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Immunoaffinity Chromatography for Protein Purification and Analysis

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Abstract

Immunoaffinity chromatography (IAC) is a type of liquid chromatography that uses immobilized antibodies or related binding agents as selective stationary phases for sample separation or analysis. The strong binding and high selectivity of antibodies have made IAC a popular tool for the purification and analysis of many chemicals and biochemicals, including proteins. The basic principles of IAC are described as related to the use of this method for protein purification and analysis. The main factors to consider in this technique are also presented, under a discussion of the general strategy to follow during the development of a new IAC method. Protocols, as illustrated using human serum albumin (HSA) as a model protein, are provided for the use of IAC in several formats. This will include both the use of IAC with traditional low-performance supports such as agarose, for off-line immunoextraction, and supports used in high-performance immunoaffinity chromatography (HPIAC), for on-line immunoextraction. The use of IAC for protein analysis as a flow-based or chromatographic immunoassay is also discussed and described, using HSA and a competitive binding assay format as an example.

Basic Protocol 1: Protocol for off-line immunoextraction by traditional IAC

Basic Protocol 2: Protocol for on-line immunoextraction by HPIAC

Basic Protocol 3: Protocol for competitive binding chromatographic immunoassay

Keywords: immunoaffinity chromatography; immunoextraction; protein purification; antibodies; chromatographic immunoassay

INTRODUCTION

Biological samples are often complex mixtures and can present a challenge when the goal is to purify a specific chemical or biochemical, such as a particular protein, in this type of matrix. The use of antibodies as binding agents can be valuable in this situation for purification or analysis due to their high affinity and selectivity towards their given target or antigen. Immunoaffinity chromatography (IAC) is a type of affinity chromatography and liquid chromatography in which an antibody or antibody-related binding agent is used as a stationary phase for target isolation or analysis (Moser & Hage, 2010). This method has been used as a purification tool for many years in biochemistry and medicinal chemistry (Beyer et al., 2009; Fitzgerald et al., 2016; Moser & Hage, 2010). IAC has also seen growing use over the last few decades as a method for the analysis of specific proteins and other targets in a wide variety of samples (Moser & Hage, 2010).

A key component in IAC is the antibody or related agent that is used as the stationary phase for binding to the desired target (Moser & Hage, 2010). Antibodies are glycoproteins that are produced by the body in response to foreign agents, or antigens (Hage, 1998; Hage & Phillips, 2006; Moser & Hage, 2010). A typical structure of an antibody is shown in Figure 1, using immunoglobin G (IgG) as an example. An IgG-class antibody consists of four polypeptide chains. Two of these polypeptides are identical heavy chains, and the other two are identical light chains. The four chains are linked through disulfide bonds to give a Y- or T-shaped structure. The lower portion of an antibody is referred to as the F_c region; this region is highly conserved from one type of antibody to the next (Hage, 1998; Hage & Phillips, 2006; Moser & Hage, 2010). Each of the upper arms of an antibody is known as a F_{ab} region. The two F_{ab} regions in an IgG-class antibody are identical to one another in antibodies produced by the same immune cell line and are the locations of the binding sites for the antibody with its target (Hage, 1998; Hage & Phillips, 2006; Moser &

Hage, 2010). The selectivity and binding strength for a target with a given antibody are determined by the amino acid sequence and structure of each F_{ab} region. It is through this region that an antibody can undergo multiple non-covalent interactions with its target, resulting in typical association equilibrium constants on the order of 10^5-10^{12} M^{-1} (Hage, 1998; Hage & Phillips, 2006; Moser & Hage, 2010).

The portion of an antigen that is recognized and bound to a specific type of antibody is known as the epitope (Hage & Phillips, 2006). Large antigens like proteins, bacteria, and viral particles can have many epitopes and are able to produce an immune response in an animal to which they are a foreign agent. Although low mass compounds do not typically illicit an immune response on their own, it is possible to produce antibodies against a smaller molecule by first attaching this target to a larger carrier agent, such as a protein. In these cases, the small molecule that is bound to the larger carrier and that is used to produce antibodies is known as a hapten (Hage & Phillips, 2006). Antibodies can also be modified to form fragments that can be used in IAC or other applications. Examples include the production of F_{ab} fragments by the use of chemical reagents such as dithiothreitol and diethanolamine or some enzymes to cleave specific bonds in antibodies and generate these smaller fragments (Davis & Smith, 2006; Gergely et al., 1967; Hage & Phillips, 2006; Noelken et al., 1965).

Figure 2 shows the most common format by which antibodies or related binding agents are used with IAC to carry out a separation or analysis of proteins and other targets (Hage, 1998; Hage & Phillips, 2006; Moser & Hage, 2010). First, a column is prepared that contains antibodies or related ligands that are immobilized or adsorbed onto a solid support. A sample containing targets that can bind to these ligands is then applied to this column under conditions in which strong association is obtained. This strong association is achieved by using an application buffer that is

at or near a physiological pH (i.e., pH 7-7.4). As the sample is applied, the high specificity of the IAC column causes the desired analytes to be bound while other (i.e., weakly retained or non-retained) chemicals are washed through the column. A second buffer is later applied to the IAC column to elute the retained analyte. This elution can typically be obtained by a change in pH of the buffer, although the use of a change in polarity, temperature, or addition of denaturation or competing agents may also be utilized in some cases (Hage & Phillips, 2006). As they elute from the column, the target analytes can be collected for further use and analysis or monitored directly by an on-line detector. The IAC column can then be placed back into the presence of the initial application buffer to allow the antibody-based ligands to regenerate prior to use of the column with another sample (Moser & Hage, 2010).

Several formats can be used with IAC for target detection and analysis (Moser & Hage, 2010). The simplest approach is to capture and elute a target analyte, followed by passage through an on-line detector or collection for further use and off-line detection. In this format, IAC is also referred to as immunoextraction or immunoaffinity extraction (Jackson, Sobansky, & Hage, 2012; Pfaunmiller et al., 2015). Immunoextraction is popular for protein purification because of its ease-of-use and simplicity, as it can often be done in only one or two steps. Another advantage is that direct detection can be used for either a specific target, as is accomplished through use of a highly specific antibody, or for detection of a broader class of compounds, by using a more general group-specific antibody for these targets.

Immunoextraction can be carried out either off-line or on-line with a second analytical method (Hage, 1998). Examples of on-line and off-line immunoextraction are provided in Basic Protocols 1 and 2, respectively. The choice between these two options will depend on the overall goal of the analysis and compatibility of the immunoextraction step with the second analysis method. For

example, when IAC is coupled with gas chromatography (GC), off-line extraction is often selected because IAC often uses aqueous buffers while GC needs a more volatile matrix. In this situation, the IAC column acts as a solid-phase extraction (SPE) cartridge. If needed, a solute fraction that is isolated and collected by off-line immunoextraction can also be easily derivatized before it is examined and used by another technique. On the other hand, when IAC is coupled with reversed-phase liquid chromatography (RPLC), an on-line method may be preferred because of the aqueous mobile phases of IAC act as a weak mobile phase for RPLC, allowing effective target concentration and recapture as it elutes from the IAC column. In addition, the use of on-line immunoextraction often allows for higher precision and greater ease of automation than off-line methods. In either case, the use of immunoextraction gives higher selectivity and less interference than traditional SPE, in which analytes are often isolated based primarily on their polarity or charge (Hage, 1998; Moser & Hage, 2010).

Two other forms of IAC and immunoextraction are immunodepletion and ultrafast immunoextraction. In immunodepletion (or immunoaffinity depletion), one or more antibodies are used in an IAC column to remove specific, and often abundant, components from a complex sample; this method makes it easier to then analyze the remaining minor sample components by a second method (Moser & Hage, 2010; Qian et al., 2008). This method has been mostly used in proteomics to examine low-abundance proteins in samples such as serum, plasma or cerebrospinal fluid (Selvaraju & El Rassi, 2012; Zolotarjova et al., 2005); however, immunodepletion has been also used in quantitative studies of a specific low-abundance target (Liu et al., 2012). Ultrafast immunoextraction is a special type of IAC that is carried out with microscale columns and used to bind a target compound on the time scale of a few seconds or less (Clarke et al., 2001, 2005; Jiang et al., 2005). Ultrafast immunoextraction has been used as an analytical tool for the rapid capture

of drugs and hormones and to measure the free, non-bound forms of these compounds in samples such as serum or plasma (Clarke et al., 2001, 2005; Ohnmacht et al., 2006; Schiel et al., 2011).

Indirect detection of a target in IAC can be achieved by either observing how the target interacts with a labeled binding agent or by looking at how this target prevents a labeled analog of the same compound from interacting with antibodies in an IAC system (Hage, 1998; Jackson et al., 2012; Pfaunmiller et al., 2015). The use of indirect detection in IAC is often known as a chromatographic immunoassay or flow injection immunoassay (Hage, 1998; Matsuda et al., 2015a,b; Moser & Hage, 2006, 2010). This technique has been used not only with proteins and peptides, but also for detecting bacteria, drugs, hormones, and herbicides (Delaunay-Bertoncini & Hennion, 2004; Frutos & Regnier, 1993; Hage & Nelson, 2001; Hage & Phillips, 2006; Jackson et al., 2012; Lua & Chou, 2002; Moser & Hage, 2006, 2010; Weller, 2000). One common format for a chromatographic immunoassay is a competitive binding mode, as will be illustrated under Basic Protocol 3. Other formats that may also be used are a non-competitive binding immunoassay, one-site binding immunometric assay, homogeneous immunoassay, and sandwich immunoassay (Moser & Hage, 2006).

IAC has traditionally been carried out with low-performance and non-rigid supports such as agarose for biomolecule purification (Jackson et al., 2012; Pfaunmiller et al., 2015). When IAC is combined with more rigid and efficient supports that are compatible with high-performance liquid chromatography (HPLC), this results in a method known as high-performance immunoaffinity chromatography (HPIAC). HPIAC can allow measurements with precisions in the range of 1%-5% to be obtained in as little as a few minutes. Thus, HPIAC provides greater speed, precision, and ease of automation than traditional IAC, making it more suitable for analytical applications (Jackson et al., 2012; Pfaunmiller et al., 2015). An example of a low-performance IAC method is

provided in Basic Protocol 1. Examples of IAC methods that are based on HPIAC and instead use more rigid and efficient support materials are given in Basic Protocols 2 and 3. In addition, methods by which antibodies can be attached or adsorbed to such supports for IAC are provided in Basic Protocols 2 and 3. All the protocols will be illustrated by using human serum albumin (HSA) as a representative protein. However, the same general approaches and formats can also be adapted for use in the isolation, detection, or analysis of other proteins or other types of targets.

NOTE: All work with biological samples in the preparation or use of IAC should be conducted using sterile micropipette tips, sterile microcentrifuge tubes, and nuclease-free water. Appropriate protective eyewear, gloves, and labware should also be worn in work with any samples or materials that may potentially contain substances that are chemical or biological hazards. Work with potentially infectious samples should be carried out using appropriate facilities (e.g., biosafety level-2 facilities for work with clinical samples that may contain bloodborne pathogens).

STRATEGIC PLANNING

There are several factors to consider in the creation of a new method for immunoaffinity chromatography or immunoextraction. One key item needed is a flow-compatible support that has the antibodies or related agents attached to it in a way that does not significantly alter their binding activity or accessibility to a target (Kim & Hage, 2006). The supports used in traditional IAC are usually carbohydrate-based materials like agarose or organic polymers like polyacrylamide beads (Gustavsson & Larsson, 2006). These materials often must be employed at low pressures, such as in the presence of gravity-induced flow or peristaltic flow, due to their limited mechanical stability (Hage, 1998). However, these supports tend to be inexpensive and are often compatible with a wide range of pH values, which makes them popular for applications such as purification or sample pretreatment by IACs (Gustavsson & Larsson, 2006; Hage & Phillips, 2006).

The alternative to these low-performance materials is to use more rigid and efficient supports, as employed in HPIAC (Gustavsson & Larsson, 2006; Hage, 1998; Matsuda et al., 2015b). Examples of these materials are derivatized silica, glass, azlactone beads, methacrylate polymeric supports, monoliths, and polystyrene based perfusion media (Gustavsson & Larsson, 2006; Jackson et al., 2012; Kim & Hage, 2006). For these materials to be used in IAC, they need to have low non-specific binding and be present in a form that can be easily used or modified for antibody attachment (Fitzgerald et al., 2016; Kim & Hage, 2006; Pfaunmiller et al., 2015). Because these materials can be used in HPLC systems, they provide IAC methods that are easier to automate and that have faster separation times and sharper peaks than traditional IAC. An issue with materials like silica is their range of pH stability is not as large as it is for agarose and other carbohydrate-based supports. For both traditional and HPIAC supports, items such as the degree of non-specific binding, surface area, pore size, and overall accessibility of the target to the immobilized antibodies/agents should also be considered during the development of a new IAC method (Gustavsson & Larsson, 2006; Kim & Hage, 2006).

Many techniques are available for coupling antibodies and related agents to supports for use in IAC. This is another key factor to consider in the creation of a new IAC method, such as for protein purification or analysis. Figure 3 shows several strategies for placing antibodies onto a support for IAC. Immobilization via covalent attachment is the most common approach utilized for this purpose. An example of this approach is given in Basic Protocol 2. The use of free amine groups on an antibody is one of the easiest routes for attaching antibodies to a solid support; this can be accomplished through such techniques as the cyanogen bromide (CNBr) method, the carbonyldiimidazole (CDI) method, or the Schiff base (reductive amination) method, to name a few (Kim & Hage, 2006). However, the presence of many amine groups on each antibody can lead

to some random orientation, steric hindrance, or denaturation and actual or apparent loss of activity of antibodies that are immobilized through these residues (Kim & Hage, 2006; Kortt et al., 1997).

Antibodies can also be coupled to supports through more site-selective methods, as is shown in Figure 3. For instance, F_{ab} fragments can be released from an antibody and the free sulfhydryl groups created on these fragments that can be utilized for immobilization (Hage & Phillips, 2006; Jackson et al., 2012; Kim & Hage, 2006; Pfaunmiller et al., 2015). Antibodies can also be selectively immobilized through the carbohydrate residues that are present in their F_c regions (Hage, 2000; Keener et al., 1994; Kim & Hage, 2006). This may be accomplished by converting the carbohydrate residues of antibodies into aldehyde groups through mild oxidation and then and allowing these groups to couple with a hydrazide- or amine-containing support (Jackson et al., 2012; Pfaunmiller et al., 2015; Ruhn et al., 1994). This latter method is also described in the procedure given in Basic Protocol 3.

