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Peak decay analysis and biointeraction studies of immunoglobulin binding and dissociation on protein G affinity microcolumns

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ABSTRACT

Protein G can be a valuable binding agent for antibodies and immunoglobulins in methods such as immunosensors, chromatographic-based immunoassays, and immunoaffinity chromatography. This report used the method of peak decay analysis along with frontal analysis and zonal elution studies to characterize the binding, elution and regeneration properties of affinity microcolumns that contained immobilized protein G. Frontal analysis was employed with rabbit immunoglobulin G (IgG) to characterize the binding capacity of these affinity microcolumns. Zonal elution experiments looking at the retained peaks for small injections of labeled rabbit IgG were used to optimize the column regeneration conditions. Peak decay analysis was then used to look at the effects of flow rate and elution pH on the release of several types of IgG from the protein G microcolumns. This approach made it possible to obtain detailed information on the use and behavior of such columns, as could be used in future work to optimize the capture or analysis of IgG and antibodies by such devices. The same approach and tools that were used in this report could also be adapted for work with affinity columns that make use of other supports, binding agents or targets.

1. Introduction

Supports that contain immobilized or adsorbed antibodies have been popular for many years in analytical techniques for measuring various targets [1–10]. Examples of flow-based methods in which such supports have been used have included some types of immunosensors, chromatographic immunoassays, immunoaffinity chromatography and even some approaches for studying biological interactions [1–5]. Some attractive features of these methods are the high affinity and specificity with which antibodies can bind to their complementary targets and the ability of these agents to be employed with various types of labels and detection formats [7].

A chromatographic method that employs antibodies with supports such as HPLC-grade silica or monoliths is often referred to as high-performance immunoaffinity chromatography (HPIAC) [4,9–12]. One common approach for placing antibodies onto these supports is by using covalent immobilization; however, this method can result in some loss of an antibody's activity through multi-site attachment or incorrect orientation of the antibody [8,12]. An alternative approach that avoids or minimizes these effects is to instead use biospecific adsorption of the antibody to a secondary immobilized binding agent such as protein G [12]. Protein G is a protein found in the cell walls of group G Streptococci bacteria that can bind tightly to the constant region of many types of immunoglobulins and antibodies [8,13–14]. This feature, plus the ability to release the bound antibodies through a decrease in pH and to later apply a fresh batch of antibodies, has made protein G and related immunoglobulin-binding proteins useful as tools for the capture, analysis or utilization of antibodies in chromatographic systems [8,9,15–18].

The development and optimization of methods based on protein G supports ideally requires information on such factors as the retention and elution properties of these materials when they are employed with antibodies (or, in the more general sense, immunoglobulins). Methods that have been used to study the rates of these or other biological interactions in chromatographic systems have included the split-peak method and various methods based on peak fitting or band-broadening measurements [19–22]. This report will examine the use of a technique known as the peak decay method [23,24] to study the dissociation of various types of immunoglobulins from immobilized protein G. In this technique, a small pulse of an analyte (e.g., an antibody/immunoglobulin) is injected onto an affinity column that contains the binding agent of interest. A mobile phase is then introduced

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onto the column under conditions that disrupt binding of the analyte with the immobilized agent and prevents re-association of the analyte with the column. This type of dissociation can often be produced by changing the mobile phase pH or by adding a displacement agent to the mobile phase. As the analyte is released from the column, it produces a decay curve that can be used to determine the dissociation rate constant for the analyte from the immobilized agent under the given elution conditions [23,24]. Although this method has been used in previous work to examine antibody-antigen interactions [4], it has not been used in prior work to study the protein G and its interactions with antibodies/immunoglobulins.

This report will examine the extension and use of the peak decay method to study the elution and dissociation kinetics of various types of immunoglobulin G (IgG) from protein G that has been immobilized onto HPLC-grade silica and placed into affinity microcolumns (i.e., columns containing an immobilized binding agent and with volumes in the low-to-mid microliter range) [22]. The general principles of this method will be discussed, along with various practical factors to consider in the use of this technique. This method will then be employed to examine the elution of various types of IgG from protein G microcolumns, thus providing new fundamental information on these interactions. The effect of the elution flow rate and pH will also be considered with the goal of improving the characterization and optimization of these microcolumns for future use in antibody- or immunoassay-related applications.

