 (\pm 0.3) \times 10^6$, with a correlation coefficient of 0.999 ($n = 5$); (b) $Y = \{6.8 (\pm 0.2) \times 10^2\} X + 1.1 (\pm 0.5) \times 10^7$, with a correlation coefficient of 0.996 ($n = 5$).

SMCC-HSA column

SIA-HSA column

(a) D/L-Tryptophan

(b) R/S-Warfarin

(c) R/S-Ibuprofen

Figure 6.

Chromatograms for injections of (a) D/L-tryptophan, (b) R/S-warfarin and (c) R/S-ibuprofen on HSA columns prepared by the SMCC and SIA methods. The conditions were as follows: sample concentration, 20 μ M tryptophan, ibuprofen, or warfarin; sample volume, 20 μ L; mobile phase for tryptophan, pH 7.4, 0.067 M potassium phosphate buffer; mobile phase for ibuprofen, pH 7.0, 0.067 M potassium phosphate buffer containing 8% isopropanol and 5 mM octanoic acid; mobile phase for warfarin, pH 7.0, 0.067 M potassium phosphate buffer containing 5% isopropanol and 1 mM octanoic acid; flow rate for SMCC-HSA column, 1.5 mL/min for tryptophan, 1.0 mL/min for ibuprofen and warfarin; flow rate for SIA-HSA

column, 0.3 mL/min for tryptophan and warfarin, 0.5 mL/min for ibuprofen; column size, 5 cm $\times$ 4.6 mm I.D.; temperature, 25°C.

Retention and Separation of D- and L-Tryptophan on HSA and Control Columns made by the SMCC, SIA and Schiff Base Methods^a

Table 1

Type of Column	Results for D-Tryptophan		Results for L-Tryptophan		Selectivity factor	Resolution
	Retention factor, k	k/($\mu\text{mol HSA}$) ^b	Retention factor, k	k/($\mu\text{mol HSA}$) ^b		
SMCC (HSA)	1.52 (± 0.02)	3.0 (± 0.2)	21.6 (± 0.4)	62 (± 2)	14.3 (± 0.1)	5.8 (± 0.1)
SIA (HSA)	0.57 (± 0.09)	1.8 (± 0.6)	2.20 (± 0.12)	16 (± 1)	3.83 (± 0.03)	1.8 (± 0.1)
Schiff Base (HSA)	0.82 (± 0.02)	2.9 (± 0.6)	8.65 (± 0.06)	31 (± 1)	11.9 (± 0.2)	3.5 (± 0.1)
SMCC (Control)	0.49 (± 0.01)	N/A	0.49 (± 0.01)	N/A	N/A	N/A
SIA (Control)	0.36 (± 0.01)	N/A	0.36 (± 0.01)	N/A	N/A	N/A
Schiff Base (Control)	0.06 (± 0.01)	N/A	0.06 (± 0.01)	N/A	N/A	N/A
Aminopropyl Silica	0.34 (± 0.01)	N/A	0.34 (± 0.01)	N/A	N/A	N/A

^aThe values in the parentheses represent a range of ± 1 S.D

^bThe retention factor used in calculating the ratio "k/($\mu\text{mol HSA}$)" is corrected for the nonspecific binding noted on the control support, where $k = (k_{\text{HSA}} - k_{\text{Control}})$. The other retention factors listed in this table are the overall values measured on each type of support.

Retention and Separation of R- and S-Warfarin on HSA and Control Columns made by the SMCC, SIA and Schiff Base Methods^a

Table 2

Type of Column	Results for R-Warfarin		Results for S-Warfarin		Selectivity factor	Resolution
	Retention Factor, k	k/($\mu\text{mol HSA}$) ^b	Retention Factor, k	k/($\mu\text{mol HSA}$) ^b		
SMCC (HSA)	35.1 (± 0.9)	98 (± 5)	56.8 (± 2.0)	160 (± 7)	1.66 (± 0.01)	1.81 (± 0.02)
SIA (HSA)	7.4 (± 0.3)	61 (± 3)	10.6 (± 0.4)	89 (± 4)	1.42 (± 0.01)	1.53 (± 0.03)
Schiff Base (HSA)	12.8 (± 0.3)	43 (± 5)	26.8 (± 0.4)	91 (± 10)	2.09 (± 0.04)	1.82 (± 0.03)
SMCC (Control)	1.55 (± 0.01)	N/A	1.55 (± 0.01)	N/A	N/A	N/A
SIA (Control)	1.42 (± 0.01)	N/A	1.42 (± 0.01)	N/A	N/A	N/A
Schiff Base (Control)	0.13 (± 0.01)	N/A	0.13 (± 0.01)	N/A	N/A	N/A
Aminopropyl Silica	1.36 (± 0.01)	N/A	1.36 (± 0.01)	N/A	N/A	N/A

^aThe values in the parentheses represent a range of ± 1 S.D

^bThe retention factor used in calculating the ratio "k/($\mu\text{mol HSA}$)" is corrected for the nonspecific binding noted on the control support, where $k = (k_{\text{HSA}} - k_{\text{Control}})$. The other retention factors listed in this table are the overall values measured on each type of support.

Retention and Separation of R- and S-Ibuprofen on HSA and Control Columns Made by the SMCC, SIA and Schiff Base Methods^a

Table 3

Type of Column	Results for S-Ibuprofen		Results for R-Ibuprofen		Selectivity factor	Resolution
	Retention Factor, k	k/($\mu\text{mol HSA}$) ^b	Retention Factor, k	k/($\mu\text{mol HSA}$) ^b		
SMCC (HSA)	17.0 (± 0.2)	48 (± 2)	28.2 (± 0.3)	81 (± 3)	1.66 (± 0.01)	1.88 (± 0.01)
SIA (HSA)	4.04 (± 0.13)	32 (± 1)	5.8 (± 0.3)	47 (± 3)	1.43 (± 0.03)	1.51 (± 0.06)
Schiff Base (HSA)	5.62 (± 0.02)	19 (± 2)	9.29 (± 0.06)	31 (± 4)	1.66 (± 0.01)	1.86 (± 0.03)
SMCC (Control)	0.42 (± 0.01)	N/A	0.42 (± 0.01)	N/A	N/A	N/A
SIA (Control)	0.38 (± 0.01)	N/A	0.38 (± 0.01)	N/A	N/A	N/A
Schiff Base (Control)	0.06 (± 0.01)	N/A	0.06 (± 0.01)	N/A	N/A	N/A
Aminopropyl Silica	0.35 (± 0.01)	N/A	0.35 (± 0.01)	N/A	N/A	N/A

^aThe values in the parentheses represent a range of ± 1 S.D

^bThe retention factor used in calculating the ratio "k/($\mu\text{mol HSA}$)" is corrected for the nonspecific binding noted on the control support, where $k = (k_{\text{HSA}} - k_{\text{Control}})$. The other retention factors listed in this table are the overall values measured on each type of support.