It is further possible to non-covalently immobilize an antibody by adsorbing it to a secondary ligand that is covalently coupled to the support (Hage & Phillips, 2006). The placement of biotin as a tag on the antibodies and the use of this tag for binding to immobilized avidin or streptavidin is one way to achieve this type of immobilization (Kim & Hage, 2006). The biotin-avidin/streptavidin linkage can withstand many elution conditions; however, the coupling of biotin to antibodies through residues such as amines can again lead to a possible loss of antibody activity (O'Shannessy & Quarles, 1987). An alternative strategy is to adsorb antibodies through immobilized agents such as protein A and protein G, which can both bind the F_c regions of many types of antibodies (Hage & Phillips, 2006; Moser & Hage, 2010). The antibodies that are adsorbed to protein A and G can be later released at a mildly acidic pH, which can be useful when high antibody activity is desired or frequent replacement of adsorbed antibodies is required. The

disadvantage of this periodic replacement is it can require much more antibody for IAC than when using covalent coupling methods (Hage & Phillips, 2006; Kim & Hage, 2006).

The application and elution conditions are another group of factors to consider in the design and implementation of an IAC method (Hage & Phillips, 2006; Hage, Xuan, & Nelson, 2006). The application buffer used in IAC should ideally allow strong, selective, and fast binding of the desired targets with the immobilized antibodies. The strongest binding for most natural antibodies with their targets occurs under physiological conditions and in the presence of a neutral pH buffer (i.e., pH 7.0-7.4). The strong binding that is present under these conditions means that the bound target should have little elution in the application buffer unless only low-to-moderate affinity antibodies are present (e.g., with an association equilibrium constant of 10^5 M^{-1} or less) (Hage et al., 2006).

The elution buffer for IAC should be chosen to allow fast and effective elution of the retained target while providing conditions that are gentle enough to allow the later regeneration of the immobilized antibodies (Hage et al., 2006). The need for fast but reversible binding and regeneration is especially important when an IAC column is to be used repeatedly for a large number of samples, as may occur in HPIAC. Elution in IAC is generally carried out by temporarily lowering the effective strength of antibody binding to the target by changing the mobile phase pH or adding a chaotropic agent to the mobile phase. Other less common methods of elution in IAC elution may include adding a competitive agent, organic modifier or denaturing agent to the mobile phase, or changing the column temperature during elution (O'Shannessy & Quarles, 1987). In most cases, the elution buffer requires the use of a step gradient, although both linear and non-linear gradients for elution in IAC have been used as well (Hage, 1998; Hage & Phillips, 2006; Matsuda, et al., 2015).

BASIC PROTOCOL 1

PROTOCOL FOR OFF-LINE IMMUNOEXTRACTION BY TRADITIONAL IAC

Off-line immunoextraction is the most common form of IAC that is used for the purification of proteins and other biological targets (Hage & Phillips, 2006; Moser & Hage, 2010). This method is usually carried out by injecting or combining a sample with an IAC support under conditions that promote strong and specific binding between the solutes and immobilized ligands, as is illustrated by the on/off elution scheme in Figure 2 (Hage, 1998; Hage & Phillips, 2006; Moser & Hage, 2010). After sample components besides the target have been washed away, the IAC column or support is placed in contact with an elution buffer that will weaken the extent of binding between the target and IAC support, allowing the target to be released for collection. The collected target can then be used in the desired final application or further processed and analyzed by a second method such as HPLC, gas chromatography (GC), or an enzyme linked immunosorbent assay (ELISA) (Hage, 1998; Moser & Hage, 2010).

In this protocol, we describe the use of off-line immunoextraction for proteins using as an example the capture of HSA and glycated HSA from clinical samples using polyclonal anti-HSA antibodies attached to cross-linked agarose beads (Anguizola et al., 2013). The overall scheme for this application is summarized in Figure 4. The HSA or modified HSA that has been isolated by this approach has also been used to prepare affinity microcolumns to study the binding of drugs with these purified proteins. These HSA affinity microcolumns have been utilized to characterize the overall binding of sulfonylurea drugs with these proteins and to determine the effects of *in vivo* protein glycation on these interactions (Anguizola et al., 2013). The same general protocol that was developed for this specific application can be applied for the isolation or purification of other proteins by using suitable antibodies and IAC supports that are selective for these targets.

Materials

Sample containing target of interest (e.g., commercial serum sample made from male AB plasma obtained from Sigma-Aldrich A, product H4522, lot 039K0728; prescreened and found to be negative for HIV-1/HIV-2, hepatitis B and hepatitis C)

Application buffer for IAC column (e.g., pH 7.4, 0.10 M Tris buffer, prepared as described under Reagents and solutions)

Elution buffer for IAC column (e.g., pH 2.8, 0.10 M glycine/HCl buffer, prepared as described under Reagents and solutions)

Buffer to neutralize eluted target (e.g., pH 8.0, 0.10 M Tris buffer, prepared as described under Reagents and solutions)

Deionized and purified water suitable for use in preparing buffers and biological reagents (e.g., water from a Nanopure system (Barnstead, Dubuque, IA) or a Milli-Q Advantage A10 system (Millipore, Billerica, MA)

0.2 μm GNWP nylon filters for buffers (Millipore, Billerica, MA)

Ultrafiltration spin column (e.g., VivaSpin 6 ultrafiltration spin column, containing a polyclonal anti-HSA resin, 30 kDa, Sartorius Stedim Biotech, Göttingen, Germany)

System for mixing sample and application buffer with IAC support (e.g., rotary mixer or Labquake Shaker, Labindustries, Berkley, CA)

Centrifuge (5702RH temperature-controlled centrifuge from Eppendorf, New York, NY, with a fixed-angle centrifuge rotor from VWR, West Chester, PA)

Device to place isolated target into appropriate storage buffer (e.g., Dialysis Slide-A-Lyzer dialysis cassette, 10 kDa MW cutoff, 0.1-0.5 ml sample volume from Thermo Scientific, Rockford, IL).

Lyophilization system for preparation of isolated protein for long-term storage (e.g., FreeZone 1 Liter Benchtop Freeze Dry System from Labconco (Kansas City, MO).

Procedure for off-line immunoextraction of HSA and glycated HSA

1. Add 20 µL of the desired sample (e.g., human plasma or serum) to a Vivaspin 6 spin-filter column containing a fresh 400 µL portion of a 50% packed resin slurry with polyclonal anti-HSA antibody fragments immobilized onto cross-linked agarose beads.

   Note: This slurry is provided by the manufacturer in a proprietary pH 7.0- 7.5 Tris buffer with a low salt concentration, which acts as both the storage buffer and application buffer in the example.
2. Incubate the sample/IAC support mixture at room temperature for 15 min, such as by using a rotary mixer or similar device.
3. Centrifuge the spin-filter column containing the sample and IAC support at 400 × g for 2 min.
4. Wash the spin-filter column with 200 µL of pH 7.4, 0.10 M Tris buffer for 1 min at 400 × g.
5. Wash the spin-filter column two more times as described in Step 4 to further remove any non-retained sample components form the IAC support.
6. Wash the spin-filter column six times with 200 µL of pH 2.8, 0.10 M glycine/HCl as an elution buffer for 2 min at 400 × g and collect the target (i.e., HSA/glycated HSA, in this case) that was retained by the IAC support.
7. Adjust the pH of the collected fractions to ~pH 7.0 by slowly adding pH 8.0, 0.10 M Tris buffer.

   Note: Steps 1-7 can be repeated, as needed, with fresh portions of the IAC support to collect additional amounts of the target for the desired application or other analysis steps. Although this current procedure uses a fresh portion of the IAC support for each purification cycle, it

may be possible to regenerate and reuse the IAC support if the target is effectively removed from this material and the immobilized antibodies have a sufficient level of stability and binding after regeneration in the initial application buffer (Hage et al., 1993).

8. Use dialysis or a related method to place the collected target into an alternative buffer or solution for long term storage. In this example, the collected fractions of HSA or glycated HSA (i.e., the protein targets) that were now in pH 7.0, 0.10 M Tris buffer were next placed into a sterile Slide-A-Lyzer dialysis cassette (10 kDa MW cutoff, 0.1-0.5 ml sample volume).

   Note: An alternative procedure that can be used with the captured target from Steps 1-7 is to dialyze this target against an appropriate buffer for the final application and then to use this target solution directly in the application (e.g., for the immobilization of HSA or glycated HSA, in this specific example).

9. Place the protein target solutions in the dialysis cassettes in contact with two fresh portions of 2.5 L water (two times) while gently stirring for 2 h at room temperature.
10. Place the dialysis cassettes for a third time into contact with 2.5 L water, but now with stirring and with the dialysis being allowed to proceed at 4 °C for 14-18 h.
11. After dialysis, lyophilize the target solution and then store at -80 °C until use.

BASIC PROTOCOL 2

PROTOCOL FOR ON-LINE IMMUNOEXTRACTION BY HPIAC

On-line immunoextraction is an attractive alternative to off-line immunoextraction if the goal is to automate the overall method or to quickly isolate the desired target with a high level of precision (Hage 1998; Matsuda, Jobe, et al., 2015; Rodriguez et al., 2020). To obtain these properties, on-line immunoextraction is often done by using HPIAC and supports that are compatible with

modern high-performance liquid separation systems. An HPIAC column that is used for on-line immunoextraction can be directly coupled with many other types of HPLC columns, including those utilized in reverse-phase liquid chromatography, ion-exchange chromatography, and size exclusion. In addition, on-line immunoextraction can be combined with a variety of detection modes, such as absorbance or fluorescence detection and mass spectrometry (Hage, 1998; Hage & Phillips, 2006; Moser & Hage, 2010).

To illustrate this approach, this protocol describes the creation and use of HPIAC for the on-line immunoextraction of HSA from human serum. This example uses porous HPLC-grade silica as the support to which antibodies are attached for use in the immunoextraction process. As an alternative, other supports may also be used for this type of application, such as polymeric monolith (Jiang et al., 2005; Potter & Hilder, 2008). The antibodies in this example are attached to silica in a diol-bonded form that is then used with reductive amination, or the Schiff base method, to immobilize antibodies through amine groups (see reaction scheme in Figure 5). A description is also provided on how polyclonal antibodies that are suitable for use in making an HPIAC column can be isolated from antiserum. HPIAC columns that are made in the matter described here have been used to not only capture HSA from serum but to also measure this protein as part of a clinical sample, to use this captured protein for on-line drug binding studies, or to employ the captured protein for non-covalent immobilization (Hage & Walters, 1987; Rodriguez et al., 2020; Rodriguez et al., 2021; Ruhn et al., 1994).

Materials

Antibodies which bind to the target (e.g., polyclonal anti-HSA antibodies from goat serum, Sigma-Aldrich, Cat. no. A1151)

Target of interest (e.g., HSA, essentially fatty acid free)

Support suitable for HPIAC (e.g., Nucleosil Si-300 and Nucleosil Si-1000 silica from Macherey-Nagel (Düren, Germany), each with a particle diameter of 7 μm and pore sizes of 300 or 1000 Å, respectively, either obtained or previously converted into a diol-bonded form) (Kim & Hage, 2006).

Application buffer for HPIAC column (e.g., pH 7.4, 0.067 M potassium phosphate buffer, prepared as described under Reagents and solutions)

Elution buffer for HPIAC column (e.g., pH 2.5, 0.10 M potassium phosphate buffer, prepared as described under Reagents and solutions)

Buffer to neutralize or adjust the pH of the eluted target (e.g., pH 8.0, 0.10 M potassium phosphate buffer, prepared as described under Reagents and solutions)

Periodic acid (H_5IO_6) (CAS number 10450-60-9)

90% Acetic acid solution

Buffers for use in immobilization method (e.g., pH 6.0, 0.10 M potassium phosphate buffer for use in the Schiff base method)

Deionized and purified water suitable for use in preparing buffers and biological reagents (e.g., water from a Nanopure system (Barnstead, Dubuque, IA) or a Milli-Q Advantage A10 system (Millipore, Billerica, MA)

Sodium cyanoborohydride (CAS number 25895-60-7)

Sodium borohydride (CAS number 16940-66-2)

Reagents for protein assay (e.g., micro bicinchoninic acid, or micro BCA, assay, from Pierce, Rockford, IL)

Standard for protein assay (e.g., goat immunoglobulin G, or goat IgG)

0.2 μm GNWP nylon filters for buffers (Millipore, Billerica, MA)

Rotary mixer (e.g., Thermolyne Labquake Rotisserie Shaker 400110, Barnstead, Dubuque, IA)

Temperature-controlled centrifuge (e.g., 5702 RH Centrifuge, Eppendorf, Hamburg, Germany)

Dual Range Microbalance (Mettler Toledo XS105, Columbus, OH)

50 mL Centrifuge tube (Fisher Scientific, Marietta, OH) or equivalent

Vortex mixer (Vortex Genie 2, Fisher Scientific, Marietta, OH)

Sonicator equipped with aspirator line (e.g., Branson 5510, Cleaning Bath, Branson Ultrasonics, Brookfield, CT)

Parafilm (PM 996, Fisher Scientific, Marietta, OH) or equivalent

Aluminum foil (Fisher Scientific, Marietta, OH) or equivalent

Wrist action shaker (Wrist Action Model 95, Burrell Scientific, Pittsburgh, PA)

Centrifuge (Sorvall ST8 from ThermoScientific, Waltham, MA)

Stirring hot plate (Corning, PC-420D, Corning, NY)

Components to assemble an HPLC column (e.g., XPERTEK column tube, ¼ ” × 2.1 mm × 25 cm SS, cut to desired length, and two XPERTEK HPLC column end fittings, ¼” × 2.1 mm with 2 μm frits, from P.J. Cobert Associates, St. Louis, MO)

Column packer (e.g., Prep 24 pump system, ChromTech, Apple Valley, MN)

Drying oven (e.g., Isotemp Oven, Fisher Scientific, Marietta, OH)

Temperature-controlled water bath (e.g., Isotemp 210 Digital Water Bath, Fisher Scientific, Dubuque, IA)

UV/Vis absorbance detector and cuvettes (1 cm pathlength) (e.g., V-630 Spectrophotometer, JASCO, Tokyo, Japan)

HPLC system (e.g., this example uses a system from Jasco (Easton, MD) that consists of two PU2080 pumps, an AS-2057 autosampler, a CO-2067 column oven, a DG-2080-53 degasser,

and an UV-2075 Plus absorbance detector equipped with LCNET II/ADC Plus from Jasco and a six-port valve from Rheodyne, Cotati, CA)

Purification of polyclonal anti-HSA antibodies from antiserum

1. Dissolve a 200 mg portion of anti-HSA antiserum, as obtained or prepared in a lyophilized form, in 2.0 ml of pH 7.4, 0.067 M potassium phosphate buffer.
2. Add 0.50 g of an HSA support (in this example, 7 μm particle, 300 Å pore size silica containing HSA immobilized by the Schiff base method) to the anti-HSA antiserum solution and allow this slurry to mix at room temperature for 2 h by using a rotary mixer or similar mixing method. In general, the binding strength of an antibody for its target tends to decrease with an increase in temperature and until either the antibody or target undergoes denaturation. The degree of binding between a target and antibody is also expected to gradually increase over time and to approach a maximum as the system reaches equilibrium.

