DEVELOPMENT OF BEAD INJECTION METHODOLOGY FOR IMMUNOASSAYS

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Abstract

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The research presented in this thesis develops Bead Injection techniques for application in immunological research and with the aim of developing rapid screening methods for use in clinical settings.

A method is introduced, called label dilution, which is analogous to the well-established isotope dilution method. The principle is tested on a model system of commercially available antibodies and protein-coated Sepharose beads and implemented using Bead Injection in the lab-on-valve format. This micro-scale method uses a labeled form of the target molecule as an internal standard. Label dilution employs ratiometric measurements using the absorbance signals from the label and the target molecules for quantitative determination of an analyte. The label dilution method is shown to discriminate between selective and non-selective binding and provides a means for monitoring bioligand interactions in real time. This Bead Injection method provides a sensitive, automated technique for the determination of low-level analytes in complex samples.

The second phase of this work introduces an analytical method for the detection and study of GAD65 autoantibodies, which have been implicated in the onset of type 1 diabetes. There is a clinical need for a rapid and automated assay for GAD65

autoantibodies. Therefore, this method has been designed to exploit the advantages of Bead Injection analysis for enzyme-linked immunosorbent assays (ELISA). BI ELISA is a microscale technique that uses enzyme-labeled secondary antibodies to detect the capture of target antibodies on antigen-coated Sepharose beads. BI ELISA offers the same signal amplification and indirect detection as traditional ELISA protocols, but has the advantages of reduced assay time, fewer sample preparation steps, and complete automation of solution handling and detection.

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CHAPTER 1

INTRODUCTION TO BEAD INJECTION TECHNOLOGY

1.1 Principles of Sequential Injection

Sequential Injection (SI) is an established analytical method, developed for automating sample preparation and matrix removal procedures, as well as microchemistries. SI provides fluid-based automation driven by pumps and valves in an enclosed system. First introduced in 1990 [1], Sequential Injection is based on the method of Flow Injection Analysis developed by Ruzicka and Hansen in 1974 [2]. While Flow Injection is based on continuous forward flow, Sequential Injection employs computer-controlled forward, stopped, and reversed flow. The underlying principles of Flow Injection and SI are the injection of sample into a carrier liquid, the controlled dispersion of sample and reagent zones into one another and the carrier liquid, and reproducible timing. Precise fluidic control results in reproducible flow profiles and timing of analyses, such that every sample analyzed under the same experimental parameters experiences the same dispersion and interaction with reagents as any other sample. Because every sample is handled exactly the same for a given protocol, product measurement can be made before the reaction reaches equilibrium. Sequential Injection was originally designed as a stand-alone instrument utilizing spectroscopic or electrochemical detection, but has also evolved into a means of enhancing other analytical methods such as high performance liquid chromatography, atomic absorption spectrometry, mass spectrometry, infrared spectroscopy, microscopy, and radiometric analyses.

A Sequential Injection system uses a computer controlled, bi-directional syringe pump to aspirate and dispense samples and reagents that are clustered around a multiport valve (MPV) and deliver the resulting product to a detector (Figure 1.1). The MPV provides a means of randomly accessing the sample port, reagent reservoirs, and detection flow cell. The pivoting groove of the MPV is rotated from port to port to allow injection of the sample and a number of reagents in any desired order. The syringe pump is connected to the center of a multiport valve via the holding coil, a length of tubing with sufficient volume to prevent reagent or sample from entering the syringe pump during reversed flow. Samples and reagents are introduced and manipulated by the carrier stream, which is typically buffer or water. As small volumes (microliter to milliliter) of the sample and reagents are sequentially aspirated into the holding coil, using reversed flow, the stacked zones disperse into one another, initiating a chemical reaction (Figure 1.2). The contents of the holding coil are dispensed, using forward flow, through one of the ports to the downstream detector. The product of the reaction is monitored continuously as it passes through the detection flow cell, producing a peak whose height, width, and area are each proportional to the product concentration.

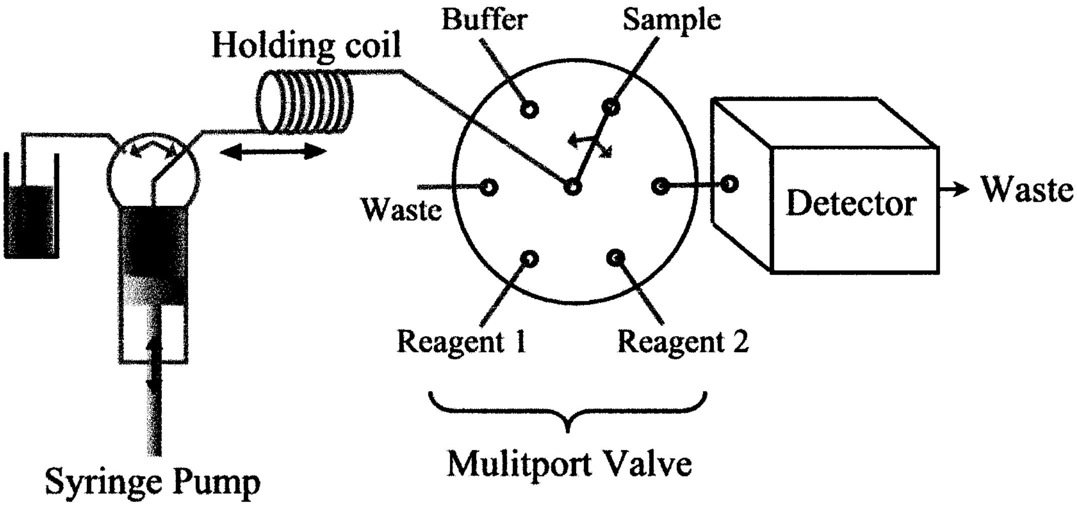

Figure 1.1 Schematic of SI system. Bi-directional syringe pump aspirates samples and reagents from positions on the multiport valve using reversed flow. Forward flow is used to push the reaction components to the detector where product formation is monitored in real time.

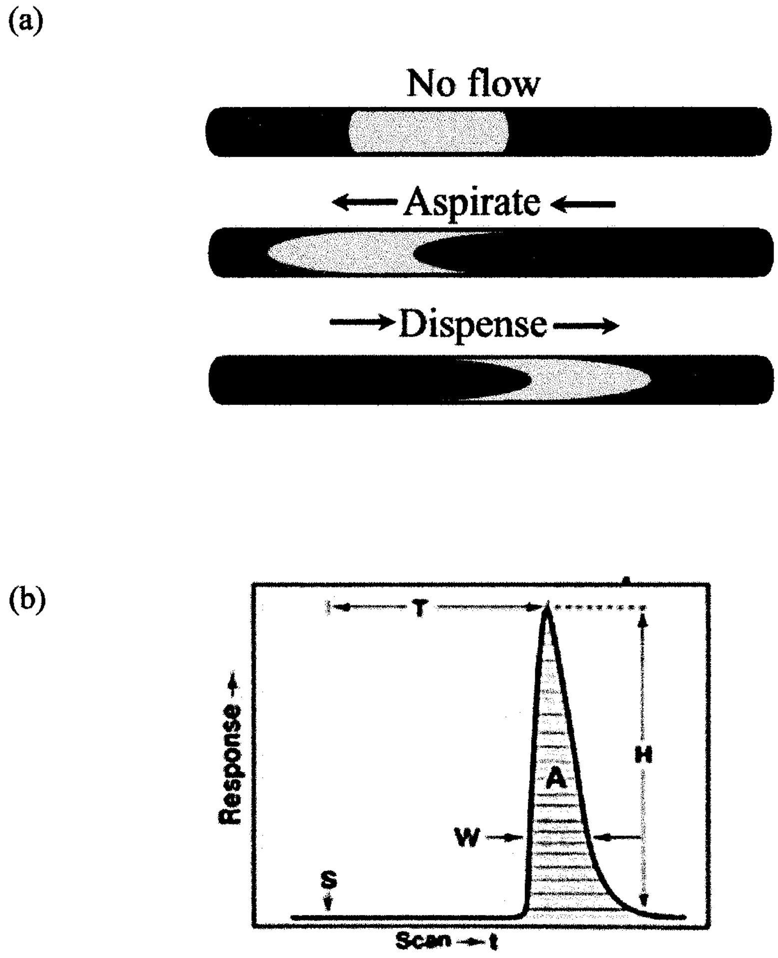

Figure 1.2 Dispersion and mixing in SI and typical SI peak. (a) Dispersion and mixing of sample and reagent zones as liquids are transported in the fluid channels. (b) Typical SI peak with height, width, and area proportional to product concentration. Reprinted with permission from Flow Injection CD, 2nd Edition [3].

As the sample and reagent zones travel through the system, dispersion and mixing occur, facilitating the chemical reaction. Dilution of the sample and reagents by dispersion is proportional to the distance that the zones travel in the system while being injected and carried to the detector. Axial dispersion is a function of the flow rates used and radial dispersion is dependent upon the shape of the transport channels. Dispersion of the fluid zones is enhanced by the rough inner surface and coiled structure of the tubing combined with changes in direction as the zones travel through the multiport valve. Increased dilution and mixing can be achieved by increasing the magnitude of flow reversal or by injecting a volume of carrier as a spacer between the reaction and the detector. While continuous flow is often used, the flow may also be stopped for a specific amount of time to allow the product of the reaction to accumulate. The flow is stopped when the highest concentration of analyte is in front of the detector, providing additional incubation time for signal amplification as well as a means of monitoring product formation as a function of time.

Fluid-based assays benefit from discontinuous multidirectional flow, as it provides great versatility in the sequencing of sample handling operations. Sequential Injection is characterized by four main attributes:

- high precision fluidic manipulation
- reproducible timing of analyses
- low sample and reagent consumption

- great flexibility in experimental design

The open architecture design of Sequential Injection systems allows for a variety of modifications to experimental protocols. The volumes and flow rates used in an experiment are dictated by the syringe size and the inner diameter and length of tubing chosen for the system. The syringe, tubing, and computer program parameters can easily be altered to create a wide variety of assays. In addition to almost any combination of flow rates and volumes, the SI system is amenable to wide range of detection methods. This has led to the use of Sequential Injection in a variety of applications, including environmental monitoring, food and beverage analysis, pharmaceutical analysis, and process control [4], as well as cytoanalysis [5,6,7], bioprocess monitoring [8], and immunoassay [9].

1.2 Principles of Bead Injection

Bead Injection (BI) represents the third generation of Flow Injection techniques. Initially referred to as a Flow Injection Renewable Surface technique, BI is a combination of solid phase chemistry and fluidic handling of microcarrier beads, allowing automated surface renewal and post analysis manipulation of the sensing surface [10]. Chemical reactions occur at the bead surfaces and are analyzed in real time, either directly on the solid phase or by monitoring the eluting liquid phase.

Microcarrier beads provide a support for the adsorption of reagents or reactive groups. Immobilization of reagents on the beads provides several advantages over SI:

- analyte can be preconcentrated in front of the detector for increased sensitivity
- the bound reactant is held in place preventing dilution
- the rate of reaction can be measured as it occurs on the surface of the beads
- the sensing surface is renewable

Bead Injection employs functionalized chromatographic beads as a solid phase support. These beads are available in a wide range of sizes and with a variety of surface derivatizations (see Chapter 2). Upon completion of the assay, the beads can be discarded, collected, or redirected for additional analysis.

Whereas Sequential Injection is a solution-based technique, Bead Injection can be envisioned as an extension of SI with the added element of a solid phase. In general, BI assays are comprised of five steps:

1) delivery of the beads to the flow cell
2) perfusion of trapped beads with carrier and establishment of spectral baseline
3) sample injection
4) removal of sample matrix or analyte elution

5) bead removal

Several configurations for capturing beads have been developed [3] and the Jet Ring Cell (JRC) [11,12] is discussed as an example. The repeatability and reliability of the Bead Injection technique necessitates a geometry for trapping beads that allows consistent packing and uniform perfusion. Using SI techniques, a precise volume of bead suspension is aspirated into the holding coil and subsequently injected into the detection chamber. The flow-through design of the chamber allows the beads to be captured, forming a small column, while carrier solution passes through to waste (Figure 1.3). The JRC consists of a solenoid valve and a stainless steel delivery tube connecting the multiport valve with a flat surface at the detector. Microcarrier beads are delivered to the tube, accumulating in front of the detector, while carrier buffer perfuses out of the tube through a small gap between the end of the tube and the flat surface. During an assay, a sample zone is delivered to the detection chamber where it perfuses through the bead column, reacting with immobilized reagent(s). Product formation can be monitored directly, in real time, as the reaction proceeds. In an assay where sample components are captured by immobilized ligands, the complex formed can be eluted for secondary detection downstream or collected for later evaluation. After the assay, the solenoid valve is actuated, lifting the tube to increase the size of the gap. The beads are flushed out using forward flow and the solenoid is turned off, returning the tube to its original position and closing the gap. The JRC is ready to be loaded with a fresh column of beads for the next assay.

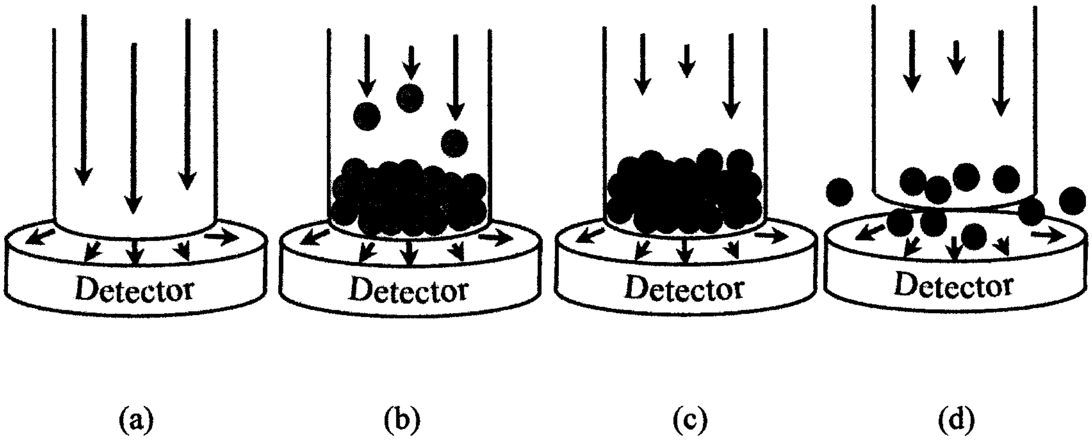

Figure 1.3 Schematic of Jet Ring Cell. (a) Buffer is delivered to the flow cell ahead of bead delivery. (b) The beads are delivered to the flow cell, while carrier buffer exits through the gap between the tube and flow cell bottom. (c) Sample is perfused through the bead column as the reaction is monitored on the bead surfaces. (d) After the assay, the solenoid is actuated, lifting the tubing, and the beads are flushed to waste.

Along with the features it shares with SI, Bead Injection is characterized by an easily renewable surface that provides a reproducible support for monitoring solid phase reactions. The beads needed for several assays are batch-prepared such that the bead column formed for each assay in a set of experiments is representative of the entire batch. On-column detection provides the ability to concentrate the immobilized components in the detection path, eliminating the effects of dilution seen in Sequential Injection and providing a means of signal amplification. In the case of an assay aimed at capturing a low-level analyte, the flow-through design of the detection chamber allows for a larger volume of sample to be used to increase the signal since unbound matrix components will be carried to waste. Bead Injection technology has been employed in the study of chemical sensors, cellular signaling [13], functional responses [14,15,16], and bioligand interactions [12,17,18] as well as for the determination of metals, radionucleotide activity, and pH [4].

