UNIVERSITY OF CINCINNATI

Date: 30-Jul-2009

I, Umadevi B Tiwari, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Chemistry

It is entitled:

Development of methods to determine the binding capacities of solid supports and improvement in immunoassay efficiency using dendrimer-modified beads

Student Signature: Umadevi B Tiwari

This work and its defense approved by:

Committee Chair: H Brian Halsall, PhD

William Heineman, PhD

Patrick Limbach, PhD

Edward Merino, PhD
Development of methods to determine the binding capacities of solid supports and improvement in immunoassay efficiency using dendrimer-modified beads

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in the Department of Chemistry, College of Arts and Sciences

November 2009

by

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B.Sc. (Chemistry) University of Mumbai, India 1999
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Committee Chair: H. Brian Halsall, Ph.D.
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Abstract

A rapid and sensitive fluorimetric assay has been developed to determine the biotin-binding capacities (BBC) of streptavidin or NeutrAvidin™ coated solid supports and therefore to estimate immobilized streptavidin/NeutrAvidin™ concentration. Biotin-4-fluorescein (b-4-f) on binding with streptavidin/NeutrAvidin™ gets quenched. A series of titrations were done using b-4-f as titrant for streptavidin/NeutrAvidin™ solid supports. Break points of titrations were used to determine total BBC. This method was successfully tested on four different solid supports - 2.8 µm streptavidin magnetic beads (4 x 10⁶ biotin/bead), 2.8 µm NeutrAvidin™ magnetic beads (2 x 10⁶ biotin/bead), 0.31 µm non-magnetic streptavidin polystyrene blue particles (6 x 10³ biotin/particle) and a 96 well streptavidin micro-titer plate (5 x 10¹³ biotin/well).

Another Fluorimetric method has been developed to determine the ligand binding capacities of streptavidin/NeutrAvidin™ coated solid supports. Streptavidin/NeutrAvidin™ coated solid supports were titrated with biotinylated antibodies followed by addition of b-4-f. Antibody concentration at the asymptote intersection (saturation point) was used to determine the ligand-binding capacity of four different solid supports as above. Streptavidin (2.8 µm) beads showed ~15fold decrease, streptavidin particles (0.31 µm) had 4fold decrease while streptavidin coated polystyrene plates, exhibited ~25fold decrease in biotinylated antibodies than their respective BBC published by manufacturers. Method can also be applied to determine the unknown concentration of biotinylated ligand in the solution. A calibration graph of ‘streptavidin high binding capacity coated plate’ vs. biotinylated antibody showed the linear range as 0.07 – 1.2 µg/well (4 - 65 nM) and a detection limit of 0.5 nM.
The effect of different generations of dendrimers on concentration of immobilized biotinylated ligands was studied. PAMAM dendrimer of generations G5.5 and G6.5 were covalently linked to tosyl beads (2.8 µM), and NeutrAvidin™ was covalently attached to the C-terminal of the dendrimer using EDC-sNHS coupling. All beads were finally coated with biotinylated antibodies. Moles of antibodies bound per bead for each generation of dendrimer vs. plain streptavidin/NeutrAvidin™ beads were compared using fluorescence. The amount of antibody bound per bead showed a trend with an increase in number of antibodies per bead from generations G0.5 to G6.5. There was an approximately 10-fold increase in the number of antibodies bound per bead for G 6.5 dendrimer (4 x 10⁶ antibodies/bead) versus plain NeutrAvidin™ coated beads (4 x 10⁵ antibodies/bead). Also, G6.5 beads showed 4.5-fold increase in biotin binding sites than plain NeutrAvidin™ beads. Finally G6.5 dendrimer-NeutrAvidin™ beads and plain NeutrAvidin™ beads (both coated with antibody) were used as capture beads for an immunoassay and G 6.5 dendrimer- NeutrAvidin™ beads gave 5-fold higher immunoassay sensitivity than plain NeutrAvidin™ beads.

The effect of different water disinfectants on immunoassay sensitivity is studied in our laboratory and it was found that oxidizing disinfectants chlorine and monochloramine had major effect on the sensitivity of MS2 bacteriophage. Hence the effect of different concentrations of monochloramine and its individual components on immunoassay sensitivity was studied for MS2 bacteriophage. It was found that decrease in immunoassay sensitivity was due to the combined or individual effect of monochloramine components (chlorine and ammonia).
ACKNOWLEDGEMENT

I would like to start my acknowledgement by thanking my advisor Dr. H. Brian Halsall for all his help, high expectations and positive criticism, which kept me going. Thanks to my co-advisor Dr. William Heineman for his immense patience, high ethics and tremendous ability to simplify complex situations.

My acknowledgement will be incomplete without thanking Dr. Anna Gudmundsdottir and Dr. Thomas H. Ridgway for all their invaluable advice, concern and support. I also extend this opportunity to thank Dr. Patrick A. Limbach for his timely help in numerous occasions throughout the years and also accepting to be a part of my Ph.D. committee in spite of his busy schedule. Thanks to Dr. Eddie Merino for serving in my committee. I am also grateful to Dr. Peter Padolik for being a supporting and helpful TA supervisor and for providing a hassle free work environment.

I am grateful to Dr. Kevin Schlueter, Agnese Jurkevica and Dr. Chamika M. Wansapura for being very helpful, especially during the start of my doctoral research. Special thanks to Sasithorn Muncharoen, Natasha Mwale, Agnese Jurkevica, Eme Amba and Tatyana Pinyayev for all your advice, sharing, yelling, fights and laughs… This journey wouldn’t be same without you guys!

Thanks to my in-laws Prema Murthy and Sethurajaraao Murthy for their understanding, patience and tolerance towards my busy schedule. Also, thanks to my guide and inspiration, my husband Dr. Rajesh S. Murthy for his unshaken faith in me and providing strength and encouragement at all times. Rajesh, as you might know, I could never have done this without you. Thanks for believing in me, even during the times when I was losing it.

Thanks to my siblings - Uttam Tiwari, Yudhishtar Tiwari and Sumati Tiwari Joshi for all your
selfless love and encouragement during my Ph.D. and throughout my life. Thanks to my wonderful father Late Balkrishana R. Tiwari for making me realize the importance of education and providing all the resources and support to the best of his ability to fulfill my ambition. Thanks to my mother Pushpa B. Tiwari for showing me the power of love, dedication, dignity and self-esteem by living the life the way you did. No word is capable of expressing my gratitude to the fullest, and for that I dedicate my Ph.D. to you!
Table of Contents

List of Figures x

List of Tables xiv

Chapter 1: Introduction to solid phase immunoassays, dendrimers and biotin binding proteins - streptavidin and NeutrAvidin™

Introduction 2

Project goals 12

References 15

Chapter 2: A fluorimetric assay to determine the biotin-binding capacities of a variety of immobilized streptavidin/ NeutrAvidin™ coated solid supports

Introduction 24

Methods and materials 26

Experimental 29

Results 33

Discussion 50

Conclusion 55

References 56

Chapter 3: A fluorimetric assay to determine the total number of biotinylated ligands bound to solid supports coated with streptavidin/ NeutrAvidin™

Introduction 61
<table>
<thead>
<tr>
<th>Chapter 4: Application of PAMAM dendrimers for NeutrAvidin™ coated solid supports</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Methods and materials</td>
</tr>
<tr>
<td>Experimental</td>
</tr>
<tr>
<td>Results and discussion</td>
</tr>
<tr>
<td>Conclusion</td>
</tr>
<tr>
<td>Future work</td>
</tr>
<tr>
<td>References</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 5: Effect of monochloramine disinfectant on MS2 bacteriophage immunoassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Methods and materials</td>
</tr>
<tr>
<td>Experimental</td>
</tr>
<tr>
<td>Results and discussion</td>
</tr>
<tr>
<td>Conclusion</td>
</tr>
<tr>
<td>References</td>
</tr>
</tbody>
</table>

| Chapter 6: Dissertation Summary                                           | 152  |
List of Figures