   Note: The HSA silica used here is prepared in the same general manner as described in this protocol for attaching anti-HSA antibodies to diol-bonded silica by the Schiff base method. However, other immobilization methods and supports containing an immobilized form of the final desired target can also be employed.
3. Centrifuge the slurry at 7500 rpm (8220 × *g*) for 3 min at room temperature to separate captured anti-HSA antibodies on the HSA support from the other components in the antiserum. Decant off the solution in the centrifuge tube. Wash the support three times with pH 7.4, 0.067 M potassium phosphate buffer and repeat the centrifugation and decanting steps.
4. Wash the support three times with 1 ml portions of pH 2.5, 0.10 M potassium phosphate buffer, followed by the same type of centrifugation and decanting steps as used in Step 3.

However, in this step now carefully collect all decanted supernatant, which will contain anti-HSA antibodies that have been released from the HSA support.

5. If the antibodies are to be used immediately for immobilization, adjust the pH for the collected and combined anti-HSA antibody fractions to the desired pH for this process (i.e., pH 6.0 for the Schiff base method) by slowly adding pH 8.0, 0.10 M potassium phosphate buffer. If the antibodies are instead to be stored before use, then adjustment to pH 7.0 may instead be used at this time and the antibodies can be exchanged into the desired buffer for immobilization at some later time.
6. The immobilized antibody support and control supports can be downward packed into a column by using slurry packer (e.g., a Prep 24 pump system). In this example, the supports were packed into 1.0 cm × 2.1 mm I.D. stainless steel columns at 4000 psi and using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. The final columns were stored in this same buffer at 4°C until use.

Immobilization of antibodies to silica by the Schiff base method

7. Determine the amount of silica that is needed for the desired application. The following procedure is for a 1.0 g portion of Nucleosil Si-1000 (7 μm particle size, 1000 Å pore size) or a similar HPLC-compatible material. The silica should be obtained either in a diol-bonded form or converted into such a form prior to use in immobilization (Kim & Hage, 2006).
8. Weigh out the desired amount of diol bonded silica into a 50 ml centrifuge tube (tube 1). A second portion of the same support should be weighed into a separate 50 ml centrifuge tube for use as a control support (tube 2).

   Note: It is important to weigh out more silica for immobilization than will be used in the construction of the column to account for silica loss during preparation and to have some

silica left over for characterization. The minimum of amount of support required for packing a column will be determined by the internal volume of the column and the packing density of the support (e.g., 0.45 g/cm^3 for the type of silica that is used in this example). It is further recommended that at least two-times this minimum amount of support be prepared or obtained to provide a sufficient amount for packing the column and for doing separate assays on the support (e.g., to determine the protein or antibody content). For the specific example used in this protocol, a two-fold excess of support consisted of about 60-70 mg silica to pack a 1.0 × 0.21 cm i.d. column and to allow a micro BCA assay to be conducted on this support.

9. Weigh out 1 g of periodic acid for every 1 g of silica to be prepared in two separate 50 ml centrifuge tubes (tubes 3 and 4).
10. Add 20 ml of a 90% acetic acid solution for every 1 g of periodic acid to tubes 3 and 4 from Step 9.
11. Vortex mix the contents of tubes 3 and 4 for 1 min or until all the periodic acid is dissolved.
12. Add the solutions of periodic acid in tubes 3 and 4 from Step 11 to the silica contained in tubes 1 and 2, respectively, from Step 8.

    Note: Periodic acid is light sensitive, so work with this reagent in the dark and cover the outside of its test tube or container with aluminum foil.
13. Vortex mix the silica/periodic acid solutions for 30 s and then sonicate these mixtures while degassing in the presence of an aspirator vacuum for 5 min.

    Note: During this step, observe the contents of the centrifuge tube carefully to make certain that the silica is not spattering into the aspirator line.
14. Tighten the lid of each centrifuge tube and wrap the lid and top of the tube with parafilm to seal it. Wrap the whole tube in aluminum foil to keep out any light.

15. Place the sealed and wrapped tube on a wrist action shaker at 300 oscillations per minute and shake the tube for 2 h at room temperature.

    Note: During this step, periodically check the contents of each tube to make sure they are adequately suspended and, if not, vortex mix the tube for 30 s to resuspend the support before placing it back on the wrist action shaker for the remainder of the 2 h.

16. After the completion of Step 15, remove the tubes from the wrist action shaker and unwrap the aluminum foil and parafilm. Place the tubes in a sufficiently balanced centrifuge and spin at 7000 rpm (7670 × *g*) for 3 min to settle the silica to the bottom of the tube.

17. Remove the supernatant from the tube and discard it to waste.

    Note: Do not unsettle the silica while removing the supernatant and be observant that as little silica is lost during this process as possible.

18. Add to each centrifuge tube containing silica 5 ml of deionized and purified water and recap the tube.

19. Vortex mix the tube for 30 s to resuspend the silica and then place the tube back into the centrifuge.

    Note: Repeat the centrifugation and decanting process described in Steps 17-19 at least five times to wash the silica.

20. Repeat the washing and decanting steps described in Steps 17-19 at least three times using pH 6.0, 0.10 M potassium phosphate buffer instead of water.

21. Add 5 ml of pH 6.0, 0.10 M potassium phosphate buffer for every 1 g silica in the centrifuge tube to create a non-viscous slurry.

22. Vortex mix the silica slurries for 30 s and degas under sonication for 5 min.

23. Add the desired antibodies to the centrifuge tube containing silica to be used to make the IAC support. No antibodies should be added to the tube with the silica that will be used as the control support.

    Note: The antibodies that are added to the silica should be dissolved in pH 6.0, 0.10 M potassium phosphate buffer. Add 100 mg of antibodies for every gram of silica. Lower concentrations of antibody can be used, but the more concentrated the antibody solution is before immobilization the higher the protein content of the silica will generally be after immobilization. An equivalent amount of the same pH 6.0 buffer, but with no antibodies present, should be added to the tube with the control silica.

24. Add 50 mg of sodium cyanoborohydride to each silica slurry for every 100 mg of antibodies that have been added to make the antibody silica.

    Note: The addition of sodium cyanoborohydride will produce toxic fumes, so this step should be performed in a fume hood.

25. Seal the centrifuge tubes with their lids and wrap each lid with parafilm.
26. Place the tubes containing the silica slurries on a rotary mixer and mix at 4 °C for 3-6 days.

    Note: The longer the silica is incubated with the antibodies, the higher the protein content of the resulting silica will become up to about 5-6 days. Incubating beyond 6 days often does not show any meaningful improvement in the final level of protein immobilization.

27. Repeat the decanting and washing procedure for the silica supports described in Steps 17-19 but now using pH 8.0, 0.10 M potassium phosphate buffer as the wash solution.
28. In a separate tube, dissolve 25 mg of sodium borohydride in 20 ml of pH 8.0, 0.10 M potassium phosphate buffer for every 1 g of silica.

29. Resuspend the silica samples in 20 ml of the sodium borohydride solution from Step 28 by using a vortex mixer.

    Note: The sodium borohydride solution will release gas, and the centrifuge tube will need to be carefully vented to prevent gas build up during this step.

30. Stir the silica slurries on a stir plate for 90 min.

    Note: Leave the cap of the centrifuge tube loosely affixed during this step so that gas can vent over the course of mixing.

31. Repeat the decanting and washing steps for the silica described in Steps 17-19 but now using pH 7.0, 0.10 M potassium phosphate buffer as the washing solution.

32. Resuspend each silica sample in 4-5 ml of pH 7.4, 0.067 M potassium phosphate buffer for storage or use. The silica can be stored in this buffer at 4°C. The final concentration of the silica in the storage buffer in this example is approximately 14-18 mg/mL, based on the amount of buffer that is used for resuspension of the silica and the initial amount of silica that was used for support preparation.

    Note: The Schiff base method outlined here can also be readily adapted for use in the immobilization of other proteins to silica, such as protein A or protein G (Hage et al., 1986; Hage & Walters, 1987; Jackson et al., 2010; Pfaunmiller et al., 2016).

Characterization of the protein content of immobilized antibody support

33. Dry small portions of the immobilized antibody support and control support in a drying oven at 60°C for use in protein assay.

    Note: For 70 mg silica, as used in this example, approximately 24 h should be used for drying.

34. Make standards for the protein assay (e.g., a micro BCA assay) by preparing a set of seven working protein standards with the following recommended concentrations of the desired type

of generic IgG (e.g., goat IgG) in pH 7.4, 0.067 M potassium phosphate buffer: 1.0, 2.5, 5.0, 7.5, 10, 15, and 20 μg/ml.

35. Prepare three samples each of the antibody support and control support by placing 0.2 mg silica/ml and 0.4 mg/ml in pH 7.4, 0.067 M potassium phosphate buffer.
36. Incubate all standards and samples solutions and slurries at 60°C in a water bath for 1 h.

    Note: Centrifugation or filtration of samples containing silica, and collection of their supernatant or filtrate for measurement can also be used when a solid support is present in the samples or standards.
37. Cool down all the solutions and slurries to room temperature and measure their absorbance at 562 nm.
38. Prepare a calibration curve using the data obtained from the standard solutions and use this curve to determine the antibody and protein concentration in each silica slurry sample. Use these results to also then find the mass of antibodies and protein per mass of silica.

Characterization of the binding capacity of immunoextraction column

39. To measure the binding capacity of the immunoextraction column by frontal analysis, place the immunoextraction or control columns on an HPLC system and equilibrate each column with pH 7.4, 0.067 M potassium phosphate buffer, or the desired application buffer.

    Note: In this specific example, this equilibration and the following binding capacity measurements were done at a flow rate of 0.10 ml/min, as illustrated in Figure 6(a).
40. Apply a fixed and known concentration of the desired target (i.e., in this example, a 5.0 μM solution of HSA) in the application buffer (e.g., pH 7.4, 0.067 M potassium phosphate buffer) while monitoring the elution of the target (e.g., at 280 nm for an applied protein target). This application should be done at a suitably low flow rate and for a sufficient amount of time to

allow a breakthrough curve with a well-defined rise and stable final plateau to be obtained (e.g., at 0.10 ml/min in this example).

Note: When the mobile phase flows through the column, the desired target (e.g., HSA) begins to saturate binding sites on the immobilized binding agent (e.g., anti-HSA antibodies), leading to an increase in the amount of unbound target that elutes from the column in the application buffer. This produces a response vs time curve that is known as a breakthrough curve. This breakthrough curve will reach a plateau as no more free binding sites are available on the column and the concentration of eluting target becomes equal to the concentration of the applied target.

41. After a breakthrough curve has been obtained on the column, apply the elution buffer selected for the given immobilized antibody and target (e.g., pH 2.5, 0.10 M potassium phosphate buffer applied at 0.1 ml/min for 30 min in this example).
42. After the retained target has been completely eluted, pass the original application buffer again through the column and allow the antibodies in the column to regenerate prior to the next application of the target.
43. Repeat Steps 39-42 as needed to determine the precision and reproducibility of the breakthrough times.
44. The binding capacity of the antibody column is determined by finding the difference in the midpoints of the breakthrough curves from the control column and antibody column for the applied target. A recommended approach for this is to use the Savitzky-Golay method to both smooth and take the first derivative of each curve, where the central moment of the first derivative represents the mean breakthrough point.

45. The measured breakthrough capacities are used to determine the maximum amount of target that can be bound to the immunoextraction column during each application cycle and the number of cycles over which the immunoextraction column can be used for target purification.

Optimization and use of immunoextraction column

46. The application, elution, and regeneration buffers to use for the purification of a target protein or solute by an immunoextraction column are typically the same as those utilized in breakthrough capacity measurements.
47. The flow rates used with these buffers during target purification may differ from the flow rates used during the breakthrough capacity measurements but may lead to a change in the apparent, dynamic binding capacity. The dynamic binding capacity would be expected to be lower as the flow rate used for application is increased. The dynamic binding capacity for other flow rates can be evaluated as described in the previous section (i.e., Steps 39-45).
48. The extraction efficiency of the immunoextraction column for a typical sample can be determined by applying the desired target concentration and sample volume (e.g., 20 µL of 5.0 µM HSA, in this example) at the selected range of possible application flow rates (e.g., 0.05–0.5 ml/min) and in the presence of the application buffer (i.e., pH 7.4, 0.067 M potassium phosphate buffer). The non-retained peak of the sample is monitored at a suitable flow rate during this process (280 nm, in this case).
49. During the measurement of extraction efficiency, the elution buffer (e.g., pH 2.5, 0.10 M potassium phosphate buffer) is next applied, followed by passage of the initial application buffer to regenerate the immunoextraction column.
50. Make similar injections of the target solution in the application buffer onto the control column as described for the immunoextraction column in Step 48, followed by the same elution and

regeneration step as in Step 49. The difference between the areas for the non-retained target peak on the control column and the immunoextraction column can be used to estimate the extraction efficiency of the immunoextraction column for the target under the given buffer and flow rate conditions. An example of such an experiment is provided in Figure 6(b).

Note: The flow rate used for target and sample application can then be adjusted as needed to lower values to increase extraction efficiency. This approach is effective if the amount of injected target is below the binding capacity of the immunoextraction column.

51. If needed, adjustments in the elution buffer and regeneration/application buffer, as well as the flow rates and times used to apply these buffers, can also be made to adjust the final binding capacity and extraction efficiency of the immunoextraction column. The effects of these changes can be determined by using procedures described previously for measuring the binding capacity or extraction efficiency.
52. Once the desired level of extraction efficiency and column reusability have been obtained, the buffers and flow rates that have been optimized for the immunoextraction column can be used for a larger set of samples.