1.3 Principles of the Lab-on-valve

The most recent development in the evolution of Sequential and Bead Injection techniques is the lab-on-valve (LOV) sample-processing manifold [19]. Aimed at downscaling reagent based assays, the LOV has been designed to minimize the distance between the sample injector and the flow cell while preserving the flexibility of SI to allow any sequence of experimental steps. Solution sampling, reagent addition, mixing, dilution, incubation, and monitoring can be performed in any desired sequence in the LOV, which integrates sample-processing channels with a multipurpose flow-through detection cell. As in conventional Sequential Injection, sample handling with the lab-on-valve can be programmed to include forward, reversed, or stopped flow, allowing for a variety of assays to be performed in the same microfluidic device. While the LOV is a microconduit structure, the other components of the instrument (syringe, tubing, fittings, fiber optics) are standard to Sequential Injection techniques. The LOV is ideally suited for bead manipulation allowing short regular paths for good repeatability.

The LOV is a meso-machined block of polymethylmethacrylate mounted atop the conventional multiport valve (MPV) of an SI system (Figure 1.4). The 1.70 mm inner diameter channels at each of the ports terminate at the perimeter of the LOV with standard threads to accommodate normal fittings and tubing with an outer diameter of 1.58 mm. The multipurpose flow cell has four channels connected to it. One channel connects the flow cell to the multiport valve and has an inner diameter of 500 μm, minimizing the volume and distance between the sample injector and the detector. The other channels connecting the flow cell with the LOV perimeter have inner diameters of 1.70 mm, which accommodate tubing, plugs, or fiber-optic casings with 1.58 mm outer diameters while leaving a gap large enough that solutions can pass by and out to waste. The positions of these components inserted into the flow cell channels define its dimensions and volume. The ability to vary the position of the

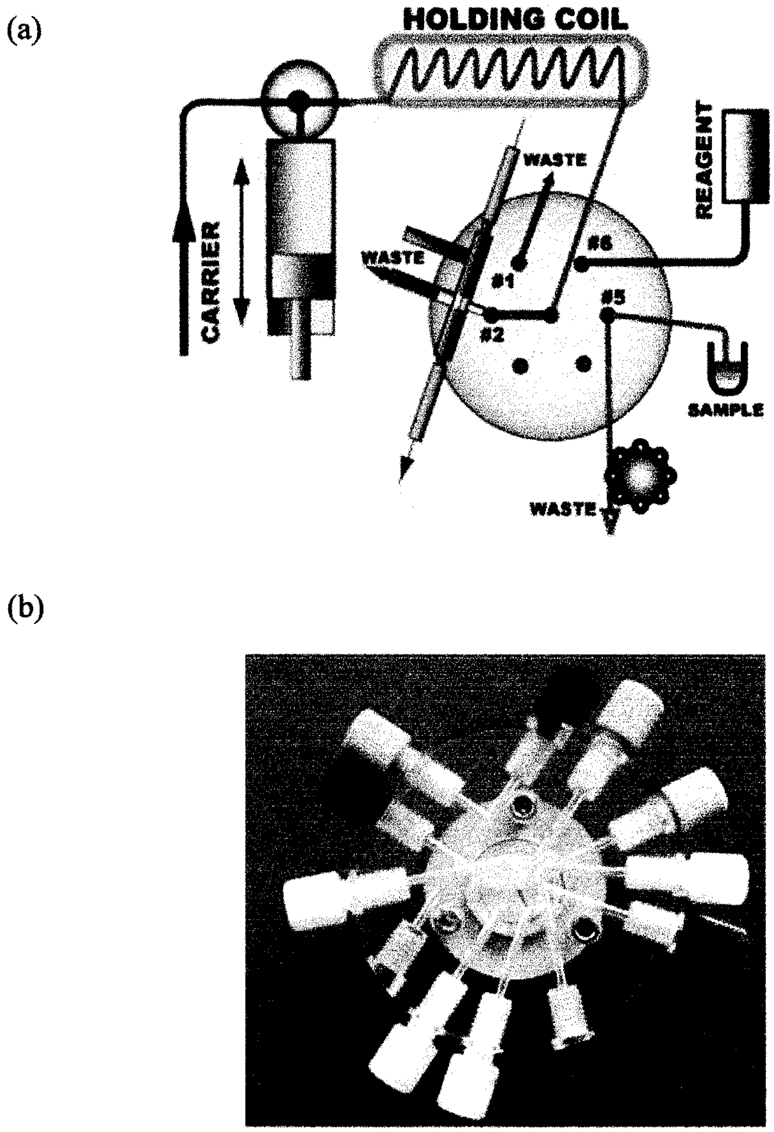

Figure 1.4 Lab-on-valve schematic and picture. Reprinted with permission [3]. (a) Schematic of SI system with LOV module, integrating the multiport valve and multipurpose flow cell (port #2). (b) Picture of LOV mounted on traditional multiport valve in the same orientation as schematic in (a). The black fittings indicate the channels in which the fiber optics can be placed to perform absorbance and/or fluorescence measurements.

fiber optics and the channels in which they are placed (see Figure 1.4) allows the multipurpose flow cell to be used for short-path absorbance, long-path absorbance, fluorescence, or simultaneous absorbance and fluorescence measurements [19]. As in SI, sample and reagent are injected through the multiport valve into the holding coil by reversed flow. Forward flow is employed to transport the sample or reagents to the flow cell, where analyte accumulation or product formation is monitored. The design of the lab-on-valve provides relatively large conduits that minimize surface contamination and clogging despite its ability to work in the micro- and nanoliter ranges.

The LOV is amenable to BI assays and provides an alternative bead capture method to the mechanically more complex Jet Ring Cell. When the bead suspension is delivered to the flow cell, fiber optics or plugs defining the volume of the flow cell efficiently trap the beads while the suspension fluid and carrier buffer are transported to waste. After an assay, the beads are effectively discarded from the LOV flow cell by a short burst of reversed flow, drawing the beads back into the holding coil, followed by forward flow through a port designated for waste.

Since its introduction, the lab-on-valve has been applied to the study of bioligand interactions [20,21], cellular metabolism [22,23], and chemical separation [24,25], as well as fermentation [26] and environmental monitoring [27]. The most significant features of the lab-on-valve sample-processing unit are:

- the close proximity of the sample injection site and detector flow cell
- the multipurpose flow cell
- the flexibility of fluidic programming
- an improved means of trapping and discarding beaded support

The shorter fluid path provided by the LOV manifold results in less dispersion of the sample and reagents as they are aspirated and delivered to the flow cell. This design also makes bead removal via reversed flow more practical than with the Jet Ring Cell and avoids the need to mechanically manipulate components of the flow cell between assays. The versatility and flexibility of the system allow for a variable flow path to meet a wide range of experimental needs. This new approach to Sequential and Bead Injection has the advantages of a compact apparatus, complete automation of entire assay protocols, and the integration of all fluidic transport and flow cell components into a single structure that ensures the reliability and reproducibility of sample processing. The development of Bead Injection methodologies using the lab-on-valve is the basis for this work.

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CHAPTER 2

SOLID PHASE SUPPORTS FOR LIGAND IMMOBILIZATION

2.1 Introduction

In the early years of immunoassay, separating complexed reactants from unbound reactants proved to be difficult. When it was realized that proteins could passively adsorb onto plastic surfaces, solid phase assays became a means of conveniently and efficiently separating bound molecules from excess reactant [1]. Since the introduction of solid phase immunoassay, the plastic test tubes originally used as solid phases have been replaced by microtiter plates, nitrocellulose and nylon membranes, beads made of polystyrene and methylmethacralate, and microparticles. Solid phases can be grouped into three categories [2] (Table 2.1):

- *Plastic labware*: tubes, microtiter plates, tissue culture plates; most commonly polystyrene
- *Beaded materials*: distinctive in chemistry and performance compared to other types of phases; both natural (carbohydrate) and synthetic (plastic) materials
- *Membranes*: large surface area, susceptible to higher non-specific binding; nitrocellulose for proteins and nylon for nucleic acids

Table 2.1 Solid supports for immunoassays [2].

Solid Phase	Bonding Force	Relative Surface Area
Plastic Labware		
Polystyrene	Hydrophobic	Modest
Irradiated Polystyrene	Hydrophobic, Hydrophilic, Covalent	Modest
Surface-functionalized Polystyrene	Hydrophobic	Modest
Beaded material		
Polystyrene	Hydrophobic	Moderate
Derivatized Polystyrene	Covalent, Hydrophobic, Hydrophilic	High
Agarose and derivatives	Hydrophilic, Covalent	High
Microparticles	Hydrophobic, Covalent	Very High
Membranes		
Nitrocellulose	Hydrophobic, Hydrophilic	Very High
Nylon	Hydrophobic	Very High
Charge modified nylon	Hydrophilic, Covalent, Hydrophobic	Very High
Functionalized nitrocellulose	Hydrophobic, Covalent, Hydrophilic	Very High
Polyvinyl difluoride	Hydrophobic	Very High

Solid phases should not be viewed merely as passive components in the process of ligand immobilization. Ligands can be immobilized on the support via noncovalent interactions, such as hydrophobic and hydrophilic forces, as well as through covalent bonding. Solid-phase antigen-antibody interactions occur in the micro-scale environments at the liquid-solid interface and, while schematics typically depict solid phases as being flat and smooth, the surfaces can be highly contoured.

2.2 Beaded Supports

For application in the biological sciences, beaded supports must be mechanically robust and have an inert surface that will not interact with biomolecules or initiate any biological response [3]. The support material needs to be amenable to chemical modification such that the surface of the beads can be easily derivatized for the immobilization of ligands appropriate for the assay.

Beaded supports are produced from both natural and synthetic materials (Table 2.2). Naturally occurring supports include dextran, agarose, and cellulose. These matrices are polysaccharide polymers that can be formed into beaded supports and often contain functional groups, such as hydroxyl, thiol, and amino groups, which can be derivatized to permit the attachment of reactive side chains. Synthetic supports include polyacrylamide, polysaccharide-acrylamide blends, polystyrene, silica, and

Table 2.2 Beaded matrices for ligand immobilization [6].

Matrix	**Chemical stability**	**pH range**	**Temperature range**	**Coupling groups available**
Agarose Beads	*Fair* Avoid chaotropic agents, extended exposure to urea or guanidine	4-10	4-30°C	-OH
Cross-linked Agarose Beads	*Good* Avoid extended exposure to chaotropic agents	3-14	4-120°C	-OH
Polyacrylamide Beads	*Excellent* Strong alkali on uncoupled beads converts amide to carboxylic	2-10	4-120°C	$-NH_2$
Copolymers of Polyacrylamide and Agarose	*Fair* Avoid chaotropic agents, extended exposure to urea or guanidine	4-10	4-30°C	-OH $-NH_2$
Polyacrylic Beads	*Excellent* Strong alkali on uncoupled beads converts amide to carboxylic	<1-11	-20-120°C	-OH

glass. In addition to the wide variety of beads available, there is an extensive selection of activation and derivatization methods for functionalizing support matrices [4,5,6] (Table 2.3).

Agarose and dextran-based beads have been employed in Bead Injection assays aimed at studying cellular signaling [7], cellular metabolism [8], and bioligand interactions [9]. Commercially available as Sepharose, Sephadex, and Cytodex, these beaded supports are offered with a variety of functional groups or ligands already attached. Cross-linked Sepharose beads from Sigma Chemical arrive pre-swollen and range from 40-165 µm in diameter. Because agarose and dextran matrices retain a lot of water and are translucent, these beads are especially amenable to spectroscopic detection. Once the bead column is packed, a spectral baseline can easily be established and the reaction can be monitored in real time as it occurs on the surface of the beads. The size and flexibility of agarose and dextran beads allows for easy handling within the confines of the lab-on-valve manifold and the Bead Injection system.

2.3 Ligand Immobilization

Solid-phase reactants, or ligands, can be immobilized via several general methods:

- Direct adsorption – most widely used with microtiter plates and nitrocellulose

Table 2.3 Reagents for activating beaded matrices [6].

Reagent	**Binding group on matrix**	**Ligand attachment**	**Stability of final linkage**
Carbonyldiimidozole	-OH	$-NH_2$	Avoid pH > 10
Cyanogen Bromide	-OH	$-NH_2$	Good, some leaching
Glutaraldehyde	$-NH_2$	$-NH_2$	Excellent
Hydroxysuccinimide	-COOH	$-NH_2$	Excellent
Tosyl Chloride	-OH	$-NH_2$, -SH	Excellent

- Covalent attachment to functionalized surface – most common with hydrophilic beads (agarose, dextran) or heavily functionalized polystyrene beads
- Immunochemical immobilization – using a capture antibody to immobilize an antigen
- Other nonadsorbent, noncovalent methods – biotin-avidin linkage and Ig-binding proteins (protein A or G)

Experimental evidence demonstrates that passive adsorption, as a means of immobilizing a ligand, results in the loss or modification of antigenic epitopes, the decrease or loss of enzymatic activity, the formation of new epitopes, definite physicochemical changes and the decrease or loss of antibody activity [2]. To avoid these problems, a spacer molecule can be used to bind the ligand to the solid phase without inducing conformational changes in the ligand and affecting the binding activity of the target complex. While there are several spacer molecules and methods that have been used for attaching ligands to the solid phase [4,5,6] (Table 2.4), bacterial Ig-binding proteins A and G and biotin-avidin linkages are especially relevant to this work.

2.3.1 Proteins A and G

Proteins A and G are bacterial cell wall components that have evolved as a means of protecting bacteria against immunological attacks [5]. These proteins bind to immunoglobulin (Ig) molecules at the C_{H2} and C_{H3} regions (see Chapter 3, Figure 3.2),

Table 2.4 Antibody immobilization methods [6].

Method	**Variations**	**Antibody oriented?**	**Coupling group on antibody**
Proteins A and G	Direct coupling	Yes	$-NH_2$
	Coupling through anti-Ig antibody	Yes	$-NH_2$
Activated Beads	Carbonyldiimidazole	No	$-NH_2$
	Cyanogen bromide	No	$-NH_2$
	Glutaraldehyde	No	$-NH_2$
	Hydroxysuccinimide	No	$-NH_2$
	Tosyl chloride	No	$-NH_2$, $-SH$
Activated Antibody	Carbodiimides	No	$-NH_2$, $-COOH$
	Condensing agents	No	$-NH_2$
	Glutaraldehyde	No	$-NH_2$
	Periodate	Yes	Sugar
Biotin-antibody on Streptavidin Beads	–	No	$-NH_2$

leaving the antigen-binding sites available to capture antigen molecules. Protein A is a peptide chain produced by numerous strains of *Staphylococcus aureus*. The native form of protein A has a molecular mass of 42 kD, contains very few carbohydrates, and has four binding sites for Ig molecules. A recombinant form of protein A has been genetically shortened, resulting in a molecular mass of 32 kD, while maintaining affinities similar to those of native protein A and reducing the nonspecific binding. Protein G is produced by group G *Streptococci* and functions in a similar manner as protein A. The native form of protein G contains binding sites for albumin, portions of the antigen-binding sites, and membrane binding regions, which can result in higher levels of nonspecific binding. Recombinant protein G has been engineered to remove the albumin binding sites and reduce nonspecific binding [10]. Unlike protein A, protein G does not bind to the human antibody classes IgM, IgD, and IgA [4]. The affinities of the various IgG subclasses vary significantly for protein A versus protein G (Table 2.5). These Ig-binding proteins are used for chromatographic separation of antibodies, removal of antibody-antigen complexes from clinical samples, and also as a means of immobilizing antibodies on solid phase supports for immunoassays [5].