2. Experimental

2.1. Materials

The 3-glycidoxypropyltrimethoxysilane, periodic acid, sodium cyanoborohydride and sodium borohydride were from Sigma-Aldrich (St. Louis, MO, USA). The following types of IgG were also obtained from Sigma–Aldrich: rabbit IgG (>95% pure), goat IgG (>95%), human IgG (>95%), and mouse IgG (>95%). The protein G (recombinant, albumin binding domains removed) was purchased from Pierce (Rockford, IL, USA). The amount of immobilized protein on each support was determined in triplicate by using a bicinchoninic acid (BCA) protein assay, which was conducted by using reagents that were also obtained from Pierce. These immunoglobulins were labeled with *N*-hydroxysuccinimide (NHS) ester-activated fluorescein from Pierce. Other sources of protein G and IgG, or related binding agents and targets, can also be used for the peak decay method, as well as other types of fluorescent or chemical labels. All running buffers and aqueous solutions that were used in this report were prepared using deionized water, as was obtained in the following examples by using an EMD MILLI-Q water purification system from Millipore (Billerica, MA, USA) and 0.2 μm GNWP nylon filters from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Apparatus

Many standard HPLC systems can be adapted for use in the peak decay measurements. The HPLC system that was used in this particular study was a Jasco 2000 system (Easton, MD, USA) that contained a DG-2080-53 three-solvent degasser, three PU-2080 isocratic pumps, an AS-2057 autosampler equipped with a 100 μL sample loop (operated in the partial loop injection mode), a UV-2075 absorbance detector, and a FP-2020 fluorescence detector. Two Advantage PF six-port switching valves (Rheodyne, Cotati, CA) were used for alternating passage of an IgG solution and acidic or neutral buffer solutions through the microcolumns during the frontal analysis studies. The system components were controlled by a Jasco LC-Net II/ADC system and a Jasco ChromNav chromatography data system. The breakthrough times for the frontal analysis data and elution profiles were examined by using PeakFit 4.12 (SeaSolve Software, San Jose, CA).

All binding studies were carried out at room temperature (25 °C) in this report. Work at other temperatures can also be conducted by using an on-line column heater or a circulating water bath and column jacket for temperature control [1]. The elution of unlabeled IgG was monitored at 280 nm. Detection of fluorescein-labeled IgG was monitored by using an excitation wavelength of 494 nm and an emission wavelength of 518 nm.

Purification of the labeled IgG was performed by using Zeba spin columns (7 kDa MW cutoff, 0.7–4 mL sample capacity) from Pierce, along with a 5702RH temperature-controlled centrifuge from Eppendorf (New York, NY, USA) and a fixed-angle centrifuge rotor from VWR (West Chester, PA, USA). The microcolumns were packed using an HPLC slurry packing system from ChromTech (Apple Valley, MN, USA); however, other column packing systems can also be used for such work.

2.3. Antibody labeling

IgG was labeled with NHS-fluorescein, according to the manufacturer's instructions. In this process, the initial protein solution was prepared by dissolving 5 mg IgG in 5 mL of pH 8.5, 0.10 M potassium phosphate buffer (i.e., giving a 1.0 mg/mL IgG solution). A 1 mg portion of NHS-fluorescein was dissolved in 100 μL dimethylformamide, and 25 μL of this NHS-fluorescein solution was added to 5 mL of the pH 8.5 IgG solution, resulting in a reaction mixture that contained 0.05 mg NHS-fluorescein per mg IgG. This mixture was allowed to shake for 1 h in the dark at room temperature.

Zeba spin columns were utilized to purify and separate the labeled IgG from any unreacted NHS-fluorescein. Prior to use, each spin column was washed three times with pH 7.4, 0.067 M potassium phosphate buffer for buffer exchange. The labeled IgG solution was then loaded into two spin columns and centrifuged at 1000×*g* for 2 min. The labeled IgG solutions that remained in the spin columns were then pooled for further use. The label/protein ratio and concentration of the final labeled IgG solution were determined by making absorbance measurements at 494 and 280 nm, according to the manufacturer's directions. The final labeled IgG solutions had the following measured concentrations: rabbit IgG, 0.76 mg/mL (5.4 μM); mouse IgG, 0.81 mg/mL (5.1 μM); goat IgG, 0.77 mg/mL (5.2 μM); and human IgG, 0.92 mg/mL (6.1 μM). The sample solutions contained 3–6 (average, 5) moles of label per mol of IgG. The labeled IgG solutions were stored at 4 °C in pH 7.4, 0.067 M potassium phosphate buffer when not in use. These labeled IgG conjugates and were stable for up to 2 weeks when protected from light and stored under these conditions [25].

2.4. Support and microcolumn preparation

The method that is described in this article can be employed with a variety of supports and immobilization schemes. The specific examples that are described here were all conducted using Nucleosil Si-1000-7 silica (7 μm particle size, 1000 Å pore size) that was purchased from Macherey-Nagel (Düren, Germany). A pore size of 1000 Å was chosen for this support to allow sufficient room for the immobilization of protein G and the later binding of this agent to antibodies. Although silica with a smaller pore size (e.g., 50–500 Å) could be employed to make protein G supports, and it has been shown that supports with the larger pore sizes that were