    Note: For complex samples such as serum or plasma, it is recommended that the samples first be passed through disposable filters (pore size, 2 μm) prior to their injection onto an HPIAC immunoextraction column. This is done to avoid clogging the column. Diluted samples may also be used for this purpose.

BASIC PROTOCOL 3

PROTOCOL FOR COMPETITIVE BINDING CHROMATOGRAPHIC IMMUNOASSAY

One type of chromatographic immunoassay that is used for indirect target detection is one that makes use of a competitive binding format (Hage, 1998; Hage & Nelson, 2001; Moser & Hage,

2010). This approach uses an analog of the target, either with a label or without one, that is measured separately from the target in the sample. This analog and target are then allowed to compete, either simultaneously or sequentially for a limited number of antibodies or related binding agents in an IAC column. The amount of target is then determined by looking at how the presence of this target affects binding by the analog with the IAC column.

This protocol illustrates the use of the sequential injection method for a competitive binding chromatographic immunoassay (Hage et al., 1993; Moser & Hage, 2006; Nelson et al., 2003). In this approach, the sample containing the target is injected first onto an IAC column that contains a small amount of antibody against the target. This is followed soon after by an injection of the analog of the target (see Figure 7). These injections are done in the presence of the application buffer and with no elution step in between. The retained target and analog are then eluted, the IAC column is regenerated, and the process is repeated. During this process the amount of non-retained or retained analog is then measured and used to determine how much target had previously been injected onto the same column. This method is illustrated in this protocol by using HSA as the target, an IAC column that contains a limited amount of anti-HSA antibodies, and a fixed amount of unlabeled HSA as the analog (Hage, Thomas & Beck, 1993; Nelson, Reiter & Hage, 2003). The same general procedure can be easily modified for use with other targets and types of labeled or unlabeled analogs (Hage, 1998; Hage & Nelson, 2001; Moser & Hage, 2010). In addition, this protocol demonstrates the use of a site-selective immobilization method for antibodies based on coupling of these agents to a hydrazide-activated support through the carbohydrate groups in the F_c region, as shown by the reaction scheme in Figure 8 (Hage, 2000; Ruhn et al., 1994; Wolfe & Hage, 1995).

Materials

Support suitable for HPIAC (e.g., Nucleosil Si-300 and Nucleosil Si-1000 silica, each with a particle diameter of 7 μm and pore sizes of 300 or 1000 Å, respectively, and previously obtained or converted into a diol-bonded form) (Kim & Hage, 2006)

90% Acetic acid solution

Periodic acid (H_5IO_6) (CAS number 10450-60-9)

Buffers for use in immobilization method (e.g., pH 5.0, 0.10 M potassium phosphate buffer for use in the preparation of hydrazide-activated silica, pH 5.0, 20 mM sodium acetate buffer containing 0.15 M sodium chloride for antibody oxidation, as well as pH 7.0, and pH 8.0, 0.10 M potassium phosphate buffers, prepared as described under Reagents and solutions)

Oxalic dihydrazide (CAS number 98996-98-5)

Sodium borohydride (CAS number 16940-66-2)

Target-specific antibodies (e.g., polyclonal anti-HSA antibodies from goat serum, Sigma-Aldrich, Cat. no. A1151)

Ethylene glycol (CAS number 98996-98-5)

Sodium chloride (CAS number 7440-23-5)

Triton X-100 (CAS number 9036-19-5)

Target of interest (e.g., HSA, essentially fatty acid free)

Analog of target (e.g., HSA, with or without a label in this example)

Application buffer for HPIAC column (e.g., pH 7.0, 0.10 M potassium phosphate buffer, prepared as described under Reagents and solutions)

Elution buffer for HPIAC column (e.g., pH 2.5, 0.10 M potassium phosphate buffer, prepared as described under Reagents and solutions)

Deionized and purified water suitable for use in preparing buffers and biological reagents (e.g., water from a Nanopure system (Barnstead, Dubuque, IA) or a Milli-Q Advantage A10 system (Millipore, Billerica, MA)

Reagents for protein assay (e.g., micro bicinchoninic acid, or micro BCA, assay, from Pierce, Rockford, IL)

Standard for protein assay (e.g., goat immunoglobulin G, or goat IgG)

0.2 μm GNWP nylon filters for buffers (Millipore, Billerica, MA)

Sonicator equipped with aspirator line (e.g., Cleaning Bath, Branson Ultrasonics, Brookfield, CT)

Wrist action shaker (Wrist Action Model 95, Burrell Scientific, Pittsburgh, PA)

Centrifuge (Sorvall ST8 from ThermoScientific, Waltham, MA)

Rotary mixer (e.g., Thermolyne Labquake Rotisserie Shaker 400110, Barnstead, Dubuque, IA)

Device to place isolated target into appropriate storage buffer (e.g., Dialysis Slide-A-Lyzer dialysis cassette, 10 kDa MW cutoff, 0.1-0.5 mL sample volume from Thermo Scientific, Rockford, IL).

Components to assemble an HPLC column (e.g., XPERTEK column tube, ¼ ” × 2.1 mm × 25 cm SS, cut to desired length, and two XPERTEK HPLC column end fittings, ¼” × 2.1 mm with 2 μm frits, from P.J. Cobert Associates, St. Louis, MO)

Column packer (e.g., Prep 24 pump system, ChromTech, Apple Valley, MN)

Drying oven (e.g., Isotemp Oven, Fisher Scientific, Marietta, OH)

Temperature-controlled water bath (e.g., Isotemp 210 Digital Water Bath, Fisher Scientific, Dubuque, IA)

UV/Vis absorbance detector and cuvettes (1 cm pathlength) (e.g., V-630 Spectrophotometer, JASCO, Tokyo, Japan)

HPLC system (e.g., this example uses a system from Jasco (Easton, MD) that consists of two PU2080 pumps, an AS-2057 autosampler, a CO-2067 column oven, a DG-2080-53 degasser, and an UV-2075 Plus absorbance detector equipped with LCNET II/ADC Plus from Jasco and a six-port valve from Rheodyne, Cotati, CA)

Preparation of hydrazide-activated silica method

1. To produce aldehyde-activated silica, suspend 1 g of diol-bonded silica in 20 ml of 90% (v/v) acetic acid-water mixture containing 1 g of periodic acid.
2. Sonicate this mixture under vacuum for about 10 to 15 min.
3. Shake this mixture for 2 h at room temperature on a wrist action shaker.
4. Wash the support four times with deionized, purified water and centrifuge at 7000 rpm (7670 × *g*) for 3 min.
5. Wash the silica four times with pH 7.0, 0.10 M potassium phosphate buffer and centrifuge as described in Step 4.
6. Add to the silica 20 ml of pH 5.0, 0.10 M phosphate buffer containing a 5-fold molar excess of oxalic dihydrazide vs. initial diol groups on the support. For example, for 1.0 g of Nucleosil Si-300 silica that has been converted into a diol form, about 177.2 mg oxalic dihydrazide is required.

   Note: The initial amount of diol groups that may be present on the support can be calculated by using the surface area of the silica and the theoretical monolayer coverage of diols on silica (i.e., 2.3 μmol/m^2). For 1.0 g of Nucleosil Si-300 silica, the amount of initial diol groups is often estimated as being 0.0003 mol diol/g silica. The amount of oxalic dihydrazide that provides a five-fold molar excess vs. these original diol groups on Nucleosil Si-300 can then be determined by using the following formula.

$$\left(\frac{0.0003\ mol\ diol}{g\ silica}\right) \times (Weight\ of\ silica,\ g) \times \left(\frac{118.10\ g\ dihydrazide}{mol\ dihydrazide}\right) \times \left(\frac{5\ mol\ dihydrazide}{mol\ diol}\right)$$

7. Shake the aldehyde silica–dihydrazide mixture on a wrist action shaker for 1 to 2 h.
8. Centrifuge at 7000 rpm (7670 × *g*) and wash the silica four times with pH 7.0, 0.10 M phosphate buffer.
9. Add to the support a 25-mol excess of sodium borohydride ($NaBH_4$) vs. the initial diol groups in 20 ml of pH 8.0, 0.10 M phosphate buffer per gram of silica. For 1.0 g Nucleosil Si-300 silica that has been converted into a diol form, about 283.7 mg of $NaBH_4$ is required.

   Note: After activation of the silica with a dihydrazide, the remaining aldehyde groups are converted into alcohol groups through the addition of $NaBH_4$. The amount of $NaBH_4$ that is needed to give at least a 25-molar excess vs. the amount of diol groups that may have been originally present on Nucleosil Si-300 can be calculated as follows.

$$\left(\frac{0.0003\ mol\ diol}{g\ silica}\right) \times (Weight\ of\ silica,\ g) \times \left(\frac{37.83\ g\ NaBH_4}{mol\ NaBH_4}\right) \times \left(\frac{25\ mol\ NaBH_4}{mol\ diol}\right)$$

10. Shake the new mixture for 90 min at room temperature. This step is used to ensure any remaining aldehyde groups on the support are reduced to alcohol groups.
11. Wash the silica four times with deionized and purified water and four times with pH 7.0, 0.10 M potassium phosphate buffer by using a centrifuge at 7000 rpm (7670 × g).

Note: Once the hydrazide-activated silica has been prepared, it should be either used immediately or dried and used within 2.5 weeks of preparation when stored under vacuum (Ruhn, et al., 1994).

Oxidation of antibodies with periodate

12. Combine 2 mg/ml of the desired antibodies with 0.02 M periodic acid in pH 5.0, 20 mM sodium acetate buffer containing 0.15 M sodium chloride.

Note: During the preparation of the periodate solution, it is important to protect this solution from exposure to light by wrapping its container in aluminum foil; this solution can be stored in the dark at room temperature for extended periods of time.

13. Cover the container of this mixture with aluminum foil and react in the dark for 2 h at room temperature while mixing on a wrist action shaker or rotary mixer.
14. Quench the reaction by adding 0.25 ml ethylene glycol per ml of solution. Allow this mixture to react for 2 min.
15. Purify the oxidized antibodies by placing 1-3 ml of the antibody solution in a dialysis bag or cartridge (e.g., Slide-A-Lyzer dialysis cassette, 10 kDa MW cutoff) and dialyze at 4 ºC for 2 h against 2 l of pH 5.0, 20 mM acetate buffer containing 0.15 M sodium chloride, followed by three additional 2 h dialysis cycles against pH 5.0, 0.10 M potassium phosphate buffer containing 0.1% (vol/vol) Triton X-100.

Immobilization of oxidized antibodies to hydrazide-activated silica

16. Combine 0.03 g of the hydrazide-activated silica with 2 ml of pH 5.0, 0.10 M potassium phosphate buffer containing 1.3 mg/ml of oxidized antibodies and 0.1% (vol/vol) Triton X-100. It is also recommended that a similar mixture be prepared containing the hydrazide-activated silica and the pH 5.0, 0.10 M potassium phosphate buffer and 0.1% (vol/vol) Triton X-100 without any antibodies added for use as a control material.
17. Gently mix each of these slurries on a rotary shaker at 4°C for 2 days.
18. Centrifuge and wash the silica several times at 7000 rpm (7670 × *g*) for 3 min with pH 7.0, 0.10 M phosphate buffer containing 2 M sodium chloride. This can be followed by similar washing steps using the final desired application or storage buffer (e.g., pH 7.4, 0.067 M potassium phosphate buffer).

19. The antibody support and control support can be downward packed into a column by using slurry packer (e.g., a Prep 24 pump system). In this example, the supports were packed into 6.35 mm × 2.1 mm I.D. stainless steel columns at 3500 psi and using pH 7.0, 0.10 M potassium phosphate buffer or the final desired buffer as the packing solution.

    Note: The final columns can be stored in this same buffer at 4°C until use.

Characterization of the IAC support and IAC column

20. If a protein assay is to be done on the support, wash a small portion of each type of silica several additional times with deionized and purified water and dry the supports under vacuum at room temperature.
21. The protein content of the dried antibody support can be determined as described in the previous section for on-line immunoextraction by HPIAC (see Basic Protocol 2, Steps 33-38).

Use of IAC in a sequential injection competitive binding immunoassay

22. Using the estimated binding capacity of the IAC column (see previous section), determine the approximate moles of the target that will provide a usable calibration range for a sequential injection assay.

    Note: In the example used for this protocol, the concentrations of HSA that were used as the standards and samples ranged from 0 to 20 g/L for a calibrated 28.5 μL injection volume, while the concentration of HSA to be added as the label can range from 0.1 to 2.0 g/L. The flow rates that were evaluated for these injections ranged from 0.25 to 4.00 ml/min.
23. To validate and use the sequential injection assay, apply standards containing the desired concentration range and injection volume of the target in the presence of the application buffer.
24. Follow each injection of a standard or sample in Step 23 by a similar injection of a fixed amount of the target analog that is to be detected.

25. Determine the fraction of the bound analog by comparing the analog's total non-retained peak area after each sample injection on the IAC column vs the total area obtained for injection of the same analog solution when only a control column or no column was present in the system (see example in Figure 9).
26. Apply the elution buffer (e.g., pH 2.5, 0.10 M potassium phosphate buffer for use with HSA and anti-HSA antibodies) to release the captured target and analog from the IAC column.
27. Apply the application buffer (e.g., pH 7.0, 0.10 M potassium phosphate buffer) to regenerate the column.
28. Repeat Steps 24-27 as needed for additional samples and standards.

Note: If needed, the injection of a large excess of the target can be made onto the IAC column prior to injection of the analog. This set of injections, and the measured non-retained area for the analog, can be used to measure and correct for non-specific binding by the analog to the system.