2.3.2 Biotin and Avidin

The interaction between biotin and avidin is one of the strongest noncovalent bonds known [11], with an affinity constant of 10^{-15} M. The specificity and strength of interaction between these molecules are routinely exploited in a variety of

Table 2.5 Binding capabilities of immunoglobulins with proteins A and G [11].

Antibody	Protein A	Protein G	Antibody	Protein A	Protein G
Human IgG	s	s	**Human IgG3**	w	s
Mouse IgG	s	s	**Human IgG4**	s	s
Rabbit IgG	s	s	**Mouse IgG1**	w	m
Goat IgG	w	s	**Mouse IgG2a**	s	s
Rat IgG	w	m	**Mouse IgG2b**	s	s
Sheep IgG	w	s	**Mouse IgG3**	s	s
Bovine IgG	w	s	**Rat IgG1**	w	m
Guinea Pig IgG	s	w	**Rat IgG2a**	nb	s
Hamster IgG	m	-	**Rat IgG2b**	nb	w
Pig IgG	s	w	**Rat IgG2c**	s	s
Horse IgG	w	s	**Bovine IgG1**	w	s
Donkey IgG	m	s	**Bovine IgG2**	s	s
Dog IgG	s	w	**Sheep IgG1**	w	s
Cat IgG	s	w	**Sheep IgG2**	s	s
Chicken IgG	nb	nb	**Goat IgG1**	w	s
Human IgM	w	nb	**Goat IgG2**	s	s
Human IgE	m	nb	**Horse IgG(ab)**	w	nb
Human IgD	nb	nb	**Horse IgG(c)**	w	nb
Human IgA	w	nb	**Mouse IgM**	nb	nb
Human IgG1	s	s	**Human IgA1**	w	nb
Human IgG2	s	s	**Human IgA2**	w	nb

s = strong; m = medium; w = weak; nb = no interaction; “-“ = not enough information immunoassays. Biotin is a vitamin, with a molecular weight of 244 D, found in every living cell. Avidin, a glycoprotein found in egg whites, is a 65 kD molecule with four identical subunits, each of which can bind a biotin molecule. The binding sites contain hydrophobic and polar residues that interact with the biotin molecule, practically burying the biotin within the avidin subunit [5]. The extremely high affinity of the avidin-biotin complex allows it to endure harsh elution and washing conditions during assays. The high isoelectric point (pI) of avidin (10-10.5), which can increse electrostatic interactions, and the presence of carbohydrates in the protein make it especially susceptible to nonspecific binding.

While the selectivity and strength of the avidin-biotin interaction are the cornerstones for its use in immunoassays, a reduction in nonspecific binding and the formation of a complex that would dissociate under milder conditions would be advantageous in several assays. These needs have led to the development of several avidin derivatives that provide varying levels of nonspecific binding and different affinity constants. Two different avidin-related molecules, streptavidin and NeutrAvidin™, have similar affinities to avidin but are characterized by lower isoelectric points and the lack of carbohydrate residues (Table 2.6).

- *Streptavidin*: a bacterial form of avidin produced by *Streptomyces avidinii*; maintains the biotin-binding properties of avidin, but with a pI of 5 and the absence of carbohydrates, experiences less nonspecific binding; contains a

Table 2.6 Characteristics of avidin derivatives with reduced nonspecific binding [11].

Protein	Isoelectric Point	Contains Carbohydrate?	Nonspecific Binding	M.W.	RYD Sequence*
Avidin	10-10.5	Yes	High	67 kD	No
Streptavidin	5	No	Low	60 kD	Yes
NeutrAvidin™ Biotin-Binding Protein	6.3	No	Ultra-low	60 kD	No

* RYD → arg-tyr-asp cell-surface recognition sequence.

cell surface recognition sequence of amino acids (arginine-tyrosine-asparagine, or RYD) which can contribute to nonspecific binding [11]

- *NeutrAvidin*™ (Pierce): modified avidin derivative with pI of 6.3, no carbohydrates, and no interaction with cell surface molecules

Monomeric avidin and iminobiotin are derivatives of avidin and biotin that participate in the avidin-biotin interaction with lower affinities. These molecules are useful for assays in which the dissociation of the avidin-biotin complex is desired.

- *Monomeric avidin*: specificity for biotin maintained, but affinity decreases to 10^{-8} M; dissociation by competitive displacement; low nonspecific binding
- *Iminobiotin*: requires mild dissociation conditions; dissociation constant of complex with avidin is pH dependent

Biotin-avidin linkages are used for separation procedures as well as for signal amplification in immunoassays. Many molecules are easily biotinylated, using a variety of reagents and kits that are readily available (Molecular Probes, Sigma-Aldrich, Pierce), without compromising biological activity. Avidin and its derivatives are often conjugated to enzyme and fluorescent labels, providing signal amplification when they bind to biotinylated probes at multiple sites [10].

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CHAPTER 3

ANTIBODIES, ANTIGENS AND IMMUNOASSAYS

3.1 Introduction to Immune Response

The cells and molecules of the immune system work together to provide a collective and coordinated response to the introduction of foreign substances into the body. The mechanisms of immunity can be divided into two main categories, innate and specific immunity. *Innate*, or *non-adaptive*, immunity provides the earliest defense against infectious agents and plays key roles in the induction of specific immune responses [1]. This type of immunity is characterized by its limited capacity to distinguish between different microbes and is mediated by cells that respond nonspecifically to foreign molecules. *Specific*, or *adaptive*, immunity develops in response to infection and is characterized by its ability to tailor the response to distinct molecules [1]. Unlike innate immunity, adaptive responses have the ability to remember specific molecules, facilitating a more rapid and vigorous response upon repeated exposure to infection.

Specific immunity can be further divided into two categories based on the means by which foreign molecules are detected and eliminated. *Cellular* immunity is mediated by T lymphocytes and involves the lysis of infected cells in order to destroy intracellular microbes [2]. *Humoral* immunity depends on circulating *antibodies* to specifically recognize and bind to extracellular microbes [1]. The bound antibodies then assist in the elimination of the foreign molecules.

There are several key components to humoral immunity. A major function of this type of immunity is the production of antibodies by B lymphocytes and plasma cells [3]. Antibodies circulate through blood and the lymph system, where they bind to the foreign molecules against which they were produced. These targets of the immune response are typically referred to as *antigens*. Antigenic molecules that are large enough to initiate an immune response are called *immunogens*. Smaller molecules that must be attached to carrier macromolecules before an immune response can be generated are called *haptens*. While the hapten cannot elicit its own response, the hapten-carrier complex can act as an immunogen. The binding of antigenic molecules or complexes by antibodies initiates a response in which the immune complexes are detected and removed by macrophages via phagocytosis [4].

Specific immunity has several key features that provide a foundation for the development of successful immunological assays [1,3].

1) *Specificity* – The immune response is specific to particular antigens and even to distinct structural features of a single antigen. (Figure 3.1)
2) *Diversity* – Each lymphocyte cell produces a receptor for a single antigen, providing an extensive repertoire of antibody and cell-surface receptors with different antigen specificities. It has been estimated that the mammalian

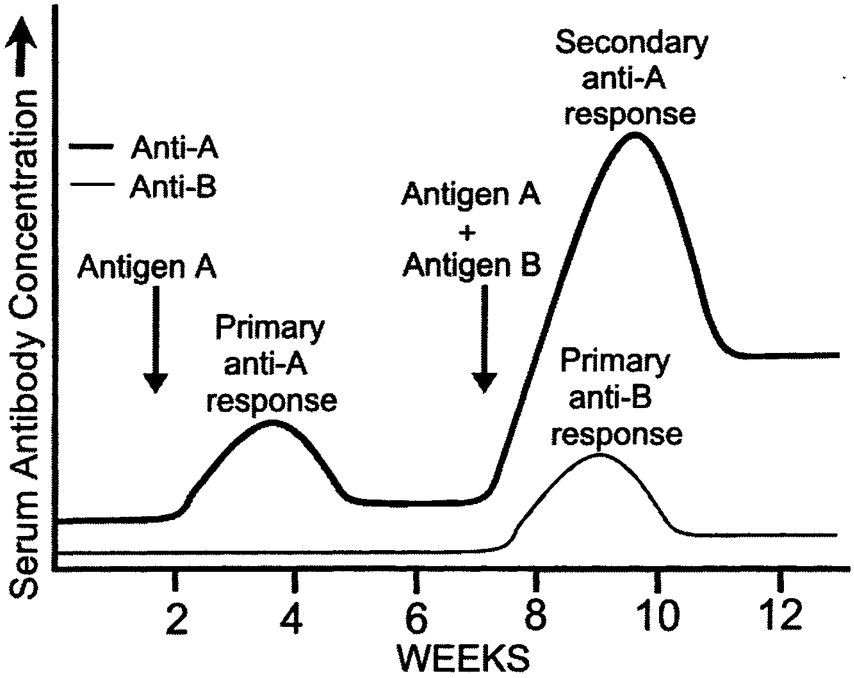


Figure 3.1 Specificity, memory, and self-limitation in an immune response [1]. Specificity: distinct responses to antigen A and antigen B; memory: secondary response is faster and stronger; self-limitation: signal decreases over time as immune system returns to a state of rest between responses.

immune system can distinguish between at least 10^9 different antibody binding sites [1].

3) *Memory* – Repeated exposure to an antigen enhances future immune responses to the same molecule. Secondary responses are typically faster, more potent, and qualitatively different from the original response. (Figure 3.1)
4) *Self-limitation* – Normal immune responses diminish over time, returning to a state of rest after fighting off an attack. (Figure 3.1)
5) *Discrimination of self from non-self* – The immune response targets and eliminates foreign substances that enter the body but does not attack endogenous species. Autoimmune diseases result from defects in this self-tolerance.

Immunological research is aimed at understanding the molecular and mechanistic origins of these fundamental properties as well as exploiting these features for the development of methods for diagnostic purposes.

3.2 Antibody Structure

Antibodies, also called immunoglobulins, are glycoproteins produced in response to foreign molecules present in the body. These glycoproteins are found throughout the body:

- in cytoplasmic membrane-bound compartments of cells and on the surface of B lymphocytes
- in blood plasma and the interstitial fluid of tissues
- bound to the surface of immune effector cells
- in secretory fluids, such as milk and mucus

Upon binding to an antigen, an antibody initiates a response in which the antigen is destroyed or eliminated.

While antibodies are produced with specificity for a wide range of antigens, all have the same fundamental structure. The core structure of each antibody is a monomer comprised of four polypeptide chains: two identical light chains and two identical heavy chains (Figure 3.2). Light chains include one variable and one constant region, while the heavy chains have one variable region and three constant regions. The variable regions at the amino terminal domains of the chains show great diversity in amino acid composition and contain the unique hypervariable sections that comprise the antigen-binding sites. The remaining amino acids at the carboxy end of each chain define the constant regions. The light chains are attached to the amino terminus of the heavy chains by noncovalent, hydrophobic interactions as well as covalent bonds in the form of disulfide bridges. The heavy chains are attached to each other via inter-chain disulfide bridges and noncovalent interactions. The hinge region of the y-

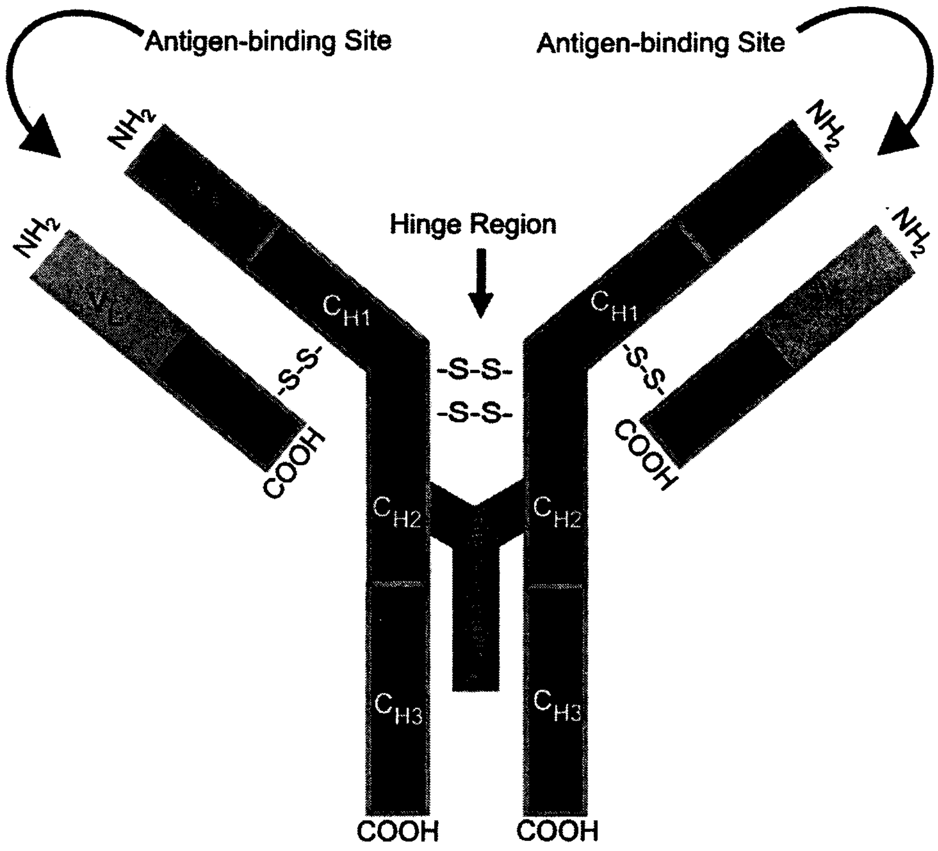


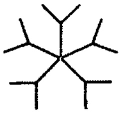
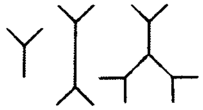
Figure 3.2 Schematic of an immunoglobulin monomer. V_H and V_L are the variable regions of the heavy and light chains, respectively. C_L is the constant region of the light chains and the three constant regions of the heavy chains are indicated by C_{H1}, C_{H2}, and C_{H3}. The disulfide bridges linking the chains together are represented by -S-S-. The hinge region provides flexibility such that the distance between the two antigen-binding sites can vary.

shaped antibody structure provides flexibility that allows the distance between the antigen binding sites to vary.

Although antibodies have the same core structure, minor differences in the overall structure and behavior of individual antibodies give rise to distinct classes and subclasses. The five primary classes, or isotypes, of immunoglobulin (Ig) molecules and the properties of human antibodies are outlined in Table 3.1. Antibodies are divided into classes and subclasses based on the type of heavy chain found in the molecule. The classes contain varying numbers of monomers and mediate different functions in immune responses.

Because antibodies are proteins, they can also be antigenic. When an antibody is produced against a foreign molecule in the body, it is called a *primary antibody.* If an animal of one species (e.g. goat) is immunized by injection of Ig molecules from a different species (e.g. mouse), the antibodies produced against the foreign Ig molecules are called *secondary antibodies.* In this example, the secondary antibody formed in the goat against the mouse immunoglobulin is referred to as a "goat, anti-mouse Ig" molecule. Secondary antibodies are especially useful in immunoassays aimed at detecting antigen-bound, non-labeled primary antibodies.

Table 3.1 Characteristics of human antibody classes.