Chapter 1

Figure 1: Homogeneous and Heterogeneous Immunoassay
Figure 2: Competitive (heterogeneous) immunoassay
Figure 3: Non-competitive (heterogeneous, sandwich) immunoassay
Figure 4: Structure of D-biotin
Figure 5: Generation 3 Dendrimer

Chapter 2

Figure 1: Structure of biotin-4-fluorescein
Figure 2: Determination of biotin binding capacities of immobilized streptavidin/ NeutrAvidin™ coated solid supports
Figure 3: Preparation of NeutrAvidin™ coated beads
Figure 4: Measurement of biotin binding capacities of streptavidin-coated beads in a 96-well micro-titer plate using biotin-4-fluorescein as a titrant
Figure 5: Optimization of incubation time for streptavidin bead – biotin-4-fluorescein (titrant) titrations
Figure 6: Measurement of biotin binding capacities of streptavidin-coated bead using streptavidin beads as titrant
Figure 7: Measurement of biotin binding capacities of NeutrAvidin™-coated beads using biotin-4-fluorescein
Figure 8: Measurement of biotin binding capacities of streptavidin-coated polystyrene blue (0.31 µm) using biotin-4-fluorescein as a titrant
Figure 9: Measurement of biotin-binding capacities of streptavidin polystyrene particles (0.31


\[ \mu m \) using streptavidin particles as titrant

Figure 10: Measurement of biotin binding capacity of 'streptavidin high binding capacity coated plate' using biotin-4-fluorescein

Figure 11: Optimization of incubation time for 'streptavidin high binding capacity coated plate' using biotin-4-fluorescein

Figure 12: UV-vis for avidin

Figure 13: D-biotin - avidin titration for standardization of D-biotin

Figure 14: Reaction velocity of alkaline phosphatase (50 \( \mu \)M FDP): determination of biotin binding sites of NeutrAvidin™ beads

Figure 15: Measurement of biotin binding capacities of NeutrAvidin™-coated beads using biotinylated alkaline phosphatase

Figure 16: Optimization of substrate (FDP) concentration for measurement of biotin binding capacity of NeutrAvidin™ beads using biotinylated alkaline phosphatase

Figure 17: Measurement of biotin binding capacity of streptavidin coated beads using biotinylated alkaline phosphatase

**Chapter 3**

Figure 1: Determination of ligand binding capacities of streptavidin coated solid supports using biotin-4-fluorescein

Figure 2: Determination of biotinylated antibody capacity for 'streptavidin high binding capacity coated plate'

Figure 3: Optimization of biotin-4-fluorescein concentration for streptavidin coated plate

Figure 4: Optimization of biotin-4-fluorescein incubation time for streptavidin coated plate

Figure 5: Optimization of primary antibody incubation time for streptavidin coated plate
Figure 6: Optimization of washing steps (after antibody incubation) for streptavidin coated plate

Figure 7: Determination of biotinylated antibody capacity for streptavidin coated beads, using biotin-4-fluorescein

Figure 8: Antibody saturation curve for streptavidin-coated beads using biotin-4-fluorescein: comparison of two different micro-titer plates

Figure 9: Determination of biotinylated antibody capacity and optimization of biotin-4-fluorescein incubation time for M-280 NeutrAvidin™ coated beads

Figure 10: Determination of biotinylated antibody capacity for streptavidin polystyrene blue particles, using biotin-4-fluorescein

Figure 11: Determination of ligand binding capacity of ‘streptavidin high binding capacity coated plate’ using antibody-enzyme conjugate (m-IgG-ALP)

Figure 12: Reaction velocity of alkaline phosphatase for the determination of ligand binding capacity of NeutrAvidin™ coated beads, using antibody-enzyme conjugate (m-IgG-ALP)

Figure 13: Determination of ligand binding capacity of NeutrAvidin™ coated beads (2.8 µm) using antibody-enzyme conjugate (m-IgG-ALP)

Figure 14: Determination of ligand binding capacity of streptavidin-coated beads (2.8 µm) using antibody-enzyme conjugate (m-IgG-ALP)

Figure 15: Antibody saturation curve for streptavidin polystyrene blue using antibody-enzyme conjugate (m-IgG-ALP: 350 µg/ml conjugate)

Figure 16: Antibody saturation curve for streptavidin polystyrene blue particles using antibody-enzyme conjugate (m-IgG-ALP: 500 µg/ml conjugate)

Figure 17: Calibration curve for determination of immobilized biotinylated ligand using biotin-4-fluorescein (determination of detection limit and linear range)
Chapter 4

Figure 1: Polymer linkers for the immobilization of biotinylated ligands

Figure 2: Schematic for preparing dendrimer-coated bead

Figure 3: Antibody saturation curve to determine the capacity of G5.5 dendrimer-NeutrAvidin™ bead

Figure 4: Antibody saturation curve to determine the capacity of G6.5 dendrimer-NeutrAvidin™ bead

Figure 5: Antibody capacity studies for streptavidin, NeutrAvidin™, G0.5, G2.5, G5.5 and G6.5 dendrimer-NeutrAvidin™ beads, summary

Figure 6: Dendrimer-NeutrAvidin™ beads vs. plain NeutrAvidin™ beads

Figure 7: Determination of biotin binding capacity for G5.5 dendrimer-NeutrAvidin™ coated beads, using biotin-4-fluorescein

Figure 8: Determination of biotin binding capacity for G6.5 dendrimer-NeutrAvidin™ coated beads, using biotin-4-fluorescein

Figure 9: A sandwich immunoassay to compare dendrimer–NeutrAvidin™ beads and plain NeutrAvidin™ beads

Figure 10: Reaction velocities of alkaline phosphatase for sandwich immunoassay using G6.5 dendrimer-NeutrAvidin™ capture beads

Figure 11: Chart representing reaction velocity vs. Bugbead concentration for sandwich immunoassay using G6.5 dendrimer–NeutrAvidin™ beads and NeutrAvidin™ beads as solid support
Figure 12: Chart representing reaction velocity vs. Bugbead concentration for sandwich immunoassay using G6.5 dendrimer–NeutrAvidin™ beads and NeutrAvidin™ beads as solid support

Chapter 5
Figure 1: A rotating disk electrode (RDE) setup for hydrodynamic amperometric detection of the MS2 immunoassay
Figure 2: A scheme to detect the effect of monochloramine on the MS2 immunoassay
Figure 3: Log-log calibration plot for MS2 immunoassay for the comparison of fluorescence and electrochemical detection methods
Figure 4: The effect of various monochloramine concentrations on MS2 bacteriophage (7 d incubation)
Figure 5: Effect of free ammonia on MS2 assay

List of Tables

Chapter 1
Table 1: Comparison of different biotin binding proteins
Table 2: Characteristics of half-generations of PAMAM dendrimers

Chapter 2
Table 1: Biotin binding capacities of four different solid supports

Chapter 3
Table 1: Biotin binding capacity vs. ligand binding capacity of streptavidin and NeutrAvidin™ coated solid supports
Table 2: Ligand binding capacities of solid support
Chapter 4

Table 1: Biotin binding capacities for streptavidin, NeutrAvidin™ and dendrimer-NeutrAvidin™ coated solid supports using the biotin-4-fluorescein method

Table 2: Estimation of surface coverage for streptavidin, NeutrAvidin™ and dendrimer-NeutrAvidin™ coated solid supports using the biotin-4-fluorescein method
Chapter 1

Introduction to solid phase immunoassays, dendrimers and biotin binding proteins - streptavidin and NeutrAvidin™
**Immunoassay**

Immunoassay is a technique that uses the high binding affinity of antibodies for complementary antigen to detect or quantitate the analyte of interest (1, 2). The non-covalent interactions of antigen-antibody binding include hydrogen bonds, ionic bonds, hydrophobic and Van der Waals interactions (3).

Immunoassays can be done with (4) or without labels (5). Labeled immunoassays can be divided into two categories – homogeneous and heterogeneous (4). Homogeneous immunoassays do not require solid supports to separate free and bound antibodies (6), as seen in Figure 1. Hence, they are simple and rapid with minimal washing steps, but prone to interferences such as high background signal, low sensitivity and cannot be used for high molecular weight analytes (6-9). Heterogeneous immunoassays require solid supports to separate antigen-antibody complexes (Figure 1), hence they need washing steps and longer incubation times, which makes the method tedious and labor-intensive (8). Due to the presence of solid supports, heterogeneous assays have lower detection limits, fewer interferences and higher sensitivity than other common assays. Hence most immunoassays are heterogeneous (6).