REAGENTS AND SOLUTIONS

Tris(hydroxymethyl)aminomethane (Tris) buffer, (pH 7.4), 0.10 M

- 250 ml deionized and purified water
- 3.26 g Tris HCl
- 0.52 g Tris base
- Store at 4 °C for up to 12 months

Glycine-HCl buffer (pH 2.8), 0.10 M

- 80 ml deionized and purified water
- 0.75 g glycine
- 0.08 g HCl
- Adjust to pH 2.8 with HCl or NaOH

- Add deionized water until the volume is 100.0 ml
- Pass the buffer through 0.2 μm GNWP nylon filter
- Store at 4 °C for up to 4 months

Tris buffer (pH 8.0), 0.10 M

- 100 ml deionized and purified water
- 4.30 g Tris HCl
- 2.75 g Tris base
- Adjust the pH to 8.0 with NaOH or HCl
- Pass the buffer through 0.2 μm GNWP nylon filter
- Store at 4 °C for up to 12 months

Potassium phosphate buffer (pH 2.5), 0.10 M

- 500 ml deionized and purified water
- 4.38 g potassium dihydrogen phosphate (KH_2PO_4)
- 1.04 mL (or, 1.75 g) 85% phosphoric acid (H_3PO_4)
- Adjust the pH to 2.5 with NaOH or HCl
- Pass the buffer through a 0.2 μm GNWP nylon filter
- Store at 4 °C for up to 12 months

Potassium phosphate buffer (pH 6.0), 0.10 M

- 500 ml deionized and purified water
- 5.92 g KH_2PO_4
- 1.15 g dipotassium hydrogen phosphate (K_2HPO_4)
- Adjust the pH to 6.0 with NaOH or HCl
- Pass the buffer through a 0.2 μm GNWP nylon filter

- Store at 4 °C for up to 12 months

Sodium acetate buffer (pH 5.5), 0.1 M

- 100 ml deionized and purified water
- Add 0.82 g sodium acetate to 90 mL deionized water
- Adjust the pH to 5.5 with HCl
- Add deionized water until the volume is 100.0 ml
- Pass the buffer through a 0.2 µm GNWP nylon filter
- Store at 4 °C for up to 12 months

Potassium phosphate buffer (pH 7.0), 0.10 M

- 500 ml deionized and purified water
- 2.71 g KH_2PO_4
- 5.24 g K_2HPO_4
- Adjust the pH to 7.0 with NaOH or HCl
- Pass the buffer through a 0.2 µm GNWP nylon filter
- Store at 4 °C for up to 12 months

Potassium phosphate buffer (pH 7.4), 0.067 M

- 4000 ml deionized and purified water
- 7.55 g KH_2PO_4
- 37.72 g K_2HPO_4
- Adjust the pH to 7.4 with NaOH or HCl
- Pass the buffer through a 0.2 µm GNWP nylon filter
- Store at 4 °C for up to 12 months

Potassium phosphate buffer (pH 8.0), 0.10 M

- 500 ml deionized and purified water
- 0.42 g KH_2PO_4
- 8.17 g K_2HPO_4
- Adjust the pH to 8.0 with NaOH or HCl
- Pass the buffer through a 0.2 μm GNWP nylon filter
- Store at 4 °C for up to 12 months

Sodium acetate buffer (pH 5.0), 20 mM containing 0.15 M sodium chloride

- 1000 ml deionized and purified water
- 2.72 g sodium acetate trihydrate
- 8.77 g sodium chloride
- Adjust the pH to 5.0 with HCl
- Pass the buffer through a 0.2 μm GNWP nylon filter
- Store at 4 °C for up to 12 months

COMMENTARY

Background information

The selectivity and strong binding of antibodies for their targets are features that have made IAC popular as a purification tool for many decades in biochemistry and related fields (Delaunay-Bertoncini & Hennion, 2004; Hage & Nelson, 2001; Hage & Phillips, 2006; Jackson et al., 2012; Lua & Chou, 2002; Moser & Hage, 2006, 2010; Weller, 2000). In many cases, this method can be used to isolate a given target with a high purification yield and in a single step (Hage & Phillips, 2006). Examples of such purification schemes are presented here in Basic Protocols 1 and 2, which illustrate the use of IAC as either a low-performance method with off-line immunoextraction or IAC as a high-performance method used for on-line immunoextraction.

IAC also has seen significant growth over the last 20 years as an analytical tool for the analysis of complex biological mixtures and environmental samples (Hage & Nelson, 2001; Moser & Hage, 2006, 2010). For instance, IAC can be easily coupled with other analytical techniques to give multidimensional methods that are easily automated, sensitive, and specific with excellent reproducibility (Hage & Nelson, 2001; Hage & Phillips, 2006). There are also many formats in which IAC can be used for chemical or biochemical detection. As discussed earlier, this includes both the use of direct detection and the use of indirect detection (i.e., through use of a chromatographic immunoassay, as demonstrated in Basic Protocol 3). Another advantage of IAC is that it can be employed either as a highly selective purification and analysis tool for a single target or as a more general tool for a class of related targets with a common structural feature (Hage & Phillips, 2006).

One disadvantage of IAC is that a preparation of antibodies, or antibody-related agents, with the desired selectivity and binding strength for the target is needed for use as the stationary phase. These antibodies may be either polyclonal or monoclonal, depending on the desired application (Moser & Hage, 2010; Hage & Phillips, 2006). Polyclonal antibodies may be easier to obtain initially than monoclonal antibodies but may require some pre-purification to isolate a given subset of antibodies with the desired selectivity and/or binding strength for the target. An example of this type of pre-purification is given in Basic Protocol 1 for the isolation of anti-HSA antibodies from anti-HSA antiserum. It is also necessary to ensure a sufficient amount of a polyclonal antibody is available from a given antiserum for making IAC columns that will last the duration of the desired applications. Although monoclonal antibodies require more initial work in their development than polyclonal antibodies, the monoclonal antibodies are screened for both their selectivity and binding strength during this development process. Also, once a cell line has been

obtained that can produce a specific type of monoclonal antibody, this cell line can be grown and propagated to produce a steady and reproducible amount of this binding agent for long term use in IAC.

With regards to the use of chromatographic immunoassays for indirect target detection, there are many approaches available for this besides the competitive binding and sequential injection format that is used in Basic Protocol 3 (Moser & Hage, 2010). For instance, a two-site immunometric assay (or sandwich immunoassay) uses two different antibodies for the measurement of a target. The first antibody is on the IAC column and used to capture the target from a sample, while the second antibody is labeled and is used to place a detectable tag on the target. A sandwich immunoassay can provide a more linear response than a competitive binding assay and tends to have a better selectivity and detection limit. However, the sandwich immunoassay format works only for targets that are sufficiently large to allow simultaneous binding by two antibodies. A one-site immunometric assay avoids this last issue, at the loss of some selectivity, by using an immobilized analog of the target to capture excess labeled antibodies after such antibodies have been allowed to bind to a target in a sample. Other formats that may also be employed, each with their own strengths and weaknesses, include a simultaneous injection competitive binding immunoassay, a displacement immunoassay, a reverse displacement immunoassay, and post-column immunodetection (Hage & Nelson, 2001; Moser & Hage, 2006, 2010; Schiel et al., 2011).

Critical parameters

A key parameter in the development of any IAC method for target purification or analysis is the antibody or antibody-related agent that is used as the stationary phase. As mentioned in the previous section, the antibodies used for this application can be either polyclonal or monoclonal

(Moser & Hage, 2010; Hage & Phillips, 2006). The primary factors to consider in selecting an antibody to use for IAC are its selectivity and binding strength for the target. This includes the extent to which the antibody preparation will cross-react with compounds that are similar to the target. These items need to be considered on a case-by-case basis. Other considerations include the cost and availability of the antibody and amount that will be required by the final method (Moser & Hage, 2010; Hage & Phillips, 2006). The type of animal and class or subclass of the antibody may also be factors to consider, such as when protein A or protein G are to be used for the biospecific adsorption of the antibodies to an IAC column (De Frutos & Regnier, 1993; Hage & Phillips, 2006).

Both polyclonal and monoclonal antibodies, as well as antibodies from various species and subclasses, have been used in immunoaffinity columns over a large number of application and elution cycles (i.e., several hundred in many cases) (Hage, 1998; Jackson et al., 2012; Moser & Hage, 2010). The number of usable cycles will depend on the stability of the antibody and the type of elution buffer that is employed, as well as the type of support that is being used (Hage et al., 2006). Protein A and protein G columns are also quite stable and have frequently been used over hundreds of sample application/elution cycles for biospecific adsorption in immunoaffinity chromatography (De Frutos & Regnier, 1993; Hage & Phillips, 2006; Moser & Hage, 2010;).

The type of support that is to be used with the antibodies is another factor to consider (Gustavsson & Larrson, 2006; Moser & Hage, 2010). There are many commercial supports that can be used for either low- or high-performance immunoaffinity chromatography, and with many of these coming in pre-activated forms (e.g., see Hage & Phillips, 2006). If only a few samples are to be purified by off-line immunoextraction, then use of low-performance support such as agarose will usually be adequate (Gustavsson & Larrson, 2006). If many samples are to be

processed, a fast turn-around time is needed, and/or reuse of the antibodies is desired, then an HPIAC support such as silica should be considered (Moser & Hage, 2010). The pore sizes, chemical stabilities, and physical stabilities of the materials also need to be considered. Agarose has a large pore size and good stability over a wide pH range that make it attractive for use in purification schemes involving proteins and other large targets, including situations in which column sanitization is desired between runs by using a high pH solution. Silica is also available in pore sizes that are suitable for use in IAC and with protein targets (e.g., pore sizes of 300-500 Å up to even 1000-4000 Å) but has a much smaller range of pH stability than agarose (i.e., pH 2-8). However, silica is also more mechanically stable than agarose and easier to prepare with small particle sizes, making silica much more suitable for use in HPIAC and with HPLC systems, where operating pressures may approach 3000-4000 psi or 20.7-27.6 MPa (Gustavsson & Larrson, 2006; Moser & Hage, 2010). The size of the column that will contain this support needs to be considered as well. This size may range from more standard size low- or high-performance columns (e.g., for the purification of large amounts of target) to even microscale columns (e.g., for use in chemical analysis) (Hage & Nelson, 2001; Hage & Phillips, 2006; Zheng et al., 2014).

When using a porous support for immunoaffinity chromatography, the diameter of the pores is important to consider as it will affect the ability of the target and antibody to access the support's surface (Gustavsson & Larsson, 2006; Hage & Phillips, 2006). In the case of antibody immobilization, supports with narrow pores will have a high surface area for immobilization but only a small fraction of this area may be accessible to antibodies for coupling. Supports with wide pores will have less issues with accessibility; however, these supports will have less surface area for immobilization. As a compromise between these effects, supports with pore diameters between 300 and 500 Å are often used for antibody immobilization in immunoaffinity chromatography

(Clarke et al., 2000; Hage & Phillips, 2006). These pore sizes are approximately three- to five-times the diameter of an IgG-class antibody, which further helps to minimize restricted diffusion as the immobilized antibodies bind to targets such as proteins (Gustavsson & Larsson, 2006). In work with very large targets (i.e., agents with masses above a hundred kDa), even larger pore diameters may be required to allow access of these targets to antibodies on the support (Hage & Phillips, 2006).

The way in which the antibodies are bound or coupled to the support is a third factor to consider (Hage & Phillips, 2006; Kim & Hage, 2006). There are many covalent coupling methods based on groups such as amines. An example is the Schiff base methods that is used in Basic Protocol 2. Many of these methods are relatively easy and can often be done with commercial pre-activated supports. However, the final level of antibody activity and coupling site selectivity that is obtained with these general methods can vary significantly from one method to another (Hage et al., 1987; Kim & Hage, 2006). A higher level of activity and more consistent orientation of the antibodies can usually be obtained by using more site-selective coupling methods such as the hydrazide method employed in Basic Protocol 3 (Hage & Phillips, 2006; Kim & Hage, 2006). However, these schemes may involve more steps than simple amine-based coupling methods. A third option is the use of secondary immobilized agents such as protein A or protein G to adsorb antibodies to a support. This last approach is usually gentle but requires the use of the additional binding agent and creates an IAC column in which the antibodies may be only adsorbed under neutral pH conditions (i.e., unless cross-linking or an equivalent method is used to prevent antibody release) (Hage & Phillips, 2006).

The proper choice of application and elution conditions are critical in IAC. The application buffer should be selected to allow strong binding of the target with the IAC column and little or

no binding by other, non-desired sample components (Hage & Nelson, 2001; Hage et al., 2006). For the use of IAC in purification and off-line immunoextraction, a mild elution buffer is often desired to allow the gentle removal of a target from the IAC column and to provide easy recovery or renaturation of this target for later use. When IAC is used in HPIAC and in on-line immunoextraction or a chromatographic immunoassay, there is usually less concern about the target retaining its activity upon elution. The main goal instead is to provide rapid release of the target from the column without causing permanent harm to the immobilized antibodies or the support (Hage et al., 2006). These items must be addressed on a case-by-case basis and are particularly important to consider when the same IAC column is to be employed for many samples.

Troubleshooting

See Table 1 for a troubleshooting guide in the development and use of IAC.

Table 1 Troubleshooting of IAC

Problem	**Possible cause**	**Solution**
Poor selectivity of IAC column for target	Cross-reactivity of antibodies with other sample components	Purify the antibodies against the target (if polyclonal antibodies) or obtain a different antibody preparation.
	Weak binding by the target to the antibody	Adjust the application buffer to increase binding strength (e.g., adjust pH to that of the native environment of the antibody and

		target) or obtain an alternative antibody.
	Non-specific binding of sample components to the support or system	Include buffer additives (a non-ionic surfactant such as Triton X-100 or a blocking agent such as bovine serum albumin) to reduce non-specific binding, reduce the surface area of the support, modify the support to reduce non-specific binding sites, or change to a support with lower non-specific binding.
Poor recovery of target	Elution buffer and elution conditions are not optimal for the immunoextraction.	Adjust the composition of the elution buffer (e.g., pH, ionic strength, or polarity) to improve target release.
Low antibody activity	Loss of antibody activity due to immobilization	Change the type of covalent immobilization method that is used or switch to the use of site-selective immobilization or biospecific adsorption

Low binding capacity of IAC column	Amount of active antibody is too low	Increase the amount of antibody used for immobilization or alter the coupling scheme to increase the relative antibody activity.
	Amount of accessible antibody is too low	Adjust the spacing between the immobilized antibodies or select with a support with a combined surface area and pore size that exposes a greater amount of immobilized antibodies to the target for binding.

Understanding Results

Basic Protocol 1: The first protocol that was described used traditional IAC for the off-line immunoextraction of a protein target. The isolation of HSA and glycated HSA was used as an example of this application. One factor that should be evaluated and optimized in this type of application is the extent of immunoextraction of the target by the IAC column. For instance, the specific application that was outlined in this protocol resulted in 54% capture of applied HSA by the IAC column under the original application elution conditions. This level of capture agreed with prior observations made under similar conditions with the same type of anti-HSA resin (Anguizola et al., 2013; Gundry et al., 2009). When the number of elution steps was increased from two to six (i.e., a factor that may be used to enhance the extent of capture), the level of capture for HSA was increased to 90%.