	IgG	**IgM**	**IgA**	**IgE**	**IgD**
Structure					
Valency	2	10	2, 4 or 6	2	2
Heavy Chain Class	γ	μ	α	ε	δ
Heavy Chain Subclass	γ1, γ2, γ3, γ4	None	α1, α2	None	None
Total MW	160 kD	1000 kD	160-500 kD	160 kD	160 kD
Serum Conc. [3] **mg/mL**	10-20	0.5-2	1-4	0.0001	0-0.4
Function	Secondary response	Primary response	Mucosal membranes	Allergic response	Cell surface markers

3.3 Antibody-Antigen Interactions

The interaction between an antibody and antigen takes place at the amino terminal end of the antibody. The hypervariable sequences located in the variable regions of the heavy and light chains form the *paratope*, or antigen-binding site. The portion of the antigen that interacts with the paratope is the *epitope*, or antibody-binding site. Epitopes can be linear or conformational, depending on the location of the amino acids involved in binding with the antibody (Figure 3.3). Amino acid residues adjacent to each other in the covalent sequence of the antigen form *linear* epitopes. Amino acid residues from separate portions of a linear sequence that are brought together spatially, upon folding of the protein, form *conformational* epitopes. Antigens are often multivalent, having many different epitopes on their surfaces for the heterogeneous population of antibodies typically present in serum [4].

Antibody-antigen recognition is based on noncovalent, reversible binding. The formation of a stable complex is dependent upon the precise alignment of the paratope and epitope. The antibody-antigen complex formed is in equilibrium with free antibody and antigen and is stabilized by a combination of intermolecular forces. The interaction between the antigen and antibody is a balance between attractive and repulsive interactions at the interface between the two molecules. The types of noncovalent bonds that are involved in these interactions are electrostatic forces, hydrogen bonds, Van der Waals forces, and hydrophobic forces [5] (Figure 3.4). The

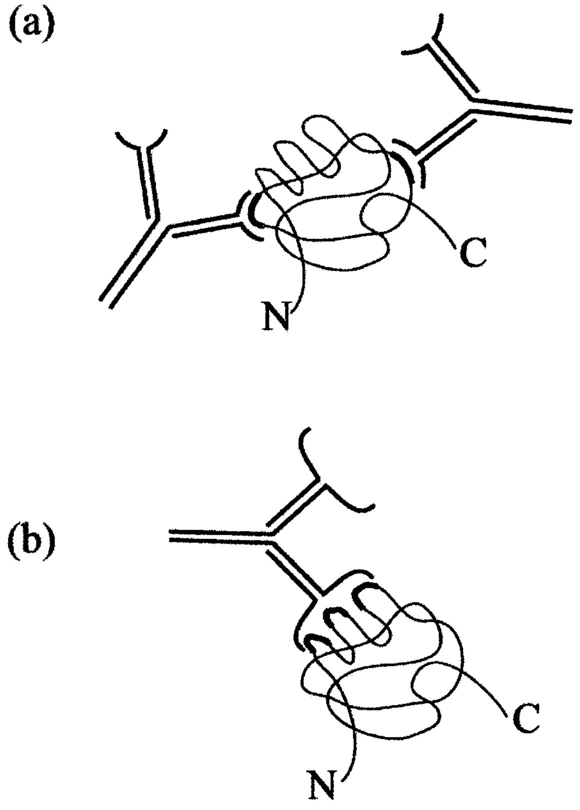


Figure 3.3 Linear and conformational epitopes of an antigen. Linear epitopes (a) consist of amino acid residues adjacent to each other in the covalent sequence of an antigen. Conformational epitopes (b) are made up of amino acid residues from separate portions of a linear sequence and are only adjacent upon folding of the protein. When an antigen is denatured and unfolds, the linear epitopes remain intact, whereas the conformational epitopes are destroyed.

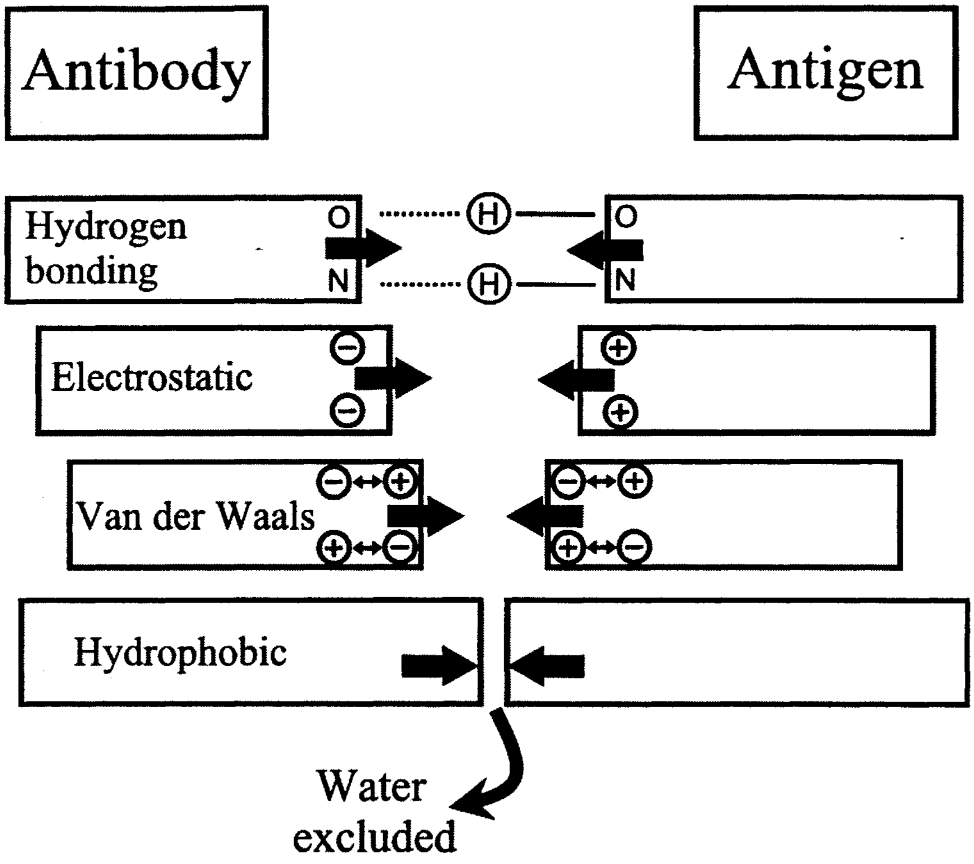


Figure 3.4 Intermolecular forces involved in antibody-antigen interactions [2].

strength of interaction between a single antibody and epitope is called *affinity*. Typical affinity constants for antibody-antigen binding [3] range from $10^5 - 10^{12}$ mol^{-1}. Because of the multivalency of both antigens and antibodies, it is possible for these molecules to be bound to each other at more than one site. *Avidity* refers to the overall strength of antibody-antigen binding, which is dictated by the intrinsic affinity of the antibody for the antigen, the valency of both molecules, and the geometric position of the complex components [3].

Several factors can adversely affect the ability of these proteins to interact with each other and form stable complexes. These factors include overlapping epitopes, allosteric effects, denaturation, and cross reactivity. If an antigen has overlapping epitopes, the binding of one antibody can obstruct a second epitope, interfering with binding of a second antibody. In the case of allosteric effects, the binding of a first antibody can cause a conformational change in the antigen, disrupting other epitopes and preventing the binding of additional antibodies. Denaturation of the antigen, which results in the unfolding of the protein, can destroy conformational epitopes. Cross reactivity can also be an issue since antibodies can bind to epitopes that they only partially recognize. Because there are a limited number of building blocks, such as amino acids, structural proteins tend to be conserved across species [2]. As a result, similar proteins produced by different organisms may elicit the same antibody recognition, which is observed as cross reactivity.

3.4 Immunoassay – Laboratory Use of Antibodies

Immunoassay was first introduced as a technique that employed antibodies as a means of investigating extremely small concentrations of target analytes in complex samples[6]. Since then, immunoassay has become a general term for a variety of analytical methods in which antibodies or antibody-related molecules are used for studying biomolecular interactions and biochemical processes [7]. These methods find application in antigen/antibody quantitation, identification and characterization of these proteins and their interactions with one another, cell surface labeling and cell separation, and localization of antigen within tissues or cells [1].

Immunoassays are based on the unique properties of antibody-antigen interactions. Antibodies can bind to a wide range of molecules, both natural and synthetic, with a high specificity such that an antigen can be separated from a sample containing many other molecules that are closely related to it. Immunoassays rely on the reversible, noncovalent binding between antibodies and antigens, but require an interaction strong enough to prevent the complex from dissociating during the many washing steps of typical assays. Current immunoassay methods include radiolabeled assays, enzyme-linked immunosorbent assays, immunoprecipitation, immunoblotting, cell sorting and immunofluorescence [1]. Immunoassays can be grouped into several categories based on (1) the detection of presence versus absence of analyte, (2) the use of a labeled component, and (3) the need for a separation step before a signal can be generated [6]. Inasmuch as the scope of this work encompasses solid phase assays using labeled and non-labeled reagents, further discussion of immunoassays will be limited to non-labeled, radiolabeled, and enzyme-linked methods.

3.4.1 Immunometric and Competitive Assays

Immunometric assays monitor analyte that binds to immobilized antibody. In this type of assay, antibody is attached in excess to the surface of a reaction chamber. The sample is introduced and incubated with the immobilized antibody. Any unbound analyte is subsequently washed out before a labeled detection antibody is added during the signal generation step. This second antibody attaches to the captured analyte and the signal generated is directly proportional to the concentration of analyte in the sample. In a competitive assay, a limited amount of capture antibody is attached to the reaction chamber surface. A labeled form of the target analyte is added to the sample, as a tracer, before the incubation step. During incubation, labeled analyte competes with the sample analyte for the limited number of binding sites. The amount of tracer that binds to the antibodies is monitored, generating a signal that is inversely proportional to the concentration of analyte in the sample.

3.4.2 Assays Using Labeled and Non-labeled Components

Some applications are aimed at investigating analytes in the nanomolar to picomolar range, such as the detection of hormones in blood samples. Because these concentrations are so low, labeled molecules are often introduced as a means of generating a detectable signal [6] (Table 3.2). The very first immunoassay was a radioimmunoassay (RIA) in which ^{125}I was used to study insulin in human serum [8]. RIAs require careful sample incubation and assay optimization, but the signal generation is simple to perform using a scintillation counter. Radiolabels have been widely used for many years and continue to find application in several laboratories. However, concerns about exposure to radiation, regulation and disposal of waste, and the instability of the labels have led to a decrease in their use.

The decline in use of RIA has also been facilitated by the increased availability of a wide variety of other types of labels, such as enzymes, fluorophores, and biotin/streptavidin derivatives. These types of labels provide an easy means of amplifying the signal and are more stable than radiolabels. In enzyme-linked immunosorbent assay (ELISA), an antibody is immobilized on the surface of a reaction chamber. After the sample is added and the analyte captured, a second antibody, labeled with an enzyme (Table 3.3), is introduced and binds to the antigen. Substrate is then added and the reaction between the enzyme and substrate produces a detectable species, often colored or fluorescent. ELISAs can be time and labor intensive because they are especially susceptible to interferences and extra attention is necessary during the incubation and signal generation steps [9,10].

Table 3.2 Possible labels for use in immunoassays [3].

Label	**Detection Method**
Iodine	Gamma-counter
Enzyme	Chromogenic substrates detected by eye or absorbance
Biotin	Avidin/streptavidin coupled to various labels
Fluorophores	UV excitation and visible emission

Table 3.3 Enzyme/substrate systems for ELISA [2].

Enzyme Label	**Substrate System**	**Reading Wavelength* (nm)**
Horseradish peroxidase (HRPO)	Ortho-phenylene diamine (OPD)	450
	Tetra-methylbenzidine (TMB)	650
	2,2'-azino di-ethylbenzothiazoline sulfonic acid (ABTS)	414
	5-aminosalicylic acid (5AS)	450
Alkaline phosphatase (AP)	Para nitrophenyl phosphate (pnpp)	405
B-galactosidase	*O*-nitrophenyl β-D-galactopyranoside (ONPG)	420

* Reading wavelengths are for non-stopped reactions.

As the repertoire of labels available for immunoassays has grown, techniques that do not require labels have also been developed. One such method is surface plasmon resonance (SPR). As a sample is passed through the flow cell of a biosensor chip, the antibodies immobilized on the sensor surface capture analyte molecules. As the mass of captured analyte increases, the refractive index of the surface layer on the other side of the sensor surface changes. A signal is generated when the light intensity at the angle of measurement changes as a result of the shifted refractive index. BIAcore™ is a commercially available SPR system that is widely used for kinetic analyses in which apparent rate constants can be determined and binding characteristics of interacting molecules are compared [11].

3.4.3 Heterogeneous and Homogeneous Assays

ELISA, RIA, and competitive assays are all examples of heterogeneous assays that require a separation step before a signal can be generated. The free analyte or tracer must be removed or the signal due to captured analyte will not be distinguishable from the background of the initial sample. A homogeneous assay can only see the analyte after it has been captured, as in SPR. Any unbound analyte in SPR is not interacting with the sensor surface and therefore is not affecting the refractive index. No washing step is required before the resonance can be measured.

Radioimmunoassay [12,13], ELISA [14,15], and surface plasmon resonance [16-18] are well-established methods of labeled and non-labeled immunoassays that offer low detection limits (nanomolar to picomolar) and high throughput [11,19]. RIA and ELISA can be performed in batch-mode and SPR has the capability of multivariate analysis. While these methods have become standard procedures in many research laboratories, they have limitations and disadvantages. ELISA is time and labor intensive while offering only limited information. Radioimmunoassays present a general hazard and safety concern that accompany the use of radioisotopes and are increasingly being phased out in many laboratories. Because SPR is only sensitive to changes in surface refractive index, it is not possible to discriminate between different molecules and detection of very small molecules or very large molecules can be difficult. The surface regeneration of the SPR sensor can be lengthy and problematic and each set of binding partners requires separate optimization of the system.

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CHAPTER 4

LABEL DILUTION METHOD

4.1 Introduction

Immunoassays are sophisticated, biochemical tools that are used to detect, quantify, and monitor biological molecules in complex matrices. The investigation and manipulation of these molecules, often present in minute concentrations, rely entirely on the specificity and strength of interaction between binding partners such as antibodies and antigens. Research has been aimed at exploiting fundamental properties of immuno-interactions as well as building on them to improve the design of immunoassays. The improvement of assay precision, the simplification of existing technology, and the automation of current assays are goals that researchers are striving to meet [1].

4.1.1 Label-free Detection

Label-free measurement of bioligand interactions is an important tool in several disciplines, including immunology, pharmacology, and molecular biology. While labels, such as fluorescent molecules, can provide higher sensitivity and a lower detection limit, attaching a label to some biomolecules may corrupt the structures of those molecules or obstruct their active sites. Since the specificity and strength of interaction between bioligands are crucial to the success of immunoassays, efforts have been aimed at the development of methods without the need for labels. Surface Plasmon Resonance (SPR) is an immunoassay method currently used for studying molecular recognition, receptor characterization, and affinity ranking. SPR features the interaction between surface-attached bioligands and biomolecules present in a solution that flows past and comes into contact with the solid surface. The binding of non-labeled bioligands is observed in real time by probing changes in the optical properties of a thin layer at which the interaction takes place (Figure 4.1). Sample solution is passed through the flow cell of a biosensor chip and immobilized bioligands capture the target molecules. As the captured molecules accumulate on the sensing surface, the increase in mass at the surface results in a change in refractive index on the other side of the chip. The shift in refractive index causes a change in the light intensity at the angle of measurement, providing a means of spectroscopically observing complex formation and dissociation.