Both homogeneous and heterogeneous immunoassays are further divided into competitive and non-competitive. In a competitive immunoassay, labeled antigens of known concentration and unknown antigens under investigation compete for the same binding sites on antibodies (Figure 2). The response produced by bound, labeled antigens will be inversely proportional to the concentration of unknown antigens. Detection limits of competitive immunoassays depend on the affinity constant of the antibodies (10). Non-competitive immunoassay (Figure 3) is also known as sandwich immunoassay, because the analyte of interest is bound (sandwiched) between two antibodies (capture antibodies and labeled antibodies).
Figure 1: Homogeneous and heterogeneous immunoassay

(Diagram not drawn to scale)
Figure 2: Competitive (heterogeneous) immunoassay

(Diagram not drawn to scale)
Figure 3: Non-competitive (heterogeneous, sandwich) immunoassay

(Diagram not drawn to scale)
Signals produced by labeled antibodies are directly proportional to the analyte concentration. Non-competitive assays have better sensitivity than competitive assays, which depends on the non-specific binding of the immunoreactants (10). Due to their higher sensitivity and better detection limits, heterogeneous, non-competitive immunoassays have been developed in our laboratory (11) and used in this research.

**Solid Support**

Solid supports play a major role in the success of heterogeneous immunoassays, and their use for immobilizing analytes and antigens dates back to 1971 (4, 12, 13). Test tubes (4, 12-14), microtiter plates/wells (14-16), column (17), capillaries (18), microspheres (beads) (11, 14), glass slides (19, 20) and particles (21, 22) are a few examples of solid supports used for immunoassays, each with its own advantages and limitations. Out of many solid supports available, the magnetic and paramagnetic beads are extremely popular. When compared with the solid supports of planner geometries, the spherical shape of microspheres (beads) provides high surface area to volume ratio resulting in more binding sites per unit surface area of the solid support (23, 24). This makes microbeads a preferred choice of solid support especially for microfluidic devices (24), which are size limited. Due to their magnetic properties, beads can be captured by a magnet, thereby facilitating pre-concentration, washing and other magnetic manipulations. Due to their homogeneous dispersion in solutions, beads reduce diffusional distances, resulting in shorter incubation times and better detection limits (4). Hence beads are preferred in this research, over other types of solid supports.

Bio-molecules can be immobilized on solid supports by physical adsorption or by covalent coupling [e.g. amine coupling, thiol coupling (25)]. Selection of the immobilization
method depends on parameters such as the reaction conditions needed for coupling, the stability and structure of the bio-molecules, the functional groups present on bio-molecules, etc. Streptavidin and biotin coupling chemistry is a popular choice for immobilization, very well known for its reproducibility, high binding capacity, chemical resistance (25), uniform distribution of biotinylated ligands on solid supports, and stronger ligand binding interactions (26).

**Biotin and biotin-binding proteins**

**Streptavidin**

In the early 1960s, Chaiet *et al.* discovered streptavidin (26-27). It is a non-glycosylated homotetrameric protein secreted by acetobacterium *Streptomyces avidinii* (26, 28-30). It is widely known for its high affinity for D-biotin (Vitamin H) \(2.5 \times 10^{13} \text{ M}^{-1}\) (31-33). Due to the exceptionally tight and specific binding, the streptavidin-biotin system has become the foundation for a wide variety of immunoassays, immunodiagnostics and purifications.

**Avidin**

Avidin, another biotin binding protein was discovered in the 1940s (26, 34). It has a similar biotin binding affinity and tetrameric structure as that of streptavidin. It is different than streptavidin in terms of its glycosylated structure and very basic pI of 10.5 (26). Due to its highly basic pI and presence of carbohydrates, avidin-biotin immobilization techniques suffer from high non-specific binding (26), making avidin less favorable than streptavidin. Differences between the biotin binding proteins - streptavidin, avidin and NeutrAvidin™ are shown in Table 1.
<table>
<thead>
<tr>
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<th>Streptavidin</th>
<th>Avidin</th>
<th>NeutrAvidin™</th>
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<tbody>
<tr>
<td>~Molecular Weight (kDa)</td>
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<td>60</td>
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<td>$5.6 \times 5.0 \times 4.0$</td>
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<tr>
<td>~pI</td>
<td>5-6</td>
<td>10.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>Low</td>
<td>High</td>
<td>Lowest</td>
</tr>
<tr>
<td>Source</td>
<td><em>Streptomyces avidinii</em></td>
<td>Hen egg white and tissues of birds, reptiles, amphibians</td>
<td>Synthesized</td>
</tr>
<tr>
<td>Biotin binding sites/molecule</td>
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<tr>
<td>Composition</td>
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<td>$2.5 \times 10^{13} \text{ M}^{-1}$</td>
<td>$1 \times 10^{15} \text{ M}^{-1}$</td>
<td>$1 \times 10^{15} \text{ M}^{-1}$</td>
</tr>
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</table>

Table 1: Comparison of different biotin binding proteins (26,35-37)
**NeutrAvidin™**

Chemically modified avidin is commercially available as NeutrAvidin™. NeutrAvidin™ is a trademark of Pierce Chemical Company (Rockford, IL, USA). It is a deglycosylated form of avidin with a nearly neutral pI (6.3), in which all biotin binding sites are preserved (38-39). It is widely used due to its lowest non-specific binding as compared to streptavidin and avidin.

**Biotin (Vitamin H)**

Biotin is a 244 Da (25) water-soluble compound, also known as Vitamin H, Vitamin B7 or Coenzyme R. It is found in various foods, plants and animals. Each biotin binding protein – streptavidin, NeutrAvidin™ and avidin has 4 biotin binding sites. Biotin consists of an ureido (tetrahydroimidizalone) ring fused with a tetrahydrothiophene ring in which a valeric acid substituent is attached to one of the carbon atoms of the tetrahydrothiophene ring (40) (Figure 4).

![Figure 4: Structure of D-biotin](image-url)
**Dendrimers**

Dendrimers [Greek: dendron – tree (41, 42)] are highly branched polymers, developed in 1985 by Donald A. Tomalija and his colleagues (43, 44). Dendrimers are extremely popular due to their well-defined, compact and reproducible structures with a large number of reactive groups on their surface (42). Each dendrimer is divided into the three subunits of core, repetitive branching units and end groups (45-49). For the first few generations, dendrimers behave like any ordinary polymer. As per molecular simulations (50), the poly (amidoamine) (PAMAM) dendrimers of generations 1 and 2 (G = 1-2) are open structures with asymmetric disk-like shapes. At higher generations (G ≥3), they start forming highly structural patterns of compact, closed, and densely packed spherical shapes (51).

Due to rapid advancements in synthetic chemistry and emerging new techniques, dendrimers of various types are synthesized. PAMAM dendrimers are one of the most popular dendrimers, known for a large number of reactive groups on the surface (52-54). They are also commercially available, highly symmetrical and have uniformly accessible sites (55) with monodisperse behavior. Characteristics of different generations of PAMAM are shown in Table 2. Michael addition of methyl acrylate (MA) on alkyl-diamine (core component) forms PAMAM dendrimers with ester terminal groups, which on further condensation (amidation), results in dendrimers with amine terminal groups (56). On repeating the stepwise procedure, dendrimers of increasing generations are formed, each with twice the number of terminal groups than its predecessor (56).