The specificity of the IAC method should also be examined. For instance, the final IAC approach that was developed in the given example to capture HSA and glycated HSA was evaluated for its specificity by using it with a 20 μL commercial sample of human serum known to contain 1020 μg protein and 840 μg HSA (Anguizola et al., 2013). The retained and isolated protein recovered from the IAC resin gave only the detectable band by SDS-PAGE, with a position that matched that seen for a band made by a standard sample of HSA (Anguizola et al. 2013; Gundry et al., 2007, 2010).

Another item to consider in IAC is the effect of the elution conditions on target recovery. For the example used in this protocol, a decrease in the recovery of HSA from over 90% to 25% was observed when the pH of the elution buffer was increased from 2.8 to 3.5. Based on these results, an elution pH of 2.8 was selected for use in all later experiments involving the off-line immunoextraction of HSA and glycated HSA (Anguizola et al., 2013).

Basic Protocol 2: The second protocol described the use of IAC for on-line immunoextraction based on HPIAC. This protocol was illustrated by using such a method for the isolation of HSA from serum. The evaluation of several key factors is also demonstrated in this example, such estimation of the binding capacity and extraction efficiency of the immunoextraction column. In the given example, the binding capacity of the immunoextraction column was found by frontal analysis to be 0.34-0.42 nmol for HSA and glycated HSA (Matsuda et al., 2015a), as determined by using data such as shown in Figure 6(a).

The extraction efficiency of the immunoextraction column in this example was determined by injecting 0.10 nmol (or ~ 6.6. μg) of HSA and glycated HSA at flow rates ranging from 0.05 to 0.5 ml/min, as illustrated in Figure 6(b) (Matsuda et al., 2015a). All non-retained sample components were passed through the immunoextraction column within 5 min at 0.05 ml/min and

within 1 min at 0.5 ml/min. The extraction efficiency for HSA was 90% at an injection flow rate of 0.05 ml/min, with a decrease in this value to approximately 60 or 70% at 0.50 or 0.25 ml/min. A similar trend was observed for glycated HSA, with a maximum extraction efficiency of 93% at 0.05 ml/min and a decrease in this value to 80% or 90% at 0.50 or 0.25 ml/min.

Basic Protocol 3: The third protocol demonstrated the use of IAC to perform a chromatographic immunoassay with a competitive binding and sequential injection format. Factors that can be varied in this type of assay include the amount of analog that is used, the binding capacity of the column, and the flow rate that is used for injection of the target and analog. In the example used here, 0.41 nmol (i.e., an amount equal to the measured IAC column binding capacity) was injected at a flow rate of 0.50 ml/min for both the target (HSA) and analog or label (also HSA) (Hage et al., 1993). The chromatogram in Figure 9(a) shows the results that were obtained when only the analog was injected on the IAC column and no prior sample or target had been passed through the same column during the same cycle in the application buffer. Under these conditions, all the antibody binding sites were available for analog, so most of this analog was captured by the column.

Figure 9(b) shows chromatograms that were obtained when sequential injections of the target and same amount of analog were next made. In this situation, the amount of analog that was bound by the IAC column was now decreased from the amount that was captured when no target was present. This occurs because the prior application of target and sample results in fewer binding sites in the IAC column being available for the analog when it is later injected (Hage et al., 1993; Moser & Hage, 2006, 2010). The total time that was required to perform one cycle of this sequential injection assay was less than 10 min/sample (Hage et al., 1993). This cycle time is in good agreement with previous results that have been reported for related assays at similar flow

rates (Nilsson et al.,1992; Cassidy et al., 1992). It was further noted that the assay time could be reduced to 2 min when using a higher injection flow rate of 4.0 ml/min. This observation was also in agreement with earlier studies (Cassidy et al., 1992) and demonstrates the potential speed of this method (Hage et al., 1993).

Time Considerations

The time required to complete Basic Protocol 1 depends on the protein extraction time by the column, time of dialysis, the number of protein fractions to be extracted, and the time of drying. The extraction procedure itself lasts less than 2 h. Overall, the protocol takes around 24-29 h based on the protein extraction by a single column.

In Basic Protocol 2, the time required to prepare the IAC support by the Schiff base method is approximately 5-6 days. However, this time can be reduced significantly lowering the time that is allowed for coupling of the antibodies the aldehyde-activated silica. The washing of the support and packing of the column takes another 3-4 h. Purification of the anti-HSA antibodies prior to their use in immobilization takes about 4-5 h. In addition, characterization of the protein content of the support takes about 4-5 h, while the binding capacity and extraction efficiency studies take about 7-8 h. Further optimization of the immunoextraction column and method may take a few additional days depending on the number of columns, mobile phases, and other conditions that are to be evaluated.

Basic Protocol 3, the preparation of the hydrazide-activated silica, antibody oxidation, and coupling of the oxidized antibodies to the silica requires a total time commitment of about 4-5 days. Washing the support and packing it into a column requires around 3-4 h, and characterization of the support's protein content takes about 4-5 h. A few additional days are then needed to select and optimize conditions for the sequential injection competitive binding immunoassay. Once the

final conditions have been selected, an assay time of less than 10 min per sample is typically expected (Hage et al., 1993)

Author Contributions

Sadia Sharmeen: Conceptualization, Methodology, Writing (original draft, review and editing); **Kyungah Suh:** Methodology, Writing (original draft and editing); **Isaac Kyei:** Methodology, Writing (original draft and editing); **Jacob Jones:** Methodology, Writing (original draft and editing); **Harshana Olupathage:** Methodology, Writing (original draft and editing); **Avery Campbell:** Writing (review and editing); **David S. Hage:** Conceptualization, Writing (review and editing), Visualization, Supervision, Project Administration, Funding Acquisition

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data and procedures that support this protocol are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. General structure of an antibody, using immunoglobulin G (IgG) as an example. This type of antibody consists of four polypeptide chains (two light chains and two heavy chains) that are linked by disulfide bonds to create a "Y"-shaped structure with two identical binding sites located in the F_{ab} regions of the structure. The lower stem of the antibody consists of the F_c region, which also often contains carbohydrate chains.

Figure 2. The on/off mode of immunoaffinity chromatography (IAC) and an example of general chromatogram that is obtained with this approach. The ovals in the applied sample represent the target analyte, while the rectangles represent non-retained sample components.

Figure 3. Common strategies for placing antibodies onto supports for use in IAC: 1) covalent immobilization through general sites on antibodies (e.g., amine groups); 2) site-selective covalent immobilization (e.g., through an antibody's carbohydrate residues in the Fc region); and 3) biospecific adsorption onto an immobilized, secondary binding agent (e.g., protein A or protein G).

Figure 4. General scheme for the off-line immunoextraction of the proteins, using the isolation of HSA and modified HSA by an anti-HSA antibody support as an example.

Figure 5. General immobilization scheme for coupling antibodies through amine groups onto diol-bonded silica by the Schiff base method**.**

Figure 6. Typical chromatograms obtained for (a) frontal analysis studies and (b) extraction efficiency experiments performed on 1.0 cm × 2.1 mm i.d. immunoextraction microcolumns containing anti-HSA polyclonal antibodies (dashed line) or a microcolumn containing an inert,

control support (solid line). The results in (a) were obtained for a 5 μM solution of normal HSA that was applied at 0.10 ml/min. The results in (b) are for 20 μl solutions containing 5 μM of normal HSA and that were injected at 0.05 ml/min. Reproduced with permission from Elsevier (Matsuda et al., 2015a).

Figure 7. Chromatographic-based competitive binding immunoassay with the sequential injection of a target and analog.

Figure 8. Site-selective immobilization scheme for coupling to immobilize the antibodies through oxidized carbohydrate groups and hydrazide-activated silica.

Figure 9. Typical chromatograms for a sequential addition, competitive binding immunoassay with (a) injection of an analog/label only (b) with the sequential injection of target followed by the analog/label. This figure is reproduced with permission from the American Chemical Society (Hage et al., 1993).

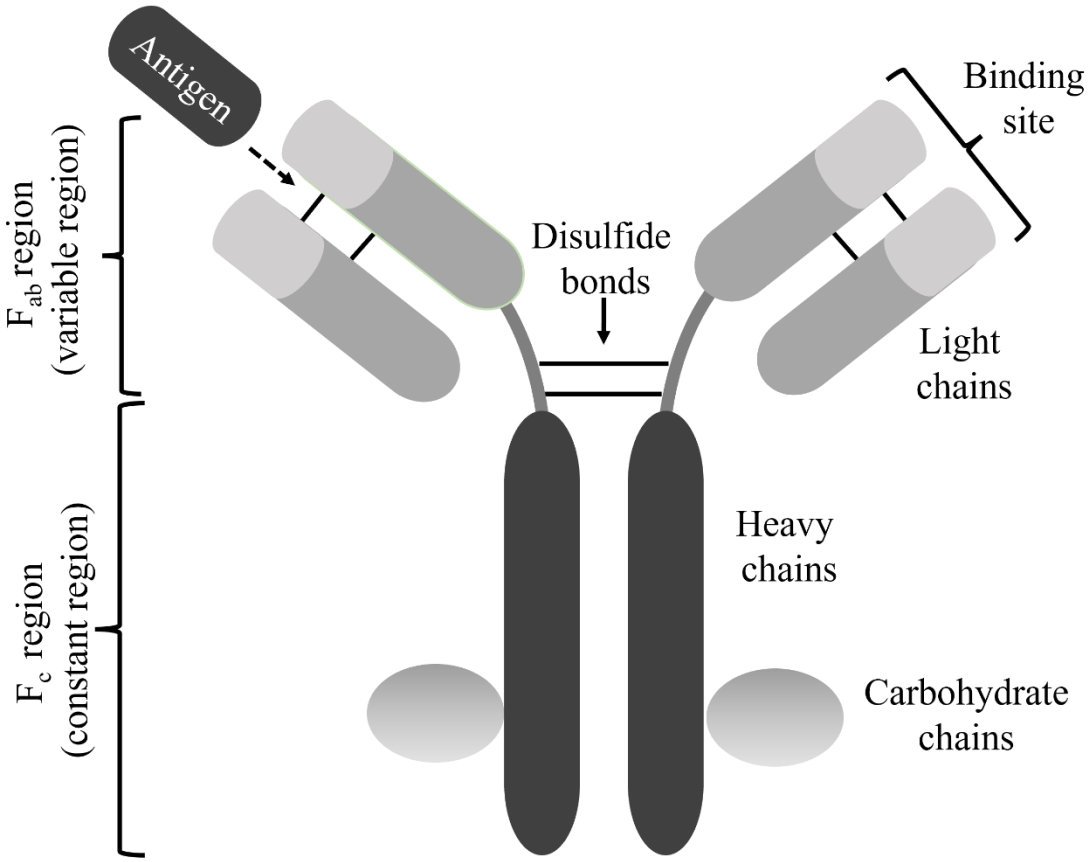
Antigen
Binding site
F_{ab} region (variable region)
Disulfide bonds
Light chains
Heavy chains
F_c region (constant region)
Carbohydrate chains

Figure 1

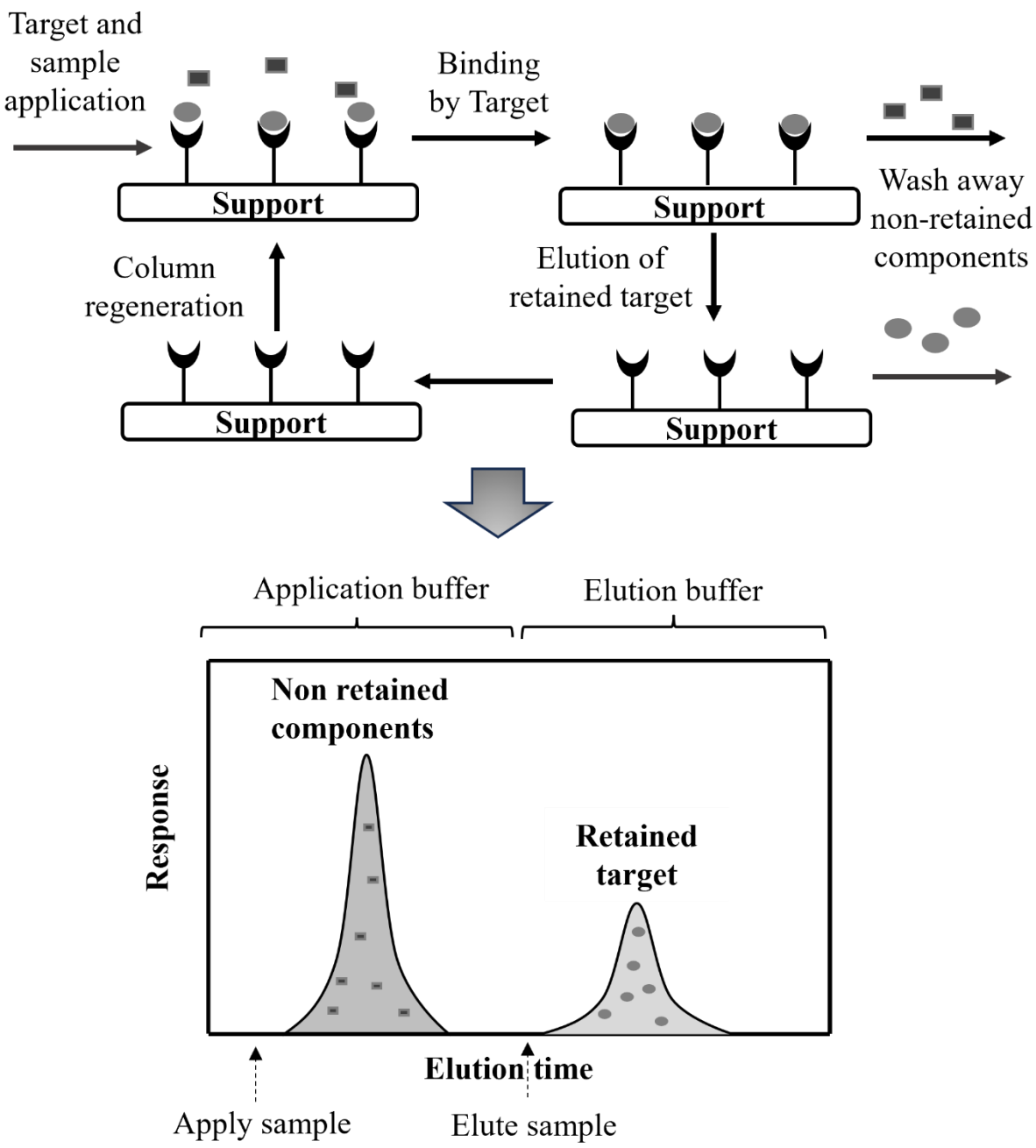
Target and sample application
Binding by Target
Support
Support
Wash away non-retained components
Column regeneration
Elution of retained target
Support
Support
Application buffer
Elution buffer
Non retained components
Retained target
Response
Elution time
Apply sample
Elute sample