While the advantages of SPR and other surface sensing transducers have been exploited, they have several significant drawbacks. The sensor chip is not trivial to produce since the layer of coating on the binding surface must be thin and perfectly uniform. The sensor chip must be activated and regenerated between assays, which can be both time consuming and problematic. Conventional biosensors employ sensing layers that require these troublesome steps while lacking any spectral resolution for identifying captured molecules. The sensitivity of SPR solely to changes in mass on the sensing surface can make detection of small molecules

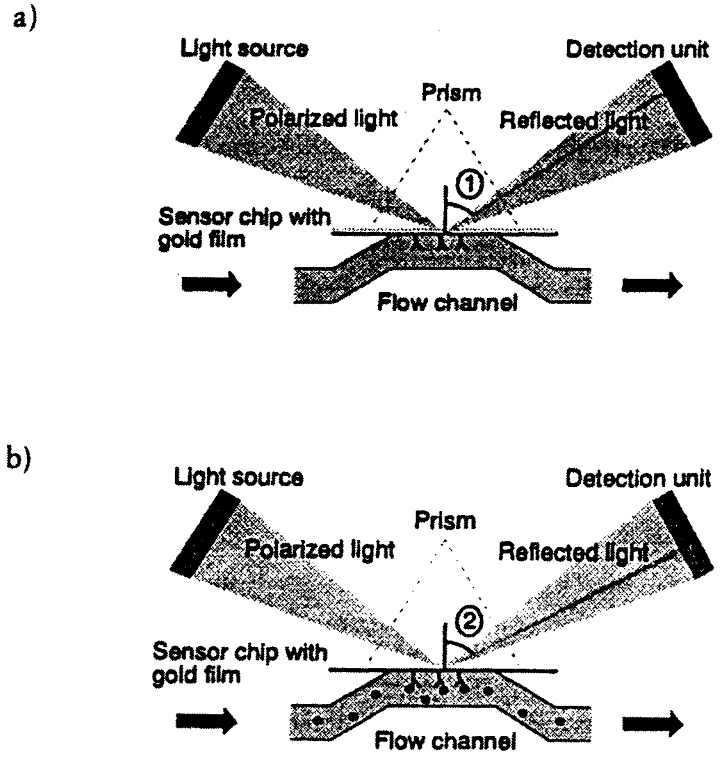

Figure 4.1 Schematic of Surface Plasmon Resonance (SPR). SPR detects changes in the refractive index of the surface layer of a solution in contact with the sensor chip. Antibodies immobilized on the sensor surface (a) capture antigen as sample solution flows past the sensor (b). The resulting change in mass on the sensor chip causes a change in the refractive index of the solution on the other side of the gold film. Reprinted from BIAcore^{TM} Methods Manual [2].

difficult and prevents discrimination between different molecules that may have been captured. Recognizing these limitations, bioligand interaction assays using Bead Injection have been explored [3,4]. Bead Injection based assays avoid these limitations by providing spectral resolution on an easily renewable surface and the ability to simultaneously detect labeled and non-labeled molecules. SPR and BI assays serve the same purpose of monitoring non-labeled bioligand interactions as they take place at the interface between the solid surface and flowing solution.

4.1.2 Theory of Label Dilution

The focus of this work is the development of a new analytical method for performing immunoassays aimed at the detection and quantitative determination of low-level analytes in complex matrices. This new method, called label dilution, is based on the established methods of Bead Injection analysis [5] and isotope dilution [6] and provides a sensitive and selective means of interrogating biomolecular interactions.

In the isotope dilution method, a known quantity of an isotope-labeled species, chemically identical to the analyte, is added to the sample as an internal standard. The sample-standard mixture undergoes exhaustive purification and a portion of the purified mixture is isolated. From the change in specific activity observed, the amount of the target molecule is calculated [6]. The isotope dilution method is a powerful technique in which potentially interfering molecules are eliminated by separation until the specific activity of the isolated fraction remains constant, ensuring that the fraction isolated for measurement is pure.

The label dilution method offers an automated, microscale alternative that is well suited for application in bioresearch and clinical settings where sample sizes are in the microliter range. This method parallels the isotope dilution method in that both techniques use labeled forms of the analyte as internal standards. Whereas the isotope dilution method uses radioactive or stable isotopes [7,8] as labels, the label dilution method employs a known quantity of the target molecule labeled with a highly sensitive chromophore. The sample, spiked with internal standard, is purified by allowing the mixture to interact with selective bioligands immobilized on a solid bead support in the Bead Injection system. As in isotope dilution, it is assumed that the labeled and non-labeled analogues of the target molecule react identically with the immobilized bioligands. As the labeled and non-labeled molecules are captured on the beads, non-retained components of the sample mixture are flushed to waste. The absorbance of the compound and the absorbance of the label are monitored and the amount of target molecule in the sample is calculated using the following equation, which parallels the isotope dilution equation [6].

$$A_{\text{non-labeled}} = A_{\text{labeled}} * ((R_{\text{labeled}} / R_{\text{diluted}}) - 1)$$

$A_{\text{non-labeled}}$ is the amount of target protein in the analyzed sample and A_{labeled} is the amount of the target protein in the labeled internal standard. R_{labeled} is the absorbance ratio of the label to the target protein in the internal standard and R_{diluted} is the absorbance ratio of the label to the target protein for the mixture of sample with a known amount of internal standard. A unique advantage of the isotope and label dilution methods is that quantitative recovery of the analyte is not necessary since both methods rely on ratiometric measurements.

With the Bead Injection format of the label dilution method, the selectivity of an assay can be evaluated and improved by changing the bioligands attached to the surface of the beads. The selectivity of capture of the analytes being investigated can be evaluated by monitoring changes in $R_{diluted}$. While the development of the method in this work has been based on absorbance spectroscopy, the Bead Injection system can be configured to monitor fluorescence as well.

4.2 Experimental

4.2.1 Apparatus

Experiments were performed using a FIAlab 3000 system (FIAlab Instruments, Inc., www.flowinjection.com) equipped with a 500 µL syringe and the lab-on-valve (LOV) central sample-processing unit (FIAlab Instruments, Inc.) (Figure 4.2). Light from a deuterium lamp (Analytical Instrument Systems, Inc.) was carried to the flow cell by a 600 µm fiber optic cable (Ocean Optics, Inc., www.oceanoptics.com). An identical cable collected the light and carried it to an Ocean Optics SD2000 fiber optic

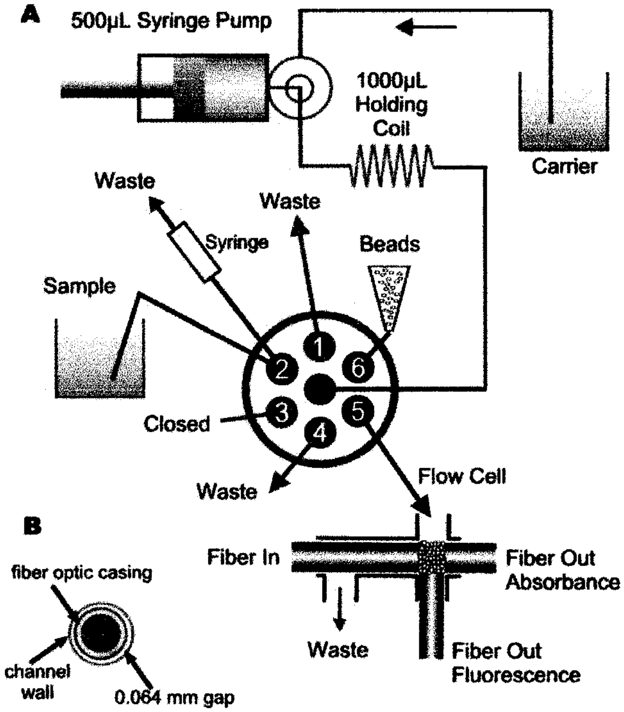

Figure 4.2 Schematic of Bead Injection system for label dilution. (A) The Bead Injection system is comprised of a 500 µL high precision syringe pump and lab-on-valve sample-processing unit. The syringe pump operates in both directions. A volume of beads or sample (analyte solution mixed with labeled internal standard) is drawn into the holding coil before the pump direction is switched and the holding coil contents are delivered to the flow cell. (B) The gap between the fiber optic and the flow channel is 64 µm, allowing solution but not beads to flow through to waste.

spectrometer. The entire system was controlled by a personal computer running FIAlab software, version 5.9.30.

4.2.2 Materials

The carrier solution was PBS (0.14M NaCl, 2mM KH_2PO_4, 16mM Na_2HPO_4, 1mM EDTA-2Na, 10mM LiCl and 0.15% v/v Tween-20). This solution was also used to dilute analyte solutions and to rinse the beads prior to use. Protein A Sepharose 4B Conjugate and recombinant protein G Sepharose 4B Conjugate were obtained from Zymed Laboratories, Inc. The biomolecules used in these assays were goat, anti-mouse IgG (Fab Specific, Product# M6898, 2.2 mg mL^{-1}) and goat, anti-mouse IgG FITC conjugate (Fab Specific, Product# F5262, 5.2 mg mL^{-1}) from Sigma-Aldrich, Inc. The bovine serum albumin standard (Product# 23209ZZ, 2 mg mL^{-1}) was obtained from Pierce Chemical Company.

4.2.3 Method

A syringe pump was used to aspirate beads, PBS carrier, or analyte from their respective ports and deliver them to the flow cell. Each assay began with the aspiration of a defined volume of bead suspension from port 6. The beads were delivered to port 5, leading to the detector, at a flow rate of 30 μL s^{-1} and trapped in the flow cell (~2.5 μL) by the fiber optics, while solution was allowed to flow past the fibers and out to waste. This was accomplished by selecting the outer diameter of the stainless steel tube housing the fiber optic cable to be 1.58 mm, while the channel accommodating the stainless steel tube had an internal diameter of 1.70 mm. The resulting gap between the channel and fiber optic of 0.064 mm did not allow beads (size 45-165 μm) to pass through. PBS carrier was subsequently perfused through the flow cell to ensure tight bead packing and a stable baseline for absorbance measurement. The capture of beads and the assay progression was continually monitored using absorbance spectroscopy.

Because the protein-coated beads absorb light at the wavelengths of interest (280 nm and 495 nm), a new spectral baseline was always established prior to the application of the sample mixture to the bead column. Ten microliters of the analyte/internal standard mixture were delivered to the beads at a flow rate of 0.5 μL s^{-1}, followed by 70 μL of PBS carrier. As the sample mixture was perfused through the bead column, the biomolecules interacted with the proteins immobilized on the beads, while any non-interacting species were carried to waste. As the analyte and internal standard were captured on the beads, the resulting spectral changes were monitored in real time. Each assay was performed three times and the averages calculated are presented in the figures below.

4.3 Results and Discussion

This work exploits the interactions between antibodies raised in goats and the bacterial proteins A and G immobilized on Sepharose beads. The analyte chosen for these experiments was goat, anti-mouse IgG and the internal standard was goat, anti-mouse IgG labeled with fluorescein isothiocyanate (FITC). The labeled protein (0.1 μg μL^{-1} FITC IgG) was premixed with varying concentrations of non-labeled IgG (ranging from 0-0.4 μg μL^{-1}). The absorbance spectrum of the mixtures includes peaks at 280 nm for the IgG and 495 nm for the FITC label (Figure 4.3). The mixtures were perfused through either protein A or protein G-coated beads (Figure 4.4). IgG molecules raised in goats interact more strongly with protein G than with protein A [9]. This difference in affinity is demonstrated by the marked dissociation of the analyte and labeled protein from the protein A-coated beads, as seen in the time interval of 45-100 seconds when the beads are perfused by PBS carrier alone (Figure 4.4 A,B). In contrast, the protein G-coated beads retain the proteins at a "steady state" level, as seen during the same time interval (Figure 4.4 C,D).

As the amount of non-labeled IgG included in the mixtures increased with each run, the signal intensity at 280 nm was expected to increase accordingly. Since the amount of FITC IgG added to each mixture was the same, the signal intensity at 495 nm should have remained constant for each mixture analyzed. However, analysis of the data (Figure 4.4) reveals that an increase in the signal at 495 nm is observed as well. Subsequent experiments to investigate this unexpected increase in signal ruled out the possibilities of reagent contamination, insufficient washing procedures, or the

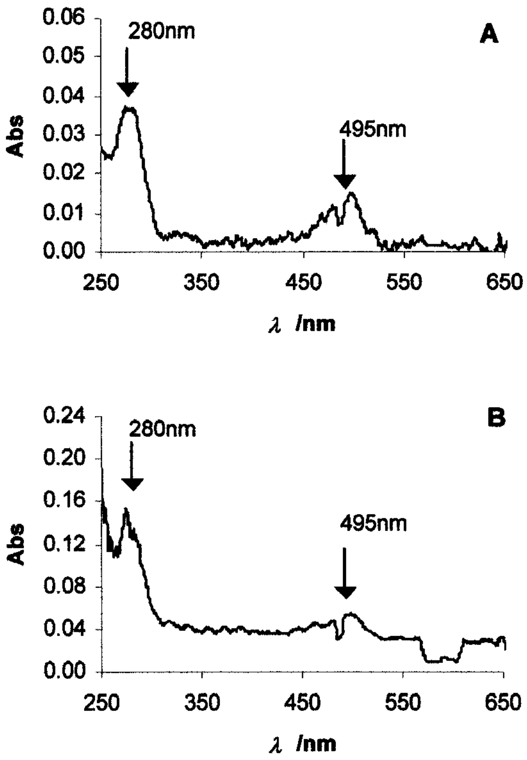

Figure 4.3 Absorbance spectra of IgG and FITC IgG in solution and on protein G beads. Full wavelength spectra recorded during stopped flow of a mixture of 0.2 μg μL^{-1} non-labeled IgG and 0.1 μg μL^{-1} FITC IgG in solution (A) and captured on protein G-coated Sepharose beads (B).

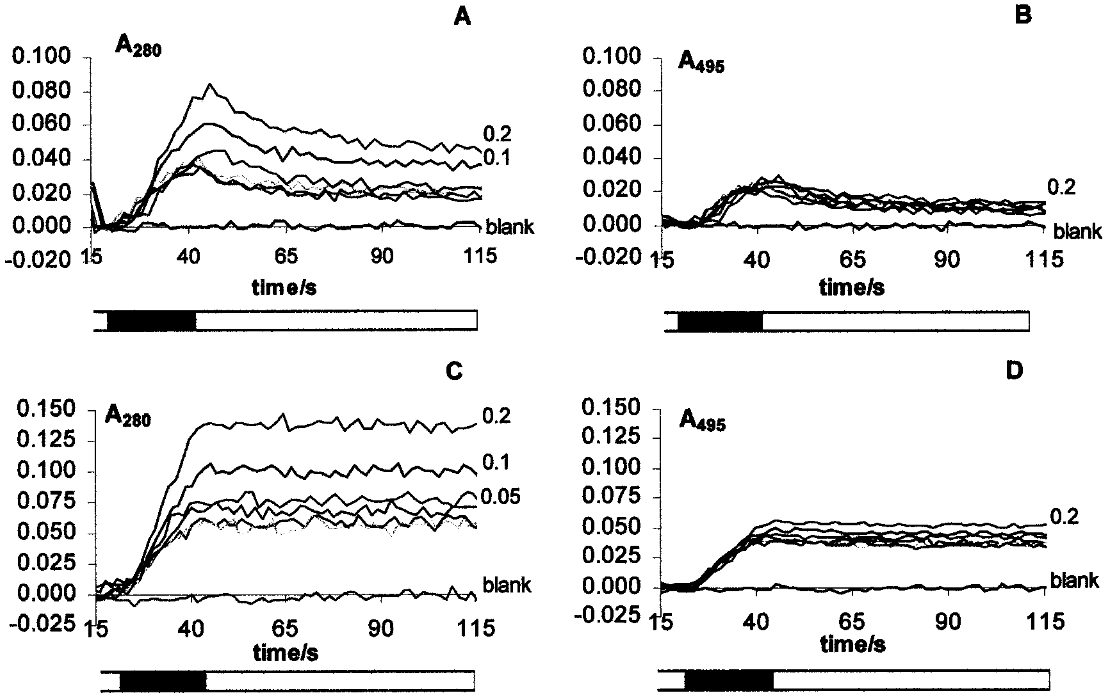

Figure 4.4 IgG and FITC IgG captured on proteins A and G during perfusion. Assay of mixtures of non-labeled IgG (0, 0.012, 0.025, 0.05, 0.1, and 0.2 μg μL^{-1}) and 0.1 μg μL^{-1} FITC IgG. 10 μL labeled IgG and non-labeled IgG were injected on protein A (A, B) and protein G (C, D) coated Sepharose beads and monitored at 280 nm as well as 495 nm. Flow rate, 0.5 μL sec^{-1}; PBS buffer with 0.15% Tween-20 as carrier. The blank run was obtained by injecting PBS buffer. The black bars below the graphs indicate the delivery of analyte solution to the beads, while the empty bars indicate the delivery of carrier buffer to wash the beads.

migration of adsorbed protein through the microcolumn and the subsequent change in its detection.