Dendrimers are classified into generations depending upon the degree of branching, where dendrimers of later generations have more branching than the earlier generations. These are further divided into half and full generation dendrimers.
<table>
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<th>Generations</th>
<th>Molecular weight (kDa)</th>
<th>No. of surface groups</th>
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<td>0.5</td>
<td>1.2</td>
<td>8</td>
</tr>
<tr>
<td>2.5</td>
<td>6.0</td>
<td>32</td>
</tr>
<tr>
<td>5.5</td>
<td>52.9</td>
<td>256</td>
</tr>
<tr>
<td>6.5</td>
<td>106.2</td>
<td>512</td>
</tr>
<tr>
<td>NeutrAvidin™</td>
<td>60</td>
<td>--</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>60</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 2: Characteristics of half-generations of PAMAM dendrimers [57-60]: Exponential increase in molecular weight and functional groups (45) can be seen with increased dendrimer generations.
If the PAMAM dendrimer is terminated at the amine group on the surface, it is known as full generation dendrimer, as shown in Figure 5. The first branching of the core unit at the terminal amine is known as dendrimer of generation zero (G 0). After this, each subsequent branching is termed as Generation 1 (G 1), Generation 2 (G 2), etc. There is ~ 1 nm increase in dendrimer diameter per generation (45). When the dendrimer structure is terminated at a carboxylic group (instead of amino group), it is known as half-generation dendrimer. Selection of a full or half generation dendrimer may depend on various factors such as the coupling chemistry chosen for dendrimer-ligand binding, the stability of the ligand under selected reaction conditions, among others.

There is a deep interest in the application-oriented research of dendrimers [45-46, 48-49]. Applications of dendrimers vary from medicine (61) and biotechnology (62) to electronics (63) and photonics (64-65), sensors (66) and nanotubes (67) to coatings and materials (68).

**Project Goals**

This research involves four different projects. The first three projects deal with method development for detection or improvement of streptavidin and NeutrAvidin™ coated solid supports. The last project reports the effects of oxidizing disinfectants on immunoassay sensitivity.

The low coverage of immobilized streptavidin or NeutrAvidin™ proteins may lead to lower number of biotinylated ligands per unit surface area and may result in assays with lower sensitivity and/or higher detection limits. In order to carry out successful assay using streptavidin/NeutrAvidin™ coated solid supports, there is a need for a method, which can detect
Figure 5: Generation 3 Dendrimer: Partial structure of G3 dendrimer is shown. A similar arrangement is present on all the four sides of two nitrogen atoms (core unit), giving an overall globular structure to the dendrimer. Termination occurred at NH$_2$ making it a full generation dendrimer (G3). If termination occurs at a carboxylic group (instead of amino group), it will form a half-generation dendrimer.
the total available biotin binding sites on these solid supports and calculate the extent to which these solid supports are covered with streptavidin or NeutrAvidin™. Therefore the aim of the first project is to develop a quick and accurate method for quantitation of total biotin binding sites on variety of immobilized streptavidin/NeutrAvidin™ coated surfaces. The aim also includes the estimation of the streptavidin/NeutrAvidin™ concentration per unit surface area of the support. A fluorescent marker biotin-4-fluorescence (b-4-f) is chosen in this research to achieve these targets.

It is not only important to have an optimum surface coverage of solid support with streptavidin or NeutrAvidin™ proteins, but also crucial to maintain an optimum coverage of biotinylated ligands on the biotin binding sites of these proteins. The degree of labeling (biotinylated ligands per unit surface area) may not only depend on total number of biotin binding sites but also on the size, charges, hydrophobic interactions, etc. between ligands and solid supports. Hence the aim of the second project is to develop a method to estimate the binding capacities of wide varieties of biotinylated ligands for different streptavidin or NeutrAvidin™ coated solid supports using b-4-f.

The quantity and quality of primary antibodies immobilized to solid supports plays a crucial role in determining the sensitivity of an immunoassay. Lower concentration of biotinylated antibodies per unit area may decrease the efficiency of capture by lowering the overall avidity of antibodies for an antigen. On the other hand, an excess of biotinylated antibodies per unit surface area may increase steric hindrance thereby restricting the conformational freedom of antibodies. The restriction in conformational freedom may decrease the affinity of antibodies to bind to the epitopes of antigen, producing lower sensitivity and/or higher detection limit for an immunoassay. In the third project dendrimers were incorporated
between solid supports and immobilized NeutrAvidin™ to target these problems. The aims of the third project were to study the effect of different generations of dendrimers on antibody binding capacity of NeutrAvidin™ coated beads, and also to compare the effect of dendrimer coated capture bead vs. plain bead (without dendrimer) on the sensitivity of an immunoassay.

The effect of different water components and disinfectants on immunoassay sensitivity is studied in our laboratory (11). The studies were done for simulants representing four different classes of microorganisms: ovalbumin (toxin), *E. coli* O157:H7 (bacteria), *B. globigii* and *B. anthracis* Stern strain (spores) and MS2 bacteriophage (virus). It was found that oxidizing disinfectants chlorine and monochloramine had major effect on the sensitivity of MS2 bacteriophage and minor effects on *B. globigii* and ovalbumin (11). Hence the aim of the fourth project was to study the effect of different concentrations of monochloramine and its individual components on immunoassay sensitivity for MS2 bacteriophage.

References


Chapter 2

A fluorimetric assay to determine the biotin-binding capacities of a variety of immobilized streptavidin/

NeutrAvidin™ coated solid supports
Introduction

Streptavidin-biotin system \([Ka = 2.5 \times 10^{13} \text{ M}^{-1}, (1-3)]\) is extensively used in bioscience and technology (4-6). The success of these systems hugely depends on the extent of labeling, i.e. number of biotin molecules attached per unit surface area of the solid support (7). The direct detection of biotin is difficult because its absorption spectra overlap with that of the proteins (7).

Few methods are available in the literature to detect the biotin-binding capacities (BBC) of streptavidin-coated plates (8, 9), microspheres (10, 11), particles (11) or other solid supports (12), each with its own limitations. Radioactive biotin provides very sensitive detection (10, 11), but is tedious and requires sophisticated instrumentation (10). Also, there is a possibility of health hazards and waste disposal problems. A spectrophotometric determination of biotin binding sites by the HABA assay (10) cannot be used for streptavidin due to the low streptavidin-HABA affinity \([Kd = 1 \times 10^{-4} \text{ M}, (2)]\) and it is even less sensitive to avidin (13) \([(Kd = 6 \times 10^{-6} \text{ M}, (2))\]. Biotinylated enzyme and enzyme inhibition assays need several optimization steps for enzyme concentration, substrate concentration, incubation time, etc. Many fluorimetric assays have lengthy procedures and/or interferences such as non-specific binding (9), background fluorescence and fluorescent sample matrices. Hence there is a need for a simple, rapid and sensitive method to detect BBC of such streptavidin/NeutrAvidin\(^\text{TM}\) coated solid supports.

This chapter explores using biotin-4-fluorescein (b-4-f) to determine the BBC of immobilized streptavidin/NeutrAvidin\(^\text{TM}\) coated solid supports. b-4-f is a conjugate of biotin and fluorescein linked by ethylene diamine, as in Figure 1.
The optimum size of ethylene diamine spacer in b-4-f results in low steric hindrance and minimum anti-cooperative binding effect (4). Hence b-4-f exhibits high affinity and fast association to streptavidin tetramer with an efficient quenching of 84-88% (14). Due to its smaller spacer length, b-4-f shares the similar binding mechanism as that of the D-biotin, where each b-4-f unit interacts individually with single streptavidin subunit resulting in four b-4-f bound per streptavidin tetramer (4, 14). Kada et al. illustrate the mechanism in detail (14).

b-4-f was first used by Kada et al. (4, 14) to detect free streptavidin in solution. Their method is considerably modified in this research, to prove its applicability in estimating immobilized streptavidin/NeutrAvidin™ concentration, and to detect active biotin-binding sites on streptavidin/NeutrAvidin™ solid supports, which play a crucial role in the detection limit, sensitivity and success of various assays using these supports.

A standard fluorimetric assay was developed to determine the BBC of a variety of streptavidin/NeutrAvidin™ coated solid supports. This method was tested on four different
solid supports - streptavidin magnetic beads (2.8 µm), NeutrAvidin™ magnetic beads (2.8 µm), non-magnetic streptavidin polystyrene blue particles (0.31 µm) and a 96 well streptavidin micro-titer plate.