Figure 2

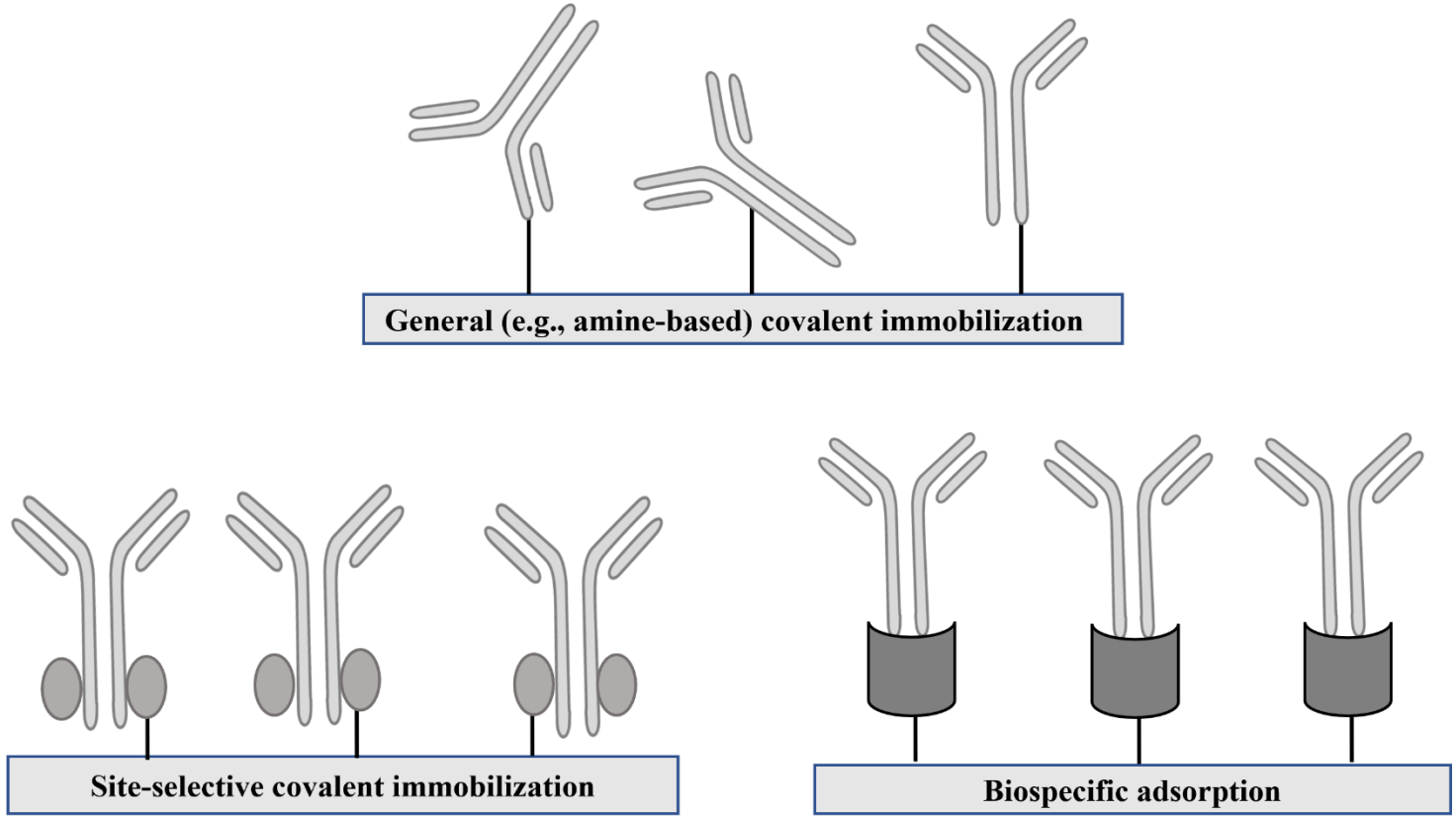
General (e.g., amine-based) covalent immobilization
Site-selective covalent immobilization
Biospecific adsorption

Figure 3

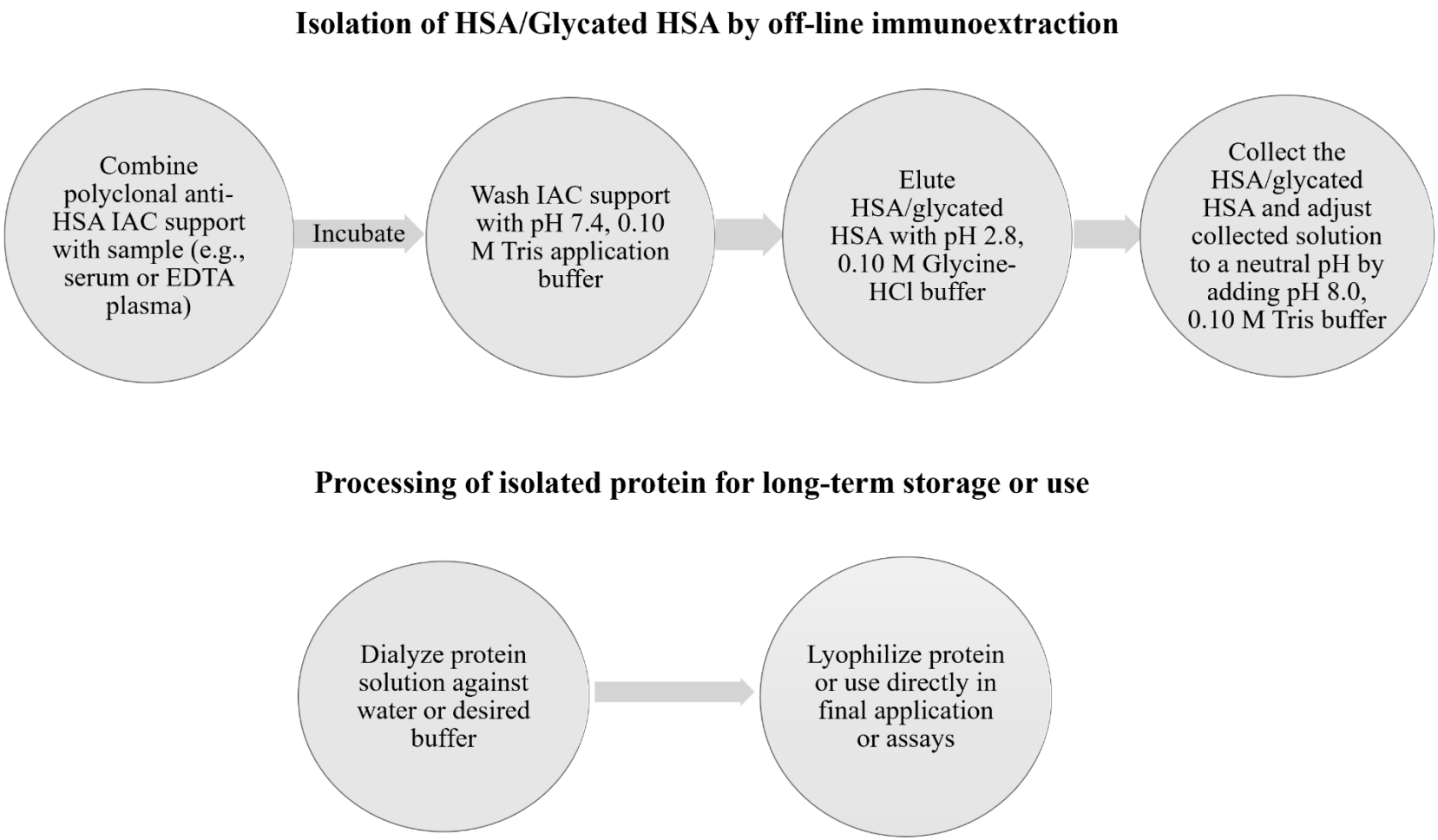
Isolation of HSA/Glycated HSA by off-line immunoextraction
Combine polyclonal anti-HSA IAC support with sample (e.g., serum or EDTA plasma)
Incubate
Wash IAC support with pH 7.4, 0.10 M Tris application buffer
Elute HSA/glycated HSA with pH 2.8, 0.10 M Glycine-HCl buffer
Collect the HSA/glycated HSA and adjust collected solution to a neutral pH by adding pH 8.0, 0.10 M Tris buffer
Processing of isolated protein for long-term storage or use
Dialyze protein solution against water or desired buffer
Lyophilize protein or use directly in final application or assays

Figure 4

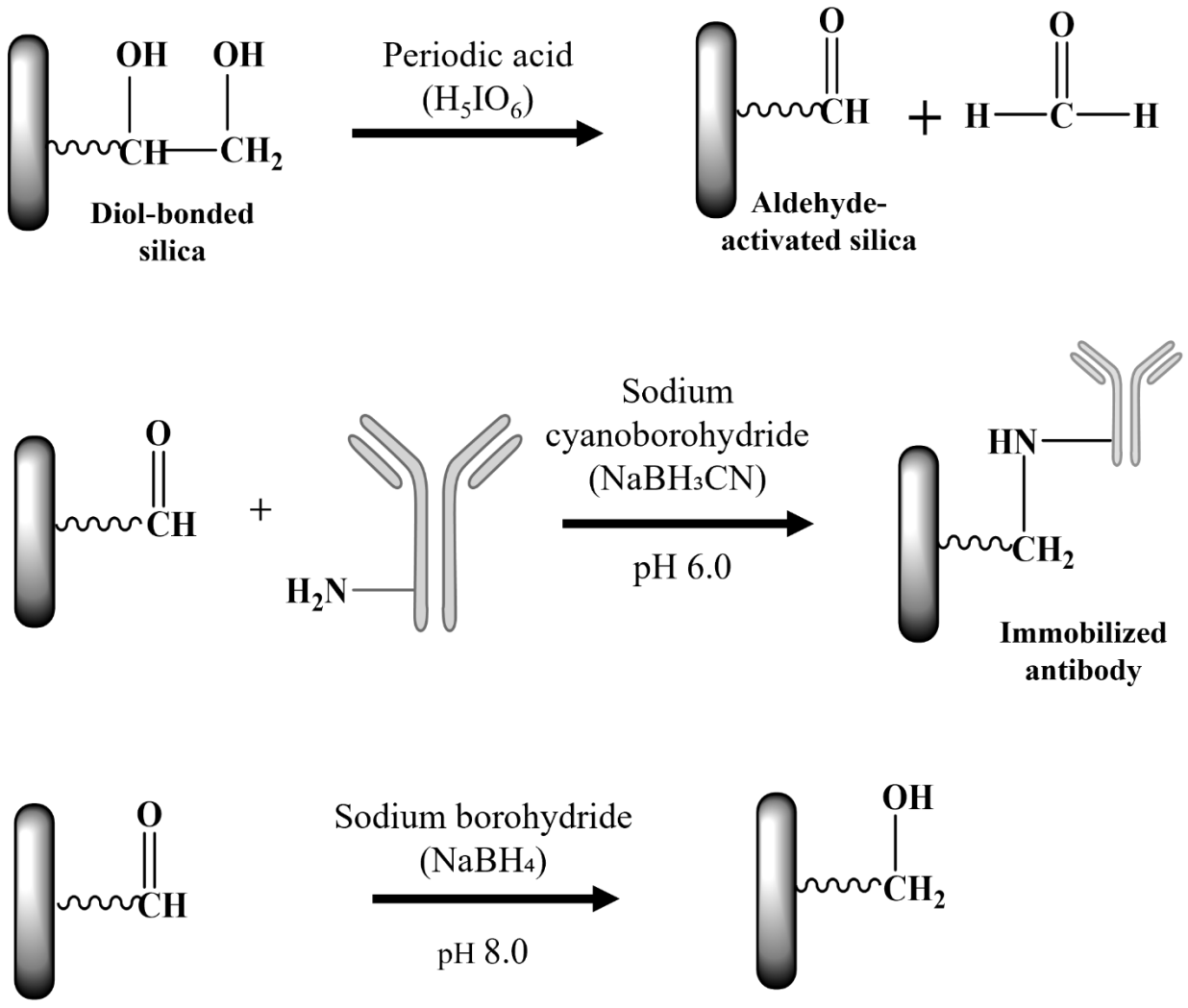
OH
OH
CH
CH2
Diol-bonded silica
Periodic acid
(H5IO6)
O
CH
Aldehyde-activated silica
O
H
C
H
O
CH
H2N
Sodium cyanoborohydride
(NaBH3CN)
pH 6.0
HN
CH2
Immobilized antibody
O
CH
Sodium borohydride
(NaBH4)
pH 8.0
OH
CH2