Finally, experiments were repeated while monitoring absorbance at two additional wavelengths (400 nm and 565 nm) at which neither the label nor the analyte should have absorbed the light. An unexpected increase in signal was observed at each of these wavelengths, indicating that the increase in absorbance, seen previously at 495 nm, was not wavelength dependent. The source of such an absorbance signal was found to be similar to what is observed in turbidimetric measurements. It is postulated that the accumulation of molecules on the surface of the beads leads to precipitate formation, which scatters light at all monitored wavelengths. Monitoring 280 nm and 495 nm wavelengths simultaneously as well as a reference wavelength at 620 nm (for baseline correction) eliminated this undesired effect (Figure 4.5).

The data was then used to calculate the ratio of $R_{labeled} : R_{diluted}$ for the label dilution equation. To determine $R_{labeled}$ and $R_{diluted}$, the corrected absorbance signals at 280 and 495 nm are averaged for the time interval between 40 and 50 seconds, where the signal reaches its maximum. The $R_{labeled}$ value comes from the solution containing only internal standard. The $R_{diluted}$ value is the ratio resulting from the simultaneous capture of analyte and internal standard. When the amounts of analyte and internal standard in the mixture are equal, the ratio of $R_{labeled} : R_{diluted}$ is expected to be two times the ratio of the signal for the pure internal standard since only the amount of

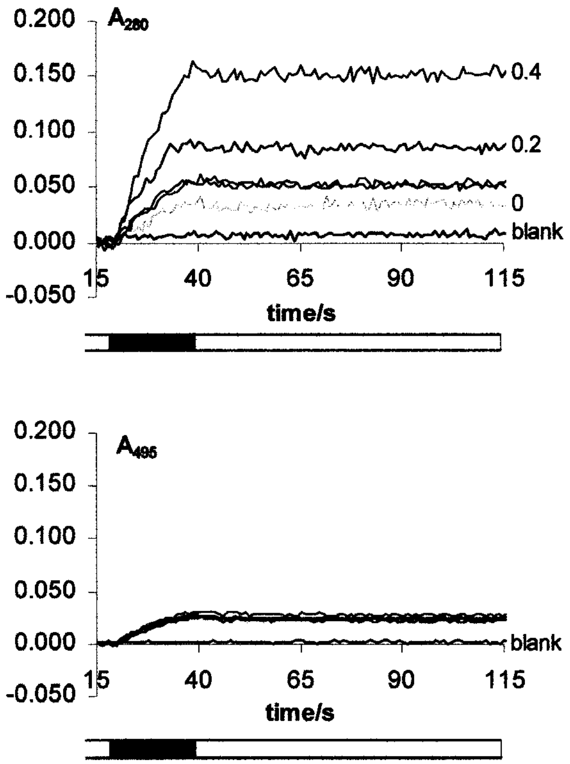

Figure 4.5 IgG and FITC IgG captured on protein G with baseline correction. Assay of mixtures of non-labeled IgG (0, 0.05, 0.1, 0.2, and 0.4 μg μL^{-1}) and 0.1 μg μL^{-1} FITC IgG. 10 μL labeled IgG and non-labeled IgG were injected on protein G-coated Sepharose beads and monitored at 280 nm as well as 495 nm. Both signals were baseline corrected using the signal monitored at 620 nm. Flow rate, 0.5 μL sec^{-1}; PBS buffer with 0.15% Tween-20 as carrier. The blank run was obtained by injecting PBS buffer. The black bars below the graphs indicate the delivery of analyte solution to the beads, while the empty bars indicate the delivery of carrier buffer to wash the beads.

non-labeled protein has increased. The ratios calculated for the corrected protein G data demonstrate the validity of the label dilution method and allow the discrimination between binding and non-binding species. The equation of the line for the mixture in solution is y = 7.71 x + 0.986 (slope +/- 0.058 and y-intercept +/- 0.012). The equation of the line for the mixture captured on protein G-coated beads is y = 6.73 x + 1.07 (slope +/- 0.24 and y-intercept +/- 0.050). (Figure 4.6) The difference in slopes indicates a possible presence of undesired protein in solution.

To demonstrate the diagnostic quality of the label dilution method, mixtures of FITC IgG and bovine serum albumin (BSA), a protein that does not have an affinity for recombinant protein G, were injected into the system in the absence and presence of beads. The concentrations of BSA ranged from 0-0.8 μg μL^{-1}. In the absence of beads, typical transient signals were observed (Figure 4.7), with absorbance increasing at 280 nm as the total protein concentration increased and absorbance of the label (495 nm) remaining constant. However, the albumin does not bind to recombinant protein G beads and since the interstitial volume in the detection chamber is negligible, the BSA is not observed as it perfuses through the bead column (Figure 4.8). Therefore, the absorbance signal monitored represents the amount of analyte captured on the beads, not the total concentration of protein delivered to the flow cell. Since the response curves obtained in the presence of beads (Figure 4.8) do not change with the increasing amount of BSA, the result correctly confirms that no target analyte is

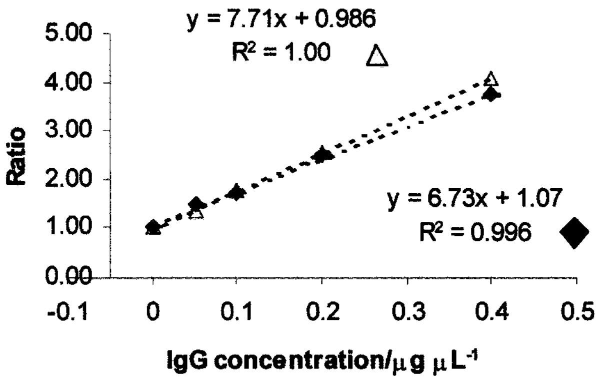

Figure 4.6 Comparison of $R_{labeled}$: $R_{diluted}$ (495 nm / 280 nm for 0.1 μg μL^{-1} FITC IgG : 495 nm / 280 nm for mixture of non-labeled IgG and FITC IgG) in solution (Δ) and captured on protein G beads (◆). Note that the slopes of the lines obtained in solution and on the beads are nearly identical, confirming the purity and identity of non-labeled and labeled IgG.

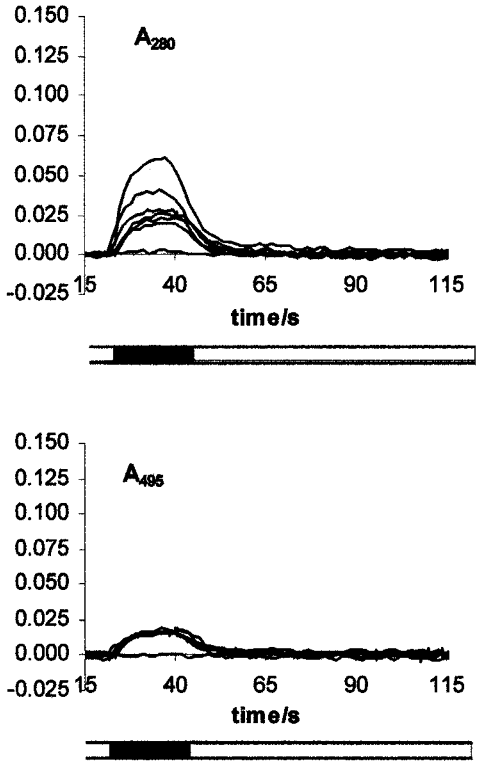

Figure 4.7 Bovine serum albumin (BSA) and FITC IgG in solution. Assay of mixtures of non-labeled BSA (0, 0.05, 0.1, 0.2, 0.4, and 0.8 μg μL^{-1}) and 0.1 μg μL^{-1} FITC IgG in solution. 10 μL labeled IgG and non-labeled BSA were injected into the flow cell NOT filled with beads and monitored at 280 nm as well as 495 nm. Note increase in absorbance at 280nm. Flow rate, 0.5 μL sec^{-1}; PBS buffer with 0.15% Tween-20 as carrier. The blank run was obtained by injecting PBS buffer. The black bars below the graphs indicate the delivery of analyte solution to the beads, while the empty bars indicate the delivery of carrier buffer to wash the beads.

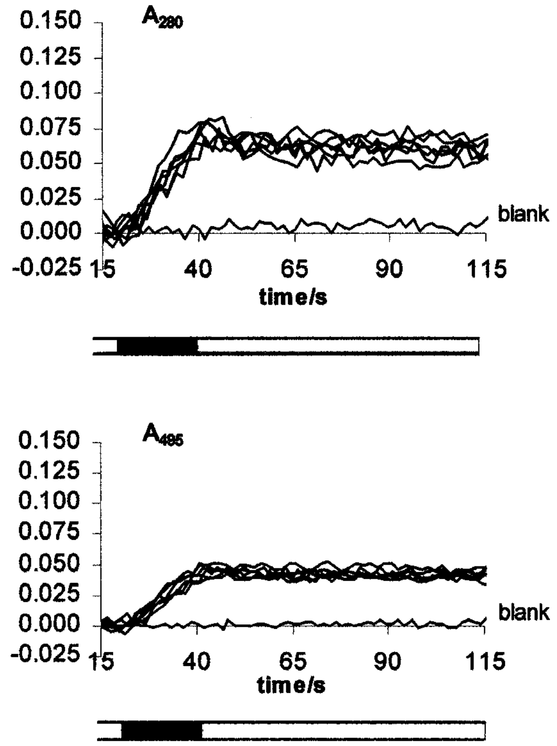

Figure 4.8 Bovine serum albumin (BSA) and FITC IgG perfused over protein G beads. Assay of mixtures of non-labeled BSA (0, 0.05, 0.1, 0.2, 0.4, and 0.8 μg μL^{-1}) and 0.05 μg μL^{-1} FITC IgG with beads. 10 μL labeled IgG and non-labeled BSA were injected on protein G-coated Sepharose beads and monitored at 280 nm as well as 495 nm. Flow rate, 0.5 μL sec^{-1}; PBS buffer with 0.15% Tween-20 as carrier. The blank run was obtained by injecting PBS buffer. The black bars below the graphs indicate the delivery of analyte solution to the beads, while the empty bars indicate the delivery of carrier buffer to wash the beads.

present in the injected sample in spite of the increasing signal at 280 nm observed in the absence of beads (Figure 4.7). The equation of the line for the mixture in solution is $y = 2.41x + 0.904$ (slope +/- 0.092 and y-intercept +/- 0.035). The equation of the line for the mixture perfused over protein G-coated beads is $y = 0.375x + 1.00$ (slope +/- 0.049 and y-intercept +/- 0.018). (Figure 4.9) The ratios calculated for the mixtures of BSA and labeled IgG demonstrate the ability of the label dilution method to detect and eliminate non-specific interactions between immobilized molecules, foreign proteins, and target analytes. In other words, the detector does not see molecules that are not captured on the surface of the beads.

4.4 Conclusion

The label dilution method is presented as an alternative immunoassay for the determination of analytes in complex matrices in their native form. This work demonstrates that the Bead Injection method, combined with measurements in solution, allows for discrimination between proteins of different binding affinities. A major advantage of the label dilution method still needs to be demonstrated by analyzing mixtures of labeled and non-labeled proteins while measuring R values in solution and after exposure to various bead-bound functional groups. This will lead to elimination of interfering molecules present in the complex matrices of real-life samples and will allow the assay of target molecules, as they will be selectively

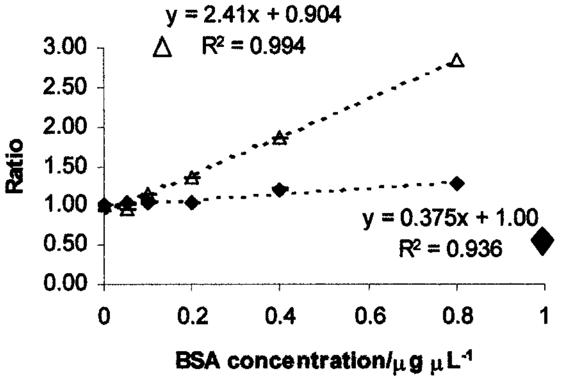

Figure 4.9 Comparison of $R_{labeled}$: $R_{diluted}$ (495 nm / 280 nm for 0.1 μg μL^{-1} FITC IgG : 495 nm / 280 nm for mixture of non-labeled BSA and FITC IgG) in solution (Δ) and captured on protein G beads (◆).

captured on the beads. Presently, the detection limit based on absorbance measurements is 470 ng of non-labeled goat, anti-mouse IgG.

In this work, bioligand interactions were monitored in real time by direct spectroscopic detection at several wavelengths, using microliter quantities of reagents and samples and on-line detection by fiber optic-based UV-VIS spectroscopy. The lab-on-valve format allows for an additional detection mode by inserting a third optical fiber into the flow cell (Figure 4.2) for fluorescence spectroscopy, which may improve the scope of the label dilution technique.

This work introduces the principles of label dilution and should be viewed as a first approximation of what this methodology can achieve. It is believed that the label dilution method is not limited to the interactions between antibodies and proteins A or G, but can be applied to the investigation of any bioligand interactions. The method can be used to evaluate receptor binding, avidin-biotin interactions, or any binding between two molecules, one of which is immobilized on the surface of beads.

4.5 Notes to Chapter 4

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CHAPTER 5

BEAD INJECTION ENZYME-LINKED IMMUNOSORBENT ASSAY

5.1 Introduction to GAD65 Autoantibodies in Insulin-Dependent Diabetes

Type 1 diabetes is an autoimmune disease that results from beta cell destruction and impaired insulin secretion. Glutamic acid decarboxylase (GAD), an enzyme found in the central nervous system and several peripheral organs, has been implicated as an autoantigen in type 1 (insulin-dependent) diabetes mellitus [1]. Autoantibodies produced against an isoform of GAD (GAD65) are the focus of intense research aimed at determining the pathogenesis of this disease. While the exact role that GAD65 and its autoantibodies play in the development of this type of diabetes is still undetermined, these antibodies have been detected in patients years before clinical onset [2]. Antibodies to insulinoma associated proteins (IA-2) and insulin (IA) are two other islet cell antibodies that have been detected in patients with type 1 diabetes, although their levels have been lower and more transient than that of antibodies to GAD65 [3]. These autoantibodies serve as immunological markers [4], indicating subclinical beta cell damage, impaired insulin secretion, future beta cell failure, and a predisposition for the eventual onset of insulin dependent diabetes. Information regarding the relative levels of these antibodies in a patient's serum may provide crucial information for proper diagnosis, estimation of disease progression, and development of appropriate treatment and prevention plans [5,6,7]. Key to the development of meaningful diagnostic methods is an understanding of the pathogenesis of type 1 diabetes. Much has been learned about GAD65 and current efforts continue to focus on the characterization of its autoantibodies [8].