Streptavidin coated solid supports can be non-cumulatively (in different wells) titrated with b-4-f. Biotin anchors fluorescein on the immobilized streptavidin. When the concentration of b-4-f increases linearly in each well containing a fixed concentration of immobilized streptavidin (non-cumulative titration), in the first few wells all/most of b-4-f added binds to the streptavidin followed by strong quenching, resulting in a plateau of lowest fluorescence [Figure 2 (A)]. A stage will be reached when all the binding sites of immobilized streptavidin are completely saturated with b-4-f (break-point). At and beyond this point [fig. 2(B)], any further b-4-f added will increase the fluorescence sharply. Hence, when a fluorescence signal is plotted against the b-4-f concentration, a plateau is followed by a sharp increase in fluorescence as shown in Figure 2. The ligand concentration at the break point is critical in determining the BBC. Similar method can be applied for NeutrAvidin™-coated beads.

**Materials and methods**

**Reagents**

Biotinylated alkaline phosphatase (β-ALP), Immunopure® Avidin, Immunopure® NeutrAvidin™ (biotin binding protein) and ‘streptavidin high binding capacity coated plates’ were from Pierce (Rockford, IL, USA). Biotin-4-fluorescein and fluorescein diphosphate (FDP) were obtained from Invitrogen™, Molecular Probes™ (Eugene, OR, USA). Streptavidin-coated M-280 Dynabeads® [~6-7 x 10^8 beads/ml, 2.8 µm diameter] and tosyl-activated M-280 Dynabeads® [~2 x 10^9 beads/ml, 2.8 µm diameter] were from Invitrogen Corporation (Carlsbad,
Figure 2: Determination of biotin binding capacities of immobilized streptavidin/NeutrAvidin™ coated solid supports.
CA, USA). Bovine serum albumin (BSA) fraction V powder (biotech grade), sodium azide, sodium chloride (ACS grade), magnesium chloride (MgCl$_2$.6H$_2$O, ACS grade), Tris (hydroxymethyl) aminomethane (THAM, laboratory grade), micro-centrifuge tubes (1.5 ml) and borosilicate culture tubes (12 x 75 mm) were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Glycine (99.5%) and potassium phosphate monobasic (ACS grade) were from Matheson Coleman Bell (Norwood, OH, USA). Potassium phosphate dibasic was from J. T. Baker Chemical Co. (Baker Analyzed Reagent; Phillipsburg, NJ, USA). The neodymium-iron-boron rare earth magnets were obtained from Radio Shack (Fort Worth, TX, USA). Non-magnetic Sphero™ streptavidin-coated polystyrene blue particles [1.0% (w/v), 0.31 µm diameter] were acquired from Spherotech Inc (Libertyville, IL, USA). Black polystyrene assay plates (96 well, flat bottom) were from Corning Inc. (Corning, NY, USA). D-biotin was from Sigma-Aldrich (Saint Louis, MO, USA). Tween 20 was from Aldrich Chemical (Milwaukee, WI, USA). All chemicals were used without further purification.

**Apparatus**

**Optical microscope:** An Olympus BX-40 microscope with a Hitachi HV-C20 camera and Scion CG-7 frame grabber software on a Macintosh G3 platform was used to count beads (15). The obtained images were processed with Scion Image Software (15).

**Luminescence spectrometer:** All fluorescence measurements were done in 96 well plates using a Perkin Elmer LS 50B luminescence spectrometer as a fluorescence plate reader. Other apparatuses used were - Incubator (Wedco Inc., Silver Spring, MD, USA), Baxter micro-centrifuge (model 3531) with Heraeus Sepatech (Abbott) fixed angle rotor and cover, Micro-titer plate shaker with orbital movement (Ika Works Inc, Wilmington, NC, USA), Diode Array UV-vis Spectrophotometer (Hewlett Packard 8452A) and Neubauer hemocytometer (Fisher
Buffers

Three aqueous buffer solutions were used. **PBS conjugation buffer**, pH 7.4: [PBS-C, 0.044 M KH$_2$PO$_4$, 0.056 M K$_2$HPO$_4$, 0.1 M NaCl, 0.02% (w/v) NaN$_3$]; **Tris buffer with BSA** pH 9.0: [0.1 M THAM, 5 mM MgCl$_2$.6H$_2$O, 10 mM glycine, 0.1% BSA, 0.02 % (w/v) NaN$_3$] and **Tris buffer without BSA** pH 9.0: [0.1 M THAM, 5 mM MgCl$_2$.6H$_2$O, 10 mM glycine, 0.02 % (w/v) NaN$_3$].

Experimental

2.1.1 Preparation of NeutrAvidin™ coated beads

The procedure for covalent binding of NeutrAvidin™ and tosyl-activated beads was adapted from the method described by Farrell (16), and Dynal Technical notes (17). In short, washed tosyl-activated beads (~ 2 x 10$^9$ beads/ml, 68 µL) were mixed with NeutrAvidin™ solution (36 µg/ml, 1 ml) in PBS-C buffer and incubated overnight at 37°C on a plate shaker with gentle agitation. Then the beads were washed and re-suspended in 400 µl of Tris buffer.

2.2.1 Determination of the biotin binding capacities of magnetic Streptavidin/NeutrAvidin™ beads using biotin-4-fluorescein

Streptavidin/NeutrAvidin™ beads (8.0 x 10$^8$ beads/ml, 200 µl) were washed twice with Tris buffer with the help of a magnet and re-suspended in 400 µl of the same buffer. Diluted streptavidin/NeutrAvidin™ suspension (4.0 x 10$^8$ beads/ml) was used as a working standard. The concentration of b-4-f was verified by UV-vis absorption at 494 nm. At 494 nm, molar extinction coefficient of b-4-f, pH 9.0 is 73,200 M$^{-1}$cm$^{-1}$ (18).

Streptavidin/NeutrAvidin™ beads in each well (4 x 10$^8$ beads/ml, 7 µl) were non-cumulatively titrated with b-4-f. The b-4-f working standard used was 2.73 x 10$^{-6}$ M (1 µl - 17
µl) for streptavidin and 1.25 x 10^{-6} M (1 µl - 20 µl) for NeutrAvidin™. The experiment was done in a 96-well black polystyrene micro-titer plate, and the volume was adjusted to 100 µl using Tris buffer containing 0.1 % BSA. Mixtures were sealed with Parafilm™ and incubated on a plate shaker for 5, 30 and 60 min and fluorescence measurements made. In a separate experiment (control), b-4-f solution (streptavidin: 2.73 x 10^{-6} M, 1 µl - 17 µl; NeutrAvidin™: 1.25 x 10^{-6} M, 1 µl - 20 µl) was added non-cumulatively as above. The total volume was adjusted to be 100 µl using Tris buffer containing 0.1 % BSA. The mixture was incubated for 5, 30 and 60 min on a plate shaker. In both cases, b-4-f was excited at 494 nm (slit width 10 nm) and emission was measured at 514 nm (slit width 10 nm).

The concentrations of streptavidin/NeutrAvidin™ beads were further verified by diluting 10 µl of streptavidin/NeutrAvidin™ working standards many-fold using PBS buffer pH 7.0 and counting beads on an optical microscope using a Neubauer hemocytometer. The calculated concentration of working standards for both the beads using this method was 4.0 x 10^{8} beads/ml.

A similar experiment was done by titrating a fixed concentration of b-4-f (4.96 x 10^{-6} M, 8 µl) with varying concentrations of streptavidin beads (8.0 x 10^{8} beads/ml, 0 µl - 14 µl).

2.2.2 Determination of biotin binding capacities of non-magnetic streptavidin coated polystyrene blue particles using biotin-4-fluorescein

The stock concentration of streptavidin polystyrene blue was calculated to be 6.1 x 10^{11} particles/ml, which was diluted 6 times to give the working standard concentration as 1.0 x 10^{11} particles/ml. Diluted particles (1.0 x 10^{11} particles/ml, 200 µl) were added to micro-centrifuge tubes (1.5 ml) and washed (13,000 rpm, minimum RCF 13,400g, 5 min) centrifugally (twice) with Tris buffer using a microcentrifuge with a fixed angle rotor (24-place x 1.5-2 ml). Particles were re-suspended in 200 µl of the same buffer.
Streptavidin-coated particles (1.0 x 10^{11} particles/ml, 8 µl) were non-cumulatively titrated with stock b-4-f (1.89 x 10^{-6} M, 0 µl - 10 µl) in 14 different centrifuge tubes. Tris buffer containing 0.1 % BSA was used to maintain the final titration volume as 100 µl. Centrifuge tubes were capped and reaction mixtures were incubated on a plate shaker (5 min). After incubation, tubes were centrifuged (13,000 rpm, 7 min) and 90 µl of each supernatant solution were transferred into fresh wells of the micro-titer plate for detection.