Figure 5

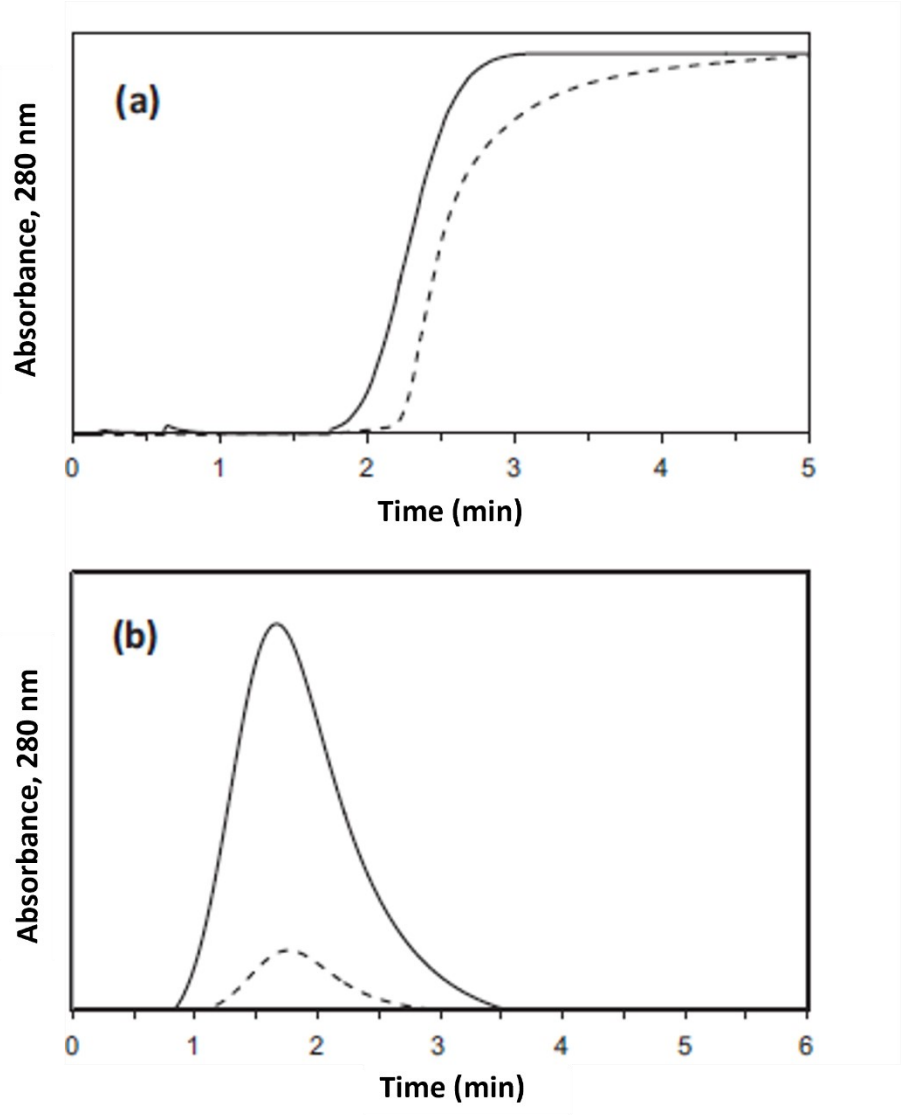
(a)
Absorbance, 280 nm
0
1
2
3
4
5
Time (min)
(b)
Absorbance, 280 nm
0
1
2
3
4
5
6
Time (min)

Figure 6

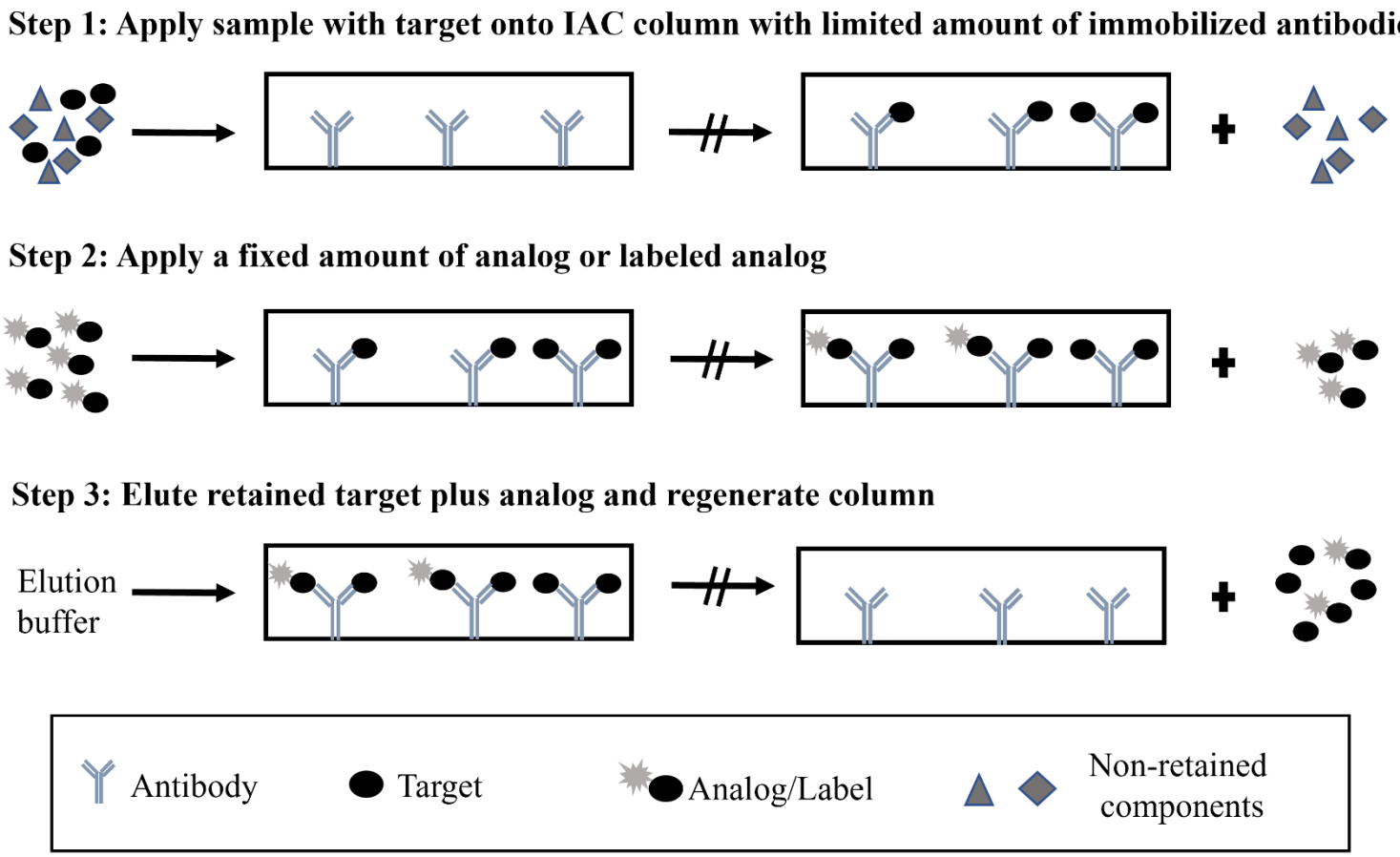
Step 1: Apply sample with target onto IAC column with limited amount of immobilized antibodies
Step 2: Apply a fixed amount of analog or labeled analog
Step 3: Elute retained target plus analog and regenerate column
Elution buffer
Antibody
Target
Analog/Label
Non-retained components

Figure 7

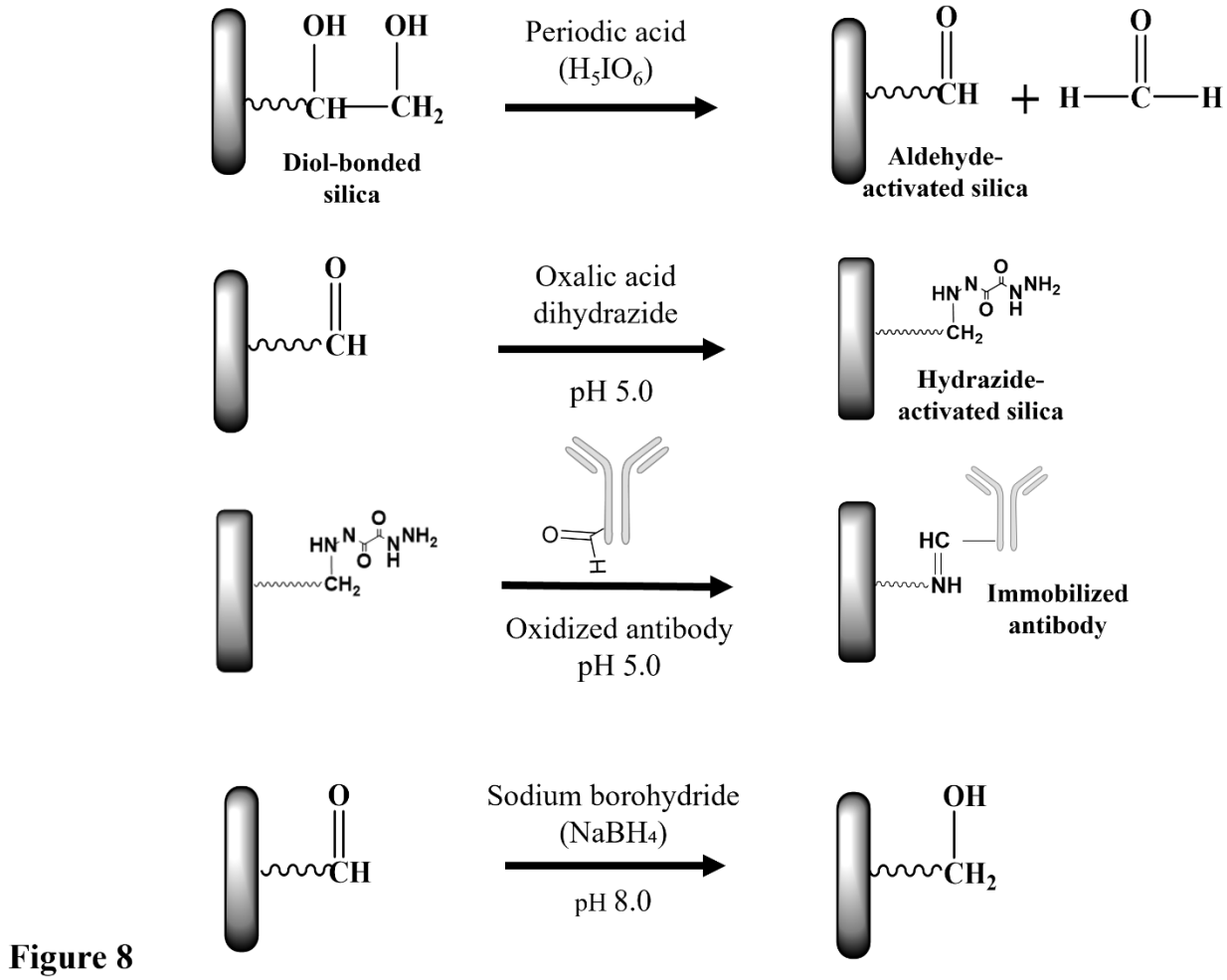
OH
OH
CH
CH2
Diol-bonded silica
Periodic acid
(H5IO6)
O
CH
+
H—C—H
Aldehyde-activated silica
Oxalic acid dihydrazide
pH 5.0
HN
N
O
NH2
CH2
Hydrazide-activated silica
O
H
Oxidized antibody
pH 5.0
HC
NH
Immobilized antibody
Sodium borohydride
(NaBH4)
pH 8.0

Figure 8

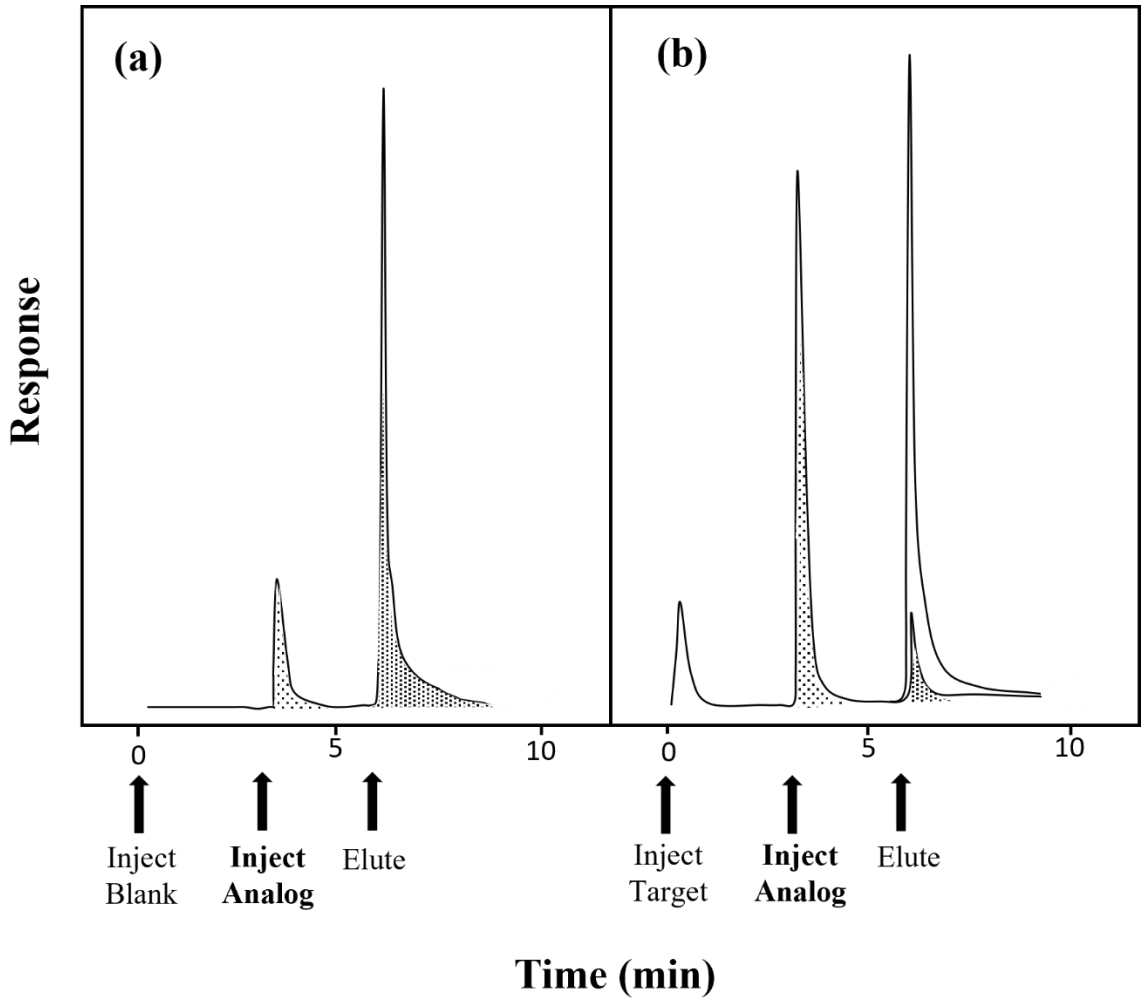
(a)
(b)
Response
0
5
10
0
5
10
Inject Blank
Inject Analog
Elute
Inject Target
Inject Analog
Elute
Time (min)

Figure 9