Clinical situations where routine assays are required for patients at risk of developing type 1 diabetes would greatly benefit from a rapid assay that can provide timely feedback to a physician. Such feedback would allow the caregiver to immediately implement secondary testing or therapeutic intervention within a single office visit. To date, no such rapid method exists for the detection of these autoantibodies, relying on more conventional approaches of radioimmunoassay [9,10], radioligand binding assay [11], and enzyme-linked immunosorbent assay (ELISA) [12,13] that typically produce test results in a matter of days. Moreover, although the radiolabeled assays are highly sensitive, the use of radioactive species presents a major disadvantage, especially with the goal of developing diagnostic methods for use in a clinical setting. While there are automated immunoassay analyzers available for a variety of analytes [14,15,16], the methods available for detecting autoantibodies to GAD65 are manual methods and thus have the disadvantages of being both time and labor intensive. None of these current methods is capable of producing routine analysis in less than thirty minutes, that is, within the time frame of a typical clinical visit. An added incentive to developing a rapid screening method is the existence of other GAD65-related autoimmune diseases (Stiff-Man Syndrome, Graves disease, Addision's disease) that could benefit from an early diagnosis [1]. With the desire for a non-radioactive method that can be performed in a short amount of time, this work describes the development of a hybrid analytical technique designed by combining Bead Injection (BI) and ELISA methodologies.

5.2 Background of Bead Injection ELISA

5.2.1 Principles of Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) exploit the inherent specificity of antibody-antigen interactions and use an enzyme attached to one of the reagents as a means of detecting target species. In a typical ELISA, one of the reagents is bound to a solid phase while other reagents are subsequently added to the reaction chamber, interacting with the immobilized species. Solid phase, heterogeneous ELISA are useful for several reasons: the samples and reagents passively adsorb to the solid phase, immobilization of the capture reagent facilitates easy separation of bound and free molecules, and the colored product formed in ELISA can be easily and rapidly read. The most common type of reaction chamber is a 200 μL well on a 96-well microtiter plate. The plates are typically made of polystyrene or polyvinyl chloride and provide a surface for the passive adsorption of proteins [17]. ELISA experimental protocols generally consist of a series of reagent addition, incubation, and washing steps, the result of which is a complex consisting of an immobilized antigen, the target antibody, and an enzyme-labeled secondary antibody. The subsequent addition of an appropriate substrate results in a color change that can be observed by the eye or read by a spectrophotometer. The color development of the enzyme-substrate product can be affected by buffer composition and pH, reaction temperature, enzyme stability, substrate concentration and stability, and the stability of the product formed.

Many assays based on ELISA have commercially available reagents and kits that are easy to use and provide detection levels of 100-1000 ng mL^{-1}, appropriate for typical diagnostic needs [17]. The use of enzyme labels provides the advantages of a stronger signal and greater sensitivity compared to a single chromophore label and greater stability than radioisotopes. The use of enzyme labels also avoids the hazards associated with using radiolabels [18]. While ELISA has become a standard procedure in many laboratories, the method continues to be both time and labor intensive, typically taking several hours to perform. A major disadvantage of ELISA is its susceptibility to interferences and changes in assay conditions during signal generation. Enzyme-substrate reactions are sensitive to time, temperature, and pH.

5.2.2 Introduction to BI ELISA

Bead Injection (BI) is a novel micro-analytical technique [19] that has been successfully used to perform automated bioassays for cellular stimulus-response [20], cellular metabolism [21], and bio-affinity studies [22]. BI uses commercially available chromatographic beads as a solid phase support for the immobilization of reagents, bioligands, and cells. This technology has the unique ability to reproducibly shuttle microliter aliquots of sample, reagent, and beaded support to and from the flow cell where reactions are monitored in real time using fiber optic absorbance spectroscopy. The renewable micro-column of derivatized beads formed in the flow cell provides a means of concentrating the target antibodies within the detection path, while unbound, non-specific matrix proteins are carried to waste. These features of BI make it amenable to heterogeneous immunoassays and for application in the study and detection of antibody-antigen interactions. BI ELISA is an automated micro-analytical technique well suited for use in clinical settings, where the sample size is a few microliters and results are desired in a short amount of time. Its application to the assay of GAD65 antibodies demonstrates the potential of BI ELISA as a novel method for immunological research and clinical screening.

5.3 Experimental

5.3.1 Instrumentation

A computer-controlled, bi-directional syringe pump is used to aspirate beads, sample, and reagent sequentially via their respective ports into the holding coil and deliver them to the flow cell (Figure 5.1). The apparatus employed for this work is similar to the lab-on-valve (LOV) Bead Injection instrument previously described [23,24]. The components include a FIAlab 3000 SI system (FIAlab Instruments, Inc., www.flowinjection.com), 500 μL Cavro syringe, and LOV integrated manifold (FIAlab Instruments, Inc.) (Figure 5.1a). The LOV manifold (Figure 5.1b)

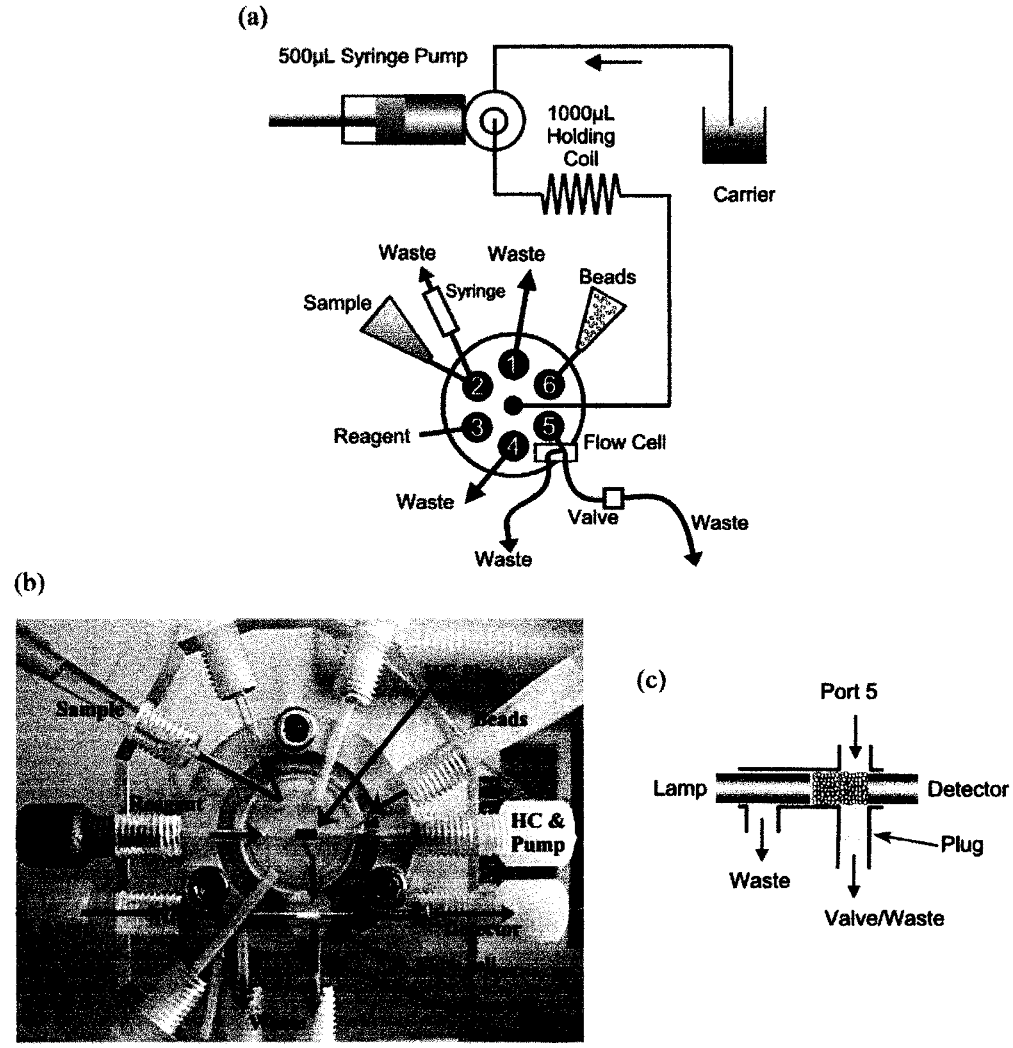


Figure 5.1 Schematic and picture of Bead Injection ELISA system. (a) The Bead Injection is system comprised of a 500 μL syringe pump, 1000 μL holding coil, and lab-on-valve sample processing unit. (b) Lab-on-valve (LOV) manifold. The Bead Injection system uses a syringe pump connected to the holding coil (HC), or central port, of the LOV manifold. A volume of sample, reagent, or beads is drawn into the HC from its respective port (2, 3, or 6). The flow is reversed and the contents of the HC are then delivered to the flow cell (5). Ports 1 and 4 are used for waste. (c) Micro-column of derivatized beads packed in the flow cell at port 5. The gaps between the fiber optics or plug and the channel walls provide enough space for solution to pass while retaining the beads in the flow cell [23].

encompasses a flow cell designed to retain and monitor the bead suspension. The 8 μL volume of the flow cell (Figure 5.1c) is defined by the distance between the fiber optic probes inserted into the LOV horizontally at port 5 [24,25]. The 600 μm fiber optics (Ocean Optics, Inc., www.oceanoptics.com) deliver light from a deuterium lamp (Analytical Instrument Systems, Inc.) to the flow cell and collect the transmitted light, carrying it to an Ocean Optics SD2000 spectrometer.

As an improvement to the previous design [24], two plugs have been inserted into the LOV, one at the end of the holding coil (HC) and the other at the bottom of the flow cell (Figure 5.1 b,c). The plugs are pieces of peek tubing, with an inner diameter of 127 μm, which are remarkably efficient and reliable at trapping the beads. The gap between the plug and channel wall is 64 μm, which allows solution, but not beads, to travel past the plugs. Upon aspiration from the bead reservoir, the beads are carried toward the HC and packed into the groove on the rotating valve head as well as the channel between the valve head and the HC plug. Once the flow is reversed, the beads are carried to the flow cell or to waste.

Another improvement is the addition of a 24-volt, direct current solenoid valve, inserted into the waste line extending from the flow cell (Figure 5.1a), to assist in bead removal. During bead loading the valve is open and the solution carrying the beads to the flow cell exits the flow cell via two available paths, tightly packing the beads between the fiber optic probes (Figure 5.1c). During analyte and reagent delivery, the valve is closed and solution is forced to perfuse through the entire bead column before unbound species are carried to waste. The valve is open during bead removal, allowing solution to enter the flow cell from two directions as the beads are aspirated from the flow cell back into the holding coil. The beads are then flushed out via the adjacent waste line (port 4). A personal computer controls the entire system using FIAlab software, version 5.9.30.

5.3.2 Materials

The carrier solution was PBS (0.14 M NaCl, 2 mM KH_2PO_4, 16 mM Na_2HPO_4, 1 mM EDTA-2Na, 10 mM LiCl and 0.15% v/v Tween-20). This solution was also used to dilute antibody solutions and to rinse the beads prior to use. Streptavidin-Sepharose 4B conjugate was obtained from Zymed Laboratories, Inc (So. San Francisco, CA). The R. H. Williams Laboratory at the University of Washington provided the primary antibodies and biotinylated GAD65 [25] (biotin-GAD65). The primary antibodies included a monoclonal antibody to GAD65 [26] (mAb144, 1 mg mL^{-1}), a non-specific antibody (mouse, anti-insulin IgG, 1 mg mL^{-1}) and normal human serum. The enzyme-labeled secondary antibody (anti-mouse IgG, H& L chain specific (goat), peroxidase conjugate, Product #401253, 1 mg mL^{-1}) was obtained from Calbiochem (San Diego, CA). 3,3',5,5' tetramethylbenzidine (TMB) substrate solution (Product #34024) was obtained from Pierce Chemical Company (Rockford, IL).

5.3.3 Method

The BI ELISA and standard ELISA methods parallel each other in chemistries and bio-interactions but have different protocols. An overall comparison of the BI ELISA with respect to the standard ELISA is illustrated in Figure 5.2. BI ELISA reactions are performed in a flow-through detector cell found on the LOV module. A renewable micro-column of derivatized Sepharose beads provides the surface for antibody capture and monitoring. The flow-through cell has the advantage of allowing antibody capture from a serum bolus to be immediately followed by a buffer wash in one continual step. Wash steps to remove nonspecific interferences in a standard ELISA require multiple batch additions and removals of buffer.

As shown in Figure 5.2, BI ELISA is only a three-step procedure, unlike the typical five-step procedure used in the standard ELISA. In the standard 96-well format, immobilization of the biotin-GAD65 on the surface of the wells must be manually prepared for each run, while BI ELISA uses the biotin-GAD65-coated Streptavidin beads as one of the reagents to be automatically renewed for each assay in a matter of seconds. In the standard ELISA, the last step (step #5) requires the addition of acid to stop the colorimetric reaction for quantitation. The precise timing found in the BI ELISA method allows for the elimination of this step, simplifying the chemistry and saving time. As a consequence of these modifications the BI ELISA assay is completed in less than 30 minutes as compared to >3 hours for a standard ELISA

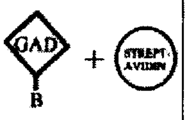
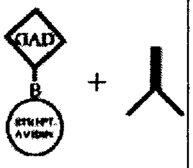
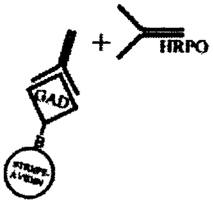
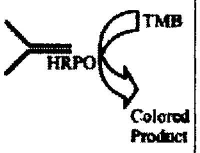
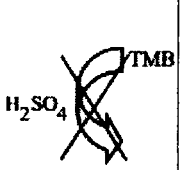
	Step #1	Step #2	Step #3	Step #4	Step #5
Reaction					
Standard ELISA	1-8+ Hours	1+ Hour 200 μL Manual	1+ Hour 200 μL Manual	5-15 Minutes 100 μL Manual	2-3 Minutes 200 μL Manual
BI ELISA	*	9 Minutes 50 μL Automated Continuous Flow	18 Minutes 50 μL Automated Pulsed Flow	3 Minutes with Detection 50 μL Automated Continuous Flow	

Figure 5.2 Comparison of procedural steps in Bead Injection ELISA with reference ELISA method. *Note: Immobilization of biotin-GAD65 on beads is carried out in a single, 30-minute batch operation, providing material for a large number of subsequent assays.

protocol. Upon completion of the assay, the beads are removed from the flow cell and replaced by a fresh aliquot for the following assay.

With the aim of developing and introducing the BI ELISA technique, optimization of the flow programming and reagent preparations was the focus of this work. Continuous flow delivery of the analyte and reagents to the bead column shortens the assay time but may be problematic for efficient capture of antibodies on the immobilized ligands. Therefore, pulsed-flow and stopped-flow were applied to provide prolonged contact time for bioligand interactions with slow kinetics. The results presented illustrate the conversion of an ELISA protocol to the BI method and the optimization of each step in the procedure.