A similar experiment was repeated with streptavidin particles as the titrant by titrating b-4-f (2.44 x 10^{-6} M, 5 µl) with varying concentrations of streptavidin particles (1.0 x 10^{11} particles/ml, 0 µl - 20 µl).

In a separate experiment (control), b-4-f (1.89 x 10^{-6} M, 0 µl - 10 µl) was used as above (without particles) with 90 µl final volume, using Tris buffer containing BSA. In all three cases, b-4-f was excited at 494 nm (slit width 10 nm) and emission was measured at 514 nm (slit width 11 nm).

**2.2.3 Determination of biotin binding capacity of streptavidin coated micro-titer plate using biotin-4-fluorescein**

The biotin binding capacity of the ‘streptavidin high binding capacity coated plate’ was evaluated. Since these plates were pre-blocked with Superblock\textsuperscript{TM} blocking buffer by the manufacturer, Tris buffer was used without BSA. b-4-f (4.28 x 10^{-6} M, 0 µL - 45 µL) was added to each well and a total volume was maintained at 120 µL using Tris buffer. The sealed reaction mixture was incubated on a plate shaker (5 and 15 min) at room temperature. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).
2.2.4 Biotin binding capacity of NeutrAvidin™ beads using biotinylated alkaline phosphatase (β-ALP)

**Standardization of D-biotin solution**

Immunopure® Avidin solution (5.79 x 10^{-6} M, 1 ml) was prepared in PBS buffer and its concentration was further verified by UV-vis absorbance. D-biotin working solution was prepared by 600-fold dilution of D-biotin stock solution (~ 5 mM) using PBS buffer. A known concentration of avidin solution (5.79 x 10^{-6}M, 4 µl) was titrated with diluted D-biotin solution (~ 8 µM, 0 µl - 27.5 µl) by maintaining the total volume at 100 µl using PBS buffer. The solution was incubated for 15 min at room temperature. D-biotin was excited at 290 nm (slit width 5 nm) and emission was measured at 340 nm (slit width 10 nm).

**Assay:** M-280 NeutrAvidin™ beads (4.0 x 10^8 beads/ml, 200 µl) were washed twice with Tris buffer (aided by a magnet) and re-suspended in 200 µl of the same buffer. NeutrAvidin™ beads in each well (2.8 x 10^6 beads, 7 µl) were titrated with D-biotin working solution (1.01 x 10^{-6}M, 0 µl - 9 µl) and the total volume was maintained at 100 µl using PBS buffer. Beads were incubated for 45 min on a plate shaker at room temperature. After incubation the excess (unbound) D-biotin was removed by washing (3 times) with Tris buffer. To these washed beads, β-ALP (60 µg/ml, 50 µl) in Tris buffer was added and incubated for 45 min. Finally beads were washed (5 times) and re-suspended in 30 µl of Tris buffer. Beads were then ready for detection.

**Detection:** Beads in each well were diluted 1:20 times with Tris buffer and a fixed volume of diluted beads (5 µl) were mixed with 5 µM FDP (85 µl). FDP was excited at 490 nm (slit width 2.5 nm) and emission was measured at 514 nm (slit width 2.5 nm). Detection was repeated using 50 µM FDP.
A similar assay was done with streptavidin-coated magnetic beads (2.8 x 10^6 beads, 7 µl) with D-biotin concentration of 1.01 x 10^{-6} M (0-17 µl).

**Results**

2.1.1 Preparation of NeutrAvidin™ beads

Tosyl beads are uniform, super-paramagnetic polystyrene beads coated with a polyurethane layer (17) and activated by tosyl chloride (p-toluene-sulphonyl chloride) (17, 20) to provide reactive tosyl groups on the bead surface. Under suitable reaction conditions, tosyl beads covalently bind to the primary amine group of NeutrAvidin™ forming NeutrAvidin™ beads. Preparation of NeutrAvidin™ beads is shown in Figure 3 (17). NeutrAvidin™ beads prepared by this method were consistent and gave no/minimum batch-to-batch variation with respect to BBC.

![Figure 3: Preparation of NeutrAvidin™ coated beads](image)

**Figure 3: Preparation of NeutrAvidin™ coated beads:** Tosyl beads (~ 2.0 x 10^9 beads/ml, 68 µL) when incubated with NeutrAvidin™ solution (36 µg/ml, 1 ml) for 12 hours at 37 °C, formed NeutrAvidin™ beads.

2.2.1 Determination of biotin binding capacities of magnetic streptavidin/NeutrAvidin™ coated beads using biotin-4-fluorescein

In a standard assay, streptavidin beads were titrated with b-4-f. Two regression lines coinciding at stoichiometric equivalence points were plotted as shown in Figure 4A, to give a streptavidin:b-4-f titration curve. In Figure 4A, the initial horizontal line of the titration (R^2 =0.9753) is
Figure 4: Measurement of biotin binding capacities of streptavidin-coated beads using biotin-4-fluorescein as a titrant: (A) Streptavidin coated magnetic beads (2.8 x 10^6 beads/well) were non-cumulatively titrated with b-4-f as a titrant (2.73 x 10^-6 M, 1 µL - 17 µL). Break point indicated 17.6 pmoles of biotin binding site/2.8 x 10^6 beads, from which number of biotin binding sites was estimated as 4 x 10^6 sites/bead. (B) Control assay had a similar concentration of b-4-f, and same total volume as ‘A’ but did not contain any streptavidin bead. The error bars are smaller than symbol heights, hence are not visible.
credited to efficient quenching of b-4-f accompanying b-4-f – streptavidin binding. Further increase in the b-4-f concentration resulted in a break point followed by a sharp, linear ($R^2 = 0.9769$) increase in fluorescence due to free, unquenched b-4-f in the solution. An approximately 21 times increase in slope ($m$) was observed for regression lines before ($m = 5.1856$) and after the equivalence point ($m = 0.2480$). The control assay showed a continuous (no break point), linear ($R^2 = 0.9953$) increase in fluorescence with increased b-4-f concentration ($m = 5.8824$), due to the absence of streptavidin beads in the solution (Figure 4B).

Identical results were obtained at 5, 15, 30 and 60 min incubation as shown in Figure 5. Hence 5 min was considered as the optimal incubation time. The moles of biotin binding sites per bead can be calculated from equation-I:

$$\text{Moles of biotin binding sites per bead} = \frac{\text{moles of b-4-f at equivalence point}}{\text{total number of beads per well}}$$  \hspace{1cm} (I)

Avogadro’s number ($6.022 \times 10^{23}$ molecules/mole) can be used to quantitate the total number of biotin binding sites per bead. The BBC of a streptavidin bead using equation-I was $6 \times 10^{-18}$ moles of b-4-f/bead or $4 \times 10^6$ b-4-f/bead.