5.3.4 Reference Method: Standard ELISA [25]

Biotin-GAD65 (300 ng mL^{-1}) was first incubated with anti-GAD65 antibody mAb144 (250 ng mL^{-1}) in 100 μL reaction buffer (TBST: 25 mM Tris, 150 mM NaCl, pH=7.2, 0.05% Tween-20). The mixture was then transferred to a 96-well Reacti-Bind NeutrAvidin coated plate (Pierce, Rockford, IL) and the plate was shaken at room temperature for one hour. After washing six times with TBST buffer, enzyme-labeled secondary antibody was added at a dilution of 1:20,000 and the plate was shaken at room temperature for one hour. After washing six times, the HRPO-substrate reaction was carried out using 1-Step Ultra TMB-ELISA (Pierce). The absorbance after adding a stop solution was measured at 450 nm in an ELISA reader. An overall time of >3 hours was needed

5.4 Results

5.4.1 Method Optimization

Optimization required that volumes, flow rate, flow mode and timing of events were varied and examined. Initially, the enzyme-substrate reaction in step #4 was monitored using stopped flow, where the bolus of substrate was delivered to the bead column and the flow was stopped when the highest concentration of substrate was parked in the detection path [27]. After a period of incubation, the flow was restarted and the product and excess substrate were pushed past the beads to waste. However, the data (Figure 5.3) indicated that the reaction rate was fast enough such that the maximum signal had already been achieved before the stopped flow period while the TMB bolus was still being delivered to the flow cell. This prompted the use of continuous flow delivery of the TMB substrate (Figure 5.4). While the resolution between the analyte and control was better using stopped flow (Figure 5.3), continuous flow provided adequate resolution with decreased assay time. Each assay was performed in triplicate and the runs are shown superimposed to illustrate the reproducibility of the assay. The leading edge of the curve represents the initial rate of reaction, where the kinetics are first order. Since data was collected throughout the

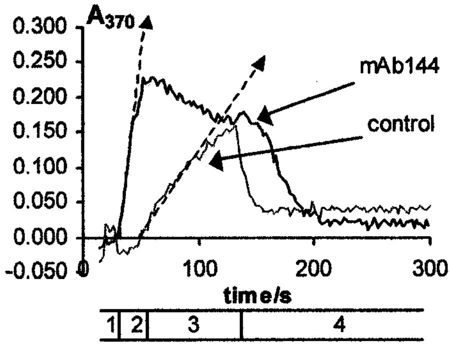


Figure 5.3 Stopped flow delivery of the TMB substrate. 20 μL of undiluted TMB were delivered to the flow cell where the biotin-GAD65/target antibody/HRPO-antibody complex was previously assembled on streptavidin-coated Sepharose beads. The progress of the substrate addition is shown in the bar below the graph: 1) delivery of PBS carrier, 2) delivery of the TMB substrate, 3) 80-second stopped-flow period, and 4) washing of the bead column with PBS carrier. The enzyme-substrate reaction was monitored at 370 nm. Flow rate, 0.5 μL s^{-1}; PBS buffer with 0.15% Tween-20 as carrier.

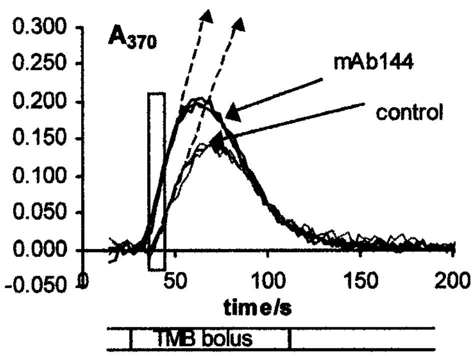


Figure 5.4 Continuous flow delivery of the TMB substrate. 20 μL of undiluted TMB were delivered to the flow cell where the biotin-GAD65/target antibody/HRPO-antibody complex was previously assembled on streptavidin-coated Sepharose beads. Triplicate runs are superimposed to illustrate the reproducibility of the assay. The rectangle indicates the time interval over which the data was integrated. The enzyme-substrate reaction was monitored at 370 nm. Flow rate, 0.5 μL s^{-1}; PBS buffer with 0.15% Tween-20 as carrier.

substrate delivery in BI ELISA, any point along the curve can be utilized. The ability to choose any portion of the curve eliminates the need for step #5 found in the reference ELISA protocol, where a stop solution was added to halt the colorimetric reaction. The rectangle inserted in Figure 5.4 indicates the 10-second time interval over which the data was integrated. The integrated values were used to produce plots of absorbance versus antibody concentration (Figures 5.5 & 5.6).

In step #3, the enzyme-labeled secondary antibody was delivered to the flow cell to bind and detect the captured primary antibody. Both stopped-flow and continuous-flow deliveries were tested, but the highest signal in the shortest time was achieved using a novel, pulsed-flow mode. In pulsed-flow mode, a bolus of HRPO-antibody was delivered to the flow cell in 9 pulses of 6 μL volumes, separated by short periods of stopped flow. After trying stopped flow periods of 6, 3, 1.5, and 0.5 minutes, a period of 1.5 minutes was found to provide the best signal within the shortest time frame.

Once the delivery format of the labeled secondary antibody had been optimized, attention was directed at determining the optimal concentration of HRPO-antibody. Using a concentration that is too high is not only expensive, but it can also result in increased non-specific binding. Several concentrations of HRPO-antibody in PBS were investigated and the averages of triplicate runs are presented (Figure 5.5) with error bars indicating one standard deviation. As the concentration of the HRPO-

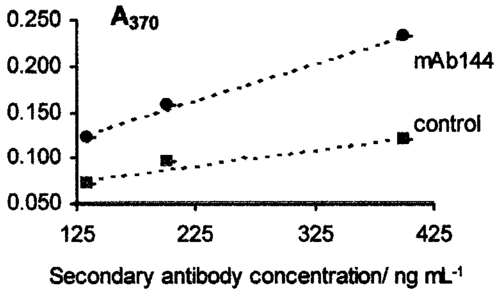


Figure 5.5 Comparison of target antibody (mAb144) to control as a function of the concentration of HRPO-labeled secondary antibody (HRPO-Ab). The average of triplicate runs is shown with error bars indicating one standard deviation. An optimal range of 250-300 ng mL-1 of HRPO-Ab was selected. This is believed to provide good sample to control resolution without sacrificing a large excess of antibody. Subsequent assays were performed using 270 ng mL^{-1} HRPO-Ab.

antibody increases, the difference between the sample and control signals increases. Since an optimal concentration is one that produces a strong signal with good resolution [28] without using a large excess of reagent, the 250 - 300 ng mL^{-1} level was determined to be a reasonable range. For subsequent experiments, the HRPO-antibody concentration was kept at 270 ng mL^{-1}.

The delivery of the primary antibody in step #2 was initially performed using the same pulsed-flow mode as for the secondary antibody, with 1.5 minute stopped flow periods. It was inadvertently discovered that the antibody could be successfully captured on the immobilized GAD65 using continuous flow at 0.5 μL s^{-1}. This change in solution delivery reduced the time needed for step #2 from 18 minutes to 9 minutes. With the flow programming established for this step, several concentrations of the primary antibody in PBS were evaluated to determine the sensitivity of the assay for mAb144. The average values of triplicate runs are presented for the mAb144 sample and the nonspecific antibody control (Figure 5.6) with error bars indicating one standard deviation. Using linear regression analysis, the equation of the line for each data set was determined (mAb144: Abs = 2e-4*[mAb144] + 0.1; control: Abs= 3e-5*[non-specific Ab] + 0.1). Three standard deviations were added to the control signal at the intersection of the lines (mAb144 and control). The limit of detection for mAb144 in buffer is 20 ng mL^{-1} based on a signal to noise ratio of three (S/N=3) over the control.

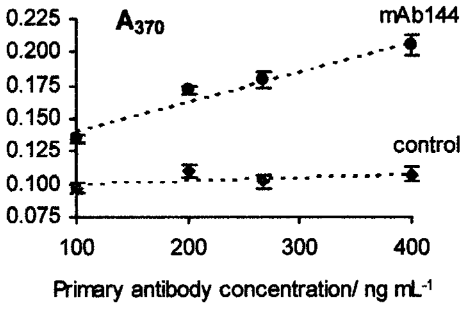


Figure 5.6 Comparison of target antibody (mAb144) to non-specific control as a function of the concentration of primary antibody added. The average of triplicate runs is shown with error bars indicating one standard deviation. The detection limit was determined to be 20 ng mL^{-1} based on a signal to noise ratio of three (S/N=3) over the control.

5.4.2 Human Serum Samples

With the flow programming and reagent concentration optimized, a series of experiments were performed using normal human serum as the control and the same serum doped with mAb144 as the sample. This was a blind study using unmarked samples. The average signals for triplicate runs are presented (Figure 5.7a) with error bars indicating one standard deviation. The ratios of the sample signals to control signal are presented in Figure 5.7b. All samples were easily identified and the data illustrate the ability of the BI ELISA to differentiate between samples with low levels of antibody in the presence of interfering non-specific antibodies and proteins typically encountered in real-life samples.

5.5 Discussion

The goal of this work was the development of a non-radioactive, ELISA-based method, using Bead Injection, for the rapid detection of GAD65 autoantibodies implicated in the pathogenesis of type 1 diabetes. The results of this preliminary work demonstrate the ability of BI ELISA to achieve good resolution using small sample volumes in a fraction of the time required for standard ELISA and RIA methods. Employing BI ELISA reduces the assay cycle time while preserving the sensitivity of the reference ELISA and maintaining the precision of both the ELISA and RIA methods typically used for GAD65 antibodies (Table 5.1).

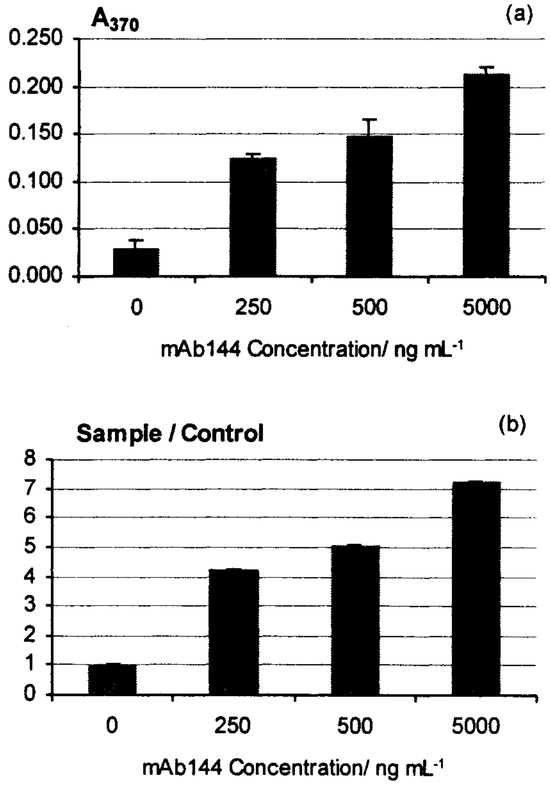


Figure 5.7 Blind study for determination of monoclonal antibody 144 using normal human serum doped with target antibody. (a) Absorbance signal for enzyme-substrate product after adding human serum samples spiked with varying amounts of mAb144. The average of triplicate runs is shown with error bars indicating one standard deviation. (b) Ratios of signals for mAb144 doped serum to non-doped serum (control). All serum samples were correctly identified.

Table 5.1 Comparison of BI ELISA with reference ELISA and RIA methods.

	BI ELISA	**ELISA**[25]	**RIA**[10]
Time	30 min	4 h	6 h
Sensitivity*	20 ng mL^{-1}	30 ng mL^{-1}	2-3 ng mL^{-1}
Reproducibility	± 2% to 5%	± 2% to 9%	± 2% to 5%

* Concentration of mAb144 in buffer.

Although tempting, further comparison between BI ELISA and established ELISA methods should be avoided since the two approaches are aimed at different research and clinical needs. The serial sample processing of BI ELISA cannot approach the throughput that is possible with the parallel processing of ELISA performed on 96-well or larger microtiter plates. Likewise, standard ELISA methodology is not suited for immediate clinical feedback where routine assays are required for "at risk patients". BI ELISA was designed not as a replacement for standard ELISA, but with the aim of enhancing current biomedical needs in clinical screening applications.

Several features in the bead injection approach make it particularly useful for immunological assays:

- Very small volumes of sample and reagent are required (0.5-100 μL).
- The enclosed manifold minimizes issues of sample contamination or evaporation and allows for prudent biohazard handling.
- The instrument is highly adaptable to a variety of assays using the same system configuration since the software program can easily be changed to alter the experimental protocol.

The design of the flow-through detection chamber allows for larger volumes of sample or reagent to be added without the worry of overfilling the reservoir. Furthermore, the captured and purified analyte, or *complex of interest,* can also be recovered by simple chemical dissociation from the beads for secondary analysis if desired. The use of reagent-coated beads in BI ELISA allows for a single batch to be used for multiple assays providing increased reproducibility and reliability between assays.

While the preliminary results presented here are encouraging, considerable improvements are needed before the full impact of BI ELISA can be realized. The assay time, while shorter than the reference methods, is still longer than ultimately desired for clinical application. Also, these experiments have been performed using a monoclonal antibody that is specific for a linear epitope of GAD65 at the N-terminus of the enzyme. Native autoantibodies associated with diabetes are specific for conformational epitopes in the middle region or at the C-terminus of GAD65. Consequently, further testing is needed with respect to epitope changes and the reliability of the BI ELISA protocol.

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CHAPTER 6

CONCLUSION

6.1 Summary

The scope of this work has been the development of Bead Injection methodologies for application in the field of immunological research. The label dilution method was conceived with the aim of exploiting the features of Bead Injection and the selective binding between antibodies and antigens for detecting low-level analytes in complex matrices. This method was introduced using a model system comprised of antibodies raised in goat, bacterial Ig-binding proteins (protein A and protein G), and bovine serum albumin as a non-specific serum protein. Using this chemical system, the attributes of the label dilution method were demonstrated. Not only does label dilution provide real-time monitoring of bioligand interactions as they occur on the bead surface, the spectral resolution offered by the BI system allows for observation of the different binding affinities of the antigen for various immobilized ligands. The ratiometric measurements performed with purified reagents demonstrate the theoretical application of the label dilution method to the quantitation of low-level analytes from complex samples.

Bead Injection ELISA was developed out of the need for a rapid, automated method for detecting GAD65 antibodies. When it became evident that direct spectroscopic detection of the antibody binding to immobilized GAD would not be feasible, it made sense to mirror an assay that had been established as a successful means of detecting the low-level antibodies. As the standard 96-well ELISA method was converted, step-by-step, to the lab-on-valve BI instrument, it became apparent that the high surface area of the bead column and the versatility of programmed flow could be exploited to decrease the overall time of the assay. The model system used for developing BI ELISA employed a monoclonal antibody raised against a linear epitope at the amino terminus of GAD that is separate and distinct from the binding site of native GAD65 antibodies.

6.2 Looking Ahead

In both cases, the methods developed have only been tested on model systems. The logical next steps in the continued development of these assays will involve the use of complex samples with endogenous, nonspecific proteins as well as polyclonal and native antibodies that will have different binding affinities and activities than these model systems. While the sequential approach to sample analysis in Bead Injection ELISA cannot rival the parallel output of multi-well plates, BI ELISA may fulfill the need for a rapid preliminary screening method. Especially with the use of a universal immobilized ligand, such as streptavidin, and a biotinylated antigen, antibody fishing may prove to be a useful preliminary assay for screening clinical samples, which can then be followed by secondary analysis for confirmation or further elucidation.

Future advancements may include performing multiple assays on a single captured sample. For example, perfusion with a mixture of secondary antibodies that are isotype specific (IgM, IgG, IgE, IgA) can be designed using different enzymatic labels for each class. The flow-through detection chamber would allow each substrate to be introduced sequentially to test for the presence of autoantibodies by isotype. This could be extended to the investigation of autoantibody subclasses (IgG_1, IgG_2, IgG_3, IgG_4), again using subclass-specific secondary antibodies that have unique enzyme labels. As an alternative to the enzyme assays, it may also be possible to derivatize these secondary antibodies with different fluorescent labels and measure the results simultaneously. The versatility of bead injection technology provides an ideal platform for the development of multiple assays for rapid clinical and biomedical testing.

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VITA

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