The effect of bead interferences on the fluorescence signal was studied by a similar experiment repeated using streptavidin beads as a titrant. Beads did not interfere with the fluorescence signal and no discrepancies in results were observed when a fixed concentration of b-4-f ($4.96 \times 10^{-6}$ M, 8 µl) was non-cumulatively titrated with varying concentrations of streptavidin beads ($8.0 \times 10^8$ beads/ml, 0 µl - 14µl) (Figure 6). Initially, when there were no streptavidin beads in the solution (control), b-4-f had the highest fluorescence due to its free, unquenched form. As the concentration of streptavidin was increased, a linear decrease in fluorescence was observed, followed by a break point ($6.1 \times 10^6$ beads).
Figure 5: Optimization of incubation time for streptavidin bead – biotin-4-fluorescein (titrant) titrations: Streptavidin coated magnetic beads (2.8 x 10^6 beads/well) were non-cumulatively titrated with b-4-f as a titrant (2.73 x 10^{-6} M, 1 µL - 17 µL). b-4-f was incubated for 5, 30 and 60 min. The error bars are smaller than symbol heights, hence are not clearly visible.
Figure 6: Measurement of biotin binding capacities of streptavidin-coated bead using streptavidin beads as titrant: b-4-f (4.96 x 10^{-6} M, 8 μl) was non-cumulatively titrated with streptavidin coated magnetic beads (8.0 x 10^8 beads/ml, 0 μl - 14μl). Break point indicated 6.1 x 10^6 beads/ 3.96 x 10^{11} moles of b-4-f, from which the number of biotin binding sites was estimated as 4 x 10^6 sites/bead.
At and beyond the break point, all/most of the b-4-f was quenched due to streptavidin binding, resulting in minimum fluorescence (Figure 6). Similar results were obtained for 5, 45 and 60 min incubation; hence 5 min was considered as the optimal incubation time.

According to Dynal, the biotin-binding capacity of a streptavidin bead is $1 \times 10^{17}$ moles of free biotin/bead or $6 \times 10^6$ molecules of free biotin/bead [1 mg of Dynabeads (6.7 x 10^7 beads) = 700 pmoles of free biotin (17)]. Thus the result obtained using the b-4-f method is in close agreement with the manufacturer’s data.

The NeutrAvidin™ bead - b-4-f titration is shown in Figure 7. Using this method, the biotin-binding capacity of NeutrAvidin™-coated beads was $4 \times 10^{18}$ moles of b-4-f/bead or $2 \times 10^6$ b-4-f/bead. The method was further verified by determining the BBC of NeutrAvidin™ beads using biotinylated alkaline phosphatase (Section 2.4: $2 \times 10^{-18}$ moles of biotin binding sites/ NeutrAvidin™ bead or $1 \times 10^6$ biotin binding sites/ NeutrAvidin™ bead).

**2.2.2 Determination of biotin binding capacity of non-magnetic streptavidin polystyrene blue particles using biotin-4-fluorescein**

Due to their smaller diameter (0.31 µm), streptavidin polystyrene blue particles were not counted on the hemocytometer, but were calculated as $6.1 \times 10^{11}$ particles/ml using the equation-II (21):

$$N = \left(\frac{6W}{3.14PD^3}\right) \times 10^{12} \quad -------\text{II}$$

where, $N$= Number of particles,

$W$ = Weight of polymer in gram ($1 \times 10^{-3}$ g),

$P$ = Density of polymer (polystyrene = 1.05 gm/cm$^3$) and

$D$ = Diameter of particles in micrometer (0.31 µm).
Figure 7: Measurement of biotin binding capacities of NeutrAvidin™-coated beads using biotin-4-fluorescein: (A) NeutrAvidin™ coated magnetic beads (2.8 x 10⁶ beads/well) were non-cumulatively titrated with b-4-f as titrant (1.25 x 10⁻⁶ M, 1 - 20 µl). Break point indicated 8.0 pmoles of biotin binding site/2.8 x 10⁶ beads, from which the number of biotin binding sites per bead was estimated as 2 x 10⁶. (B) Control assay had a similar concentration of b-4-f, and the same total volume as ‘A’ but did not contain any NeutrAvidin™ beads. The error bars are smaller than symbol heights, hence are not clearly visible.
The presence of dye imparts a dark blue color to these particles. Using supernatant solutions for fluorimetric measurements minimized dye interferences. BBC of streptavidin polystyrene blue was $1 \times 10^{-20}$ moles of b-4-f/particle or $6 \times 10^3$ b-4-f/particle (Figure 8). According to Spherotech Inc., the BBC is $6 \times 10^{-21}$ biotin-FITCl/particle or $4 \times 10^3$ biotin-FITCl/particle [$0.385 \times 10^{-9}$ moles of biotin-FITCl/mg of particle (21)]. A similar result was obtained when streptavidin particles were used as a titrant as shown in Figure 9.

2.2.3 Determination of biotin binding capacity of streptavidin coated micro-titer plate using biotin-4-fluorescein

The BBC of the ‘streptavidin high binding capacity coated plate’ was 86 pmoles of b-4-f/well or $5 \times 10^{13}$ b-4-f/well (Figure 10). Identical results were obtained for 5 and 15 min incubation times with b-4-f as shown in Figure 11, hence 5 min was considered as the optimized incubation time. Binding capacity as per the manufacturer (Piercenet) is $8 \times 10^{13}$ biotin/well (125 pmoles/well) (22). The error bars indicate fluorescence data of three independent experiments, validating the precision of the method.

2.2.4 Verification for biotin binding capacity of NeutrAvidin™ beads using biotinylated alkaline phosphatase (β-ALP)

Since NeutrAvidin™ beads were made in the laboratory, manufacturer’s data was not available to validate total biotin binding sites quantitated by the b-4-f method. Hence the experiment was validated using the β -ALP method. It is a very straightforward titration, in which beads are titrated with D-biotin followed by adding β-ALP. The concentration of D-biotin was standardized using a known concentration of avidin and a 1:4 stoichiometric ratio of avidin:biotin (19).
Figure 8: Measurement of biotin binding capacities of streptavidin-coated polystyrene blue (0.31 µm) using biotin-4-fluorescein as a titrant: (A) Non-magnetic streptavidin coated particles (8.1 x 10^8 particles/tube) were non-cumulatively titrated with b-4-f as a titrant (1.89 x 10^{-6} M, 0-10 µl). Break point indicated 8.0 pmoles of biotin binding site/8.1 x 10^8 particles, from which biotin binding sites per particle was estimated as 6 x 10^3. (B) Control assay had a similar concentration of b-4-f, and same total volume as ‘A’ but did not contain any streptavidin particle. The error bars are smaller than symbol heights, hence are not visible.
Figure 9: Measurement of biotin-binding capacities of streptavidin polystyrene particles (0.31 µm) using streptavidin particles as titrant: b-4-f (2.44 x 10⁶ M, 5 µl) was non-cumulatively titrated with streptavidin polystyrene blue particles (1.02 x 10¹¹ particles/ml, 0 µL -20 µL) as a titrant. Fluorescence of supernatant was measured. Break point indicated 1.2 x 10⁹ particles/ 1.2 x 10¹¹ moles of b-4-f, from which biotin binding sites per bead was estimated as 6 x 10³ molecules/particle. The error bars are smaller than symbol heights, hence are not clearly visible.
Figure 10: Measurement of biotin binding capacity of 'streptavidin high binding capacity coated plate' using biotin-4-fluorescein: ‘Streptavidin high binding capacity coated plate’ was non-cumulatively titrated with b-4-f as a titrant (4.28 x10^{-6} M, 0-45 µl). Break point indicated 86pmoles of biotin binding site/per well.
Figure 11: Optimization of incubation time for ‘streptavidin high binding capacity coated plate’ using biotin-4-fluorescein: ‘Streptavidin high binding capacity coated plate’ was non-cumulatively titrated with b-4-f as a titrant (4.28 x10^{-6} M, 0-45 µl). b-4-f was incubated for 5 and 15 before measuring fluorescence.
**Standardization of D-biotin solution**

The UV-vis spectrum of avidin in PBS buffer (pH 7.5) is shown in Figure 12. Using the molar extinction coefficient of avidin of 96,000 M$^{-1}$cm$^{-1}$ at 282 nm (19), the concentration of avidin in PBS buffer was determined to be 5.8 x 10$^{-6}$ M.

The volume of D-biotin added at the break point, (13.8 µl; Figure 13), concentration of avidin/well (2.3 x 10$^{-11}$ moles), dilution factor (1:600), and 1:4 stoichiometric binding of D-biotin ligands per avidin tetramer (19) were used to standardize the D-biotin stock concentration as 4.0 x 10$^{-3}$ M.

**Assay:**

As the concentration of D-biotin is increased linearly (0 - 5.1 pmoles), less of β-ALP can bind to the beads, giving a linear proportional decrease in fluorescence as shown in Figure 14. At the saturation point, (~5 pmoles) most of the biotin binding sites of the beads are occupied by D-biotin, and hence a negligible amount of β-ALP can bind to the beads due to lack of available sites, giving only a very low signal (~5.1 - 8.1 pmoles).

The slope of the fluorescence signal due to FDP hydrolysis (Figure 14) was plotted against the concentration of D-biotin as shown in Figure 15. The D-biotin concentration (4.8 pmoles) at the break point of the titration was used to determine the BBC of M-280 NeutrAvidin™ bead, and was found to be 2 x 10$^{-18}$ moles of biotin binding sites/bead or 1 x 10$^6$ biotin binding sites/bead. The BBC of the NeutrAvidin™ beads using b-4-f was 4 x 10$^{-18}$ moles of b-4-f/bead or 2 x 10$^6$ b-4-f/bead. The results are in close agreement.

An optimal substrate concentration is critical step in enzyme assays. In order to ensure a sufficient FDP concentration, the same experiment was repeated using 5 and 50 µM FDP.
Figure 12: UV-vis for avidin: UV-vis spectrum of avidin in PBS buffer (pH 7.5) was measured. Absorbance was read at 282 nm.
Figure 13: D-biotin - avidin titration for standardization of D-biotin: Known concentration of avidin ($5.8 \times 10^{-6}$M, 4 µl) was titrated with D-biotin solution (~ 8 µM, 0 µl - 27.5 µl). Break point was obtained at 13.8 µl of D-biotin working standard, from which the stock D-biotin concentration was determined as $4.0 \times 10^{-3}$M.
Figure 14: Reaction velocity of alkaline phosphatase (50 µM FDP): determination of biotin binding sites of NeutrAvidin™ beads: NeutrAvidin™-coated magnetic beads (2.8 x 10^6 beads, 7 µl) were non-cumulatively titrated with D-biotin (1.01 x 10^{-6} M, 0 - 9 µl; total volume: 100 µl) and then β-ALP (60 µg/ml, 50 µl) was added. Diluted beads (5 µl, 1:20 dilution) were added to 50 µM FDP (85 µl). FDP was excited at 490 nm (slit width 2.5 nm) and emission was measured at 514 nm (slit width 2.5 nm).
Figure 15: Measurement of biotin binding capacities of NeutrAvidin\textsuperscript(TM}-coated beads using biotinylated alkaline phosphatase: NeutrAvidin\textsuperscript(TM}-coated magnetic beads (2.8 x 10\textsuperscript{6} beads, 7 µl) were non-cumulatively titrated with D-biotin (1.01 x 10\textsuperscript{6} M, 0 - 9 µl; total volume: 100 µl) and then β-ALP (60 µg/ml, 50 µl) was added. Diluted beads (5 µl, 1:20 dilution) were added to 50 µM FDP (85 µl). FDP was excited at 490 nm (slit width 2.5 nm) and emission was measured at 514 nm (slit width 2.5 nm). Break point indicated 4.8 pmoles of D-biotin/2.8 x 10\textsuperscript{6} beads, from which biotin binding sites was estimated as 1 x 10\textsuperscript{6} per bead or 2 x 10\textsuperscript{-18} moles/bead.
Identical results were obtained for both (5 µM and 50 µM FDP) indicating that 5 µM of FDP is sufficient for maximum enzyme catalysis (Figure 16).

Using this method, the biotin-binding capacity of M-280 streptavidin bead was found to be 1 x 10^6 binding site/bead or 2 x 10^{-18} moles of sites/ bead (Figure 17).

**Discussion**

**Determination of biotin binding capacity using biotin-4-fluorescein method**

The b-4-f quenching effect on binding with streptavidin is attributable to the formation of a charge transfer complex between a b-4-f donor and one or more streptavidin residues (23). The quenching effect may also be due to communication between adjacent fluorescein moieties across the dimer-dimer interface (23, 2). Different solid supports used for this assay with their respective BBC are shown in Table 1. Results obtained by b-4-f method were in close agreement with results obtained by other methods (Table 1). The small differences in values can be attributed to the dissimilarity in sensitivities of the different methods used. In the case of streptavidin/NeutrAvidin™ beads, the dissimilar steric hindrance produced due to the wide difference in marker sizes [b-4-f: 644.69 Da (18), calf intestinal alkaline phosphatase 140 kDa (24, 25)] may contribute significantly to this variation in sensitivities. A shorter assay time can be obtained if non-cumulative titrations of b-4-f (series of tubes with different biotin/avidin ratios) are replaced by cumulative titrations (in a single cuvette) (19), assuming that there will be no/negligible bead interferences. For comparing relative data, the fluorescence intensities of cumulative titrations should be corrected for sample dilutions (14).
Figure 16: Optimization of substrate (FDP) concentration for measurement of biotin binding capacity of NeutrAvidin™ beads using biotinylated alkaline phosphatase: NeutrAvidin™-coated magnetic beads (2.8 x 10⁶ beads, 7 μl) were non-cumulatively titrated with D-biotin (1.01 x 10⁻⁶ M, 0 - 9 μl; total volume: 100 μl) and then β-ALP (60 μg/ml, 50 μl) was added. Diluted beads (5 μl, 1:20 dilution) were added to 5 and 50 μM FDP (85 μl). FDP was excited at 490 nm (slit width 2.5 nm) and emission was measured at 514 nm (slit width 2.5 nm).
Figure 17: Measurement of biotin binding capacity of streptavidin coated beads using biotinylated alkaline phosphatase: Streptavidin-coated magnetic beads were non-cumulatively titrated with D-biotin (1.01 x 10^{-6} M, 0-17 µl) and then β-ALP was added. Break point indicated 5.1 pmoles of D-biotin/2.8 x 10^6 beads, from which the number of biotin binding sites per bead was estimated as 1 x 10^6, or 2 x 10^{-18} moles/bead.
<table>
<thead>
<tr>
<th>Solid supports</th>
<th>b-4-f method</th>
<th>Other methods&lt;sup&gt;17, 21 -22&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Binding sites</td>
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<td>Moles of binding sites</td>
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<tr>
<td>Streptavidin bead (2.8 µm, magnetic)</td>
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<td>6 x 10&lt;sup&gt;-18&lt;/sup&gt;</td>
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<td>6 x 10&lt;sup&gt;-18&lt;/sup&gt;</td>
<td>6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td></td>
<td>1 x 10&lt;sup&gt;-17&lt;/sup&gt;</td>
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<tr>
<td>NeutrAvidin&lt;sup&gt;TM&lt;/sup&gt; bead (2.8 µm, magnetic)</td>
<td>2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4 x 10&lt;sup&gt;-18&lt;/sup&gt;</td>
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<td>2 x 10&lt;sup&gt;-18&lt;/sup&gt;</td>
</tr>
<tr>
<td>Streptavidin particle (0.31 µm, non-magnetic, blue)</td>
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<td>1 x 10&lt;sup&gt;-20&lt;/sup&gt;</td>
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<td>4 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>6 x 10&lt;sup&gt;-21&lt;/sup&gt;</td>
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<tr>
<td>Streptavidin plate (per well)</td>
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<td>8.6 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>8 x 10&lt;sup&gt;13&lt;/sup&gt;</td>
<td>1.25 x 10&lt;sup&gt;-10&lt;/sup&gt;</td>
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</table>

Table 1: Biotin binding capacities of four different solid supports: Streptavidin/NeutrAvidin<sup>TM</sup> immobilized on four different solid supports were quantified and results are verified using manufacturer’s data or other method.
Detection using biotinylated alkaline phosphatase method

FDP is one of the most fluorogenic alkaline phosphatase substrates (26). In the presence of alkaline phosphatase, colorless and non-fluorescent FDP is first dephosphorylated to weakly fluorescent fluorescein mono-phosphate and then to strongly fluorescent fluorescein (27, 28).

To ensure that there was a sufficient concentration of biotinylated alkaline phosphatase, the concentration of biotinylated alkaline phosphatase added was approximately double the total number of available biotin binding sites (value obtained using the b-4-f method).

To confirm the sufficiency of the substrate concentration, a similar detection was done using 50 µM FDP instead of 5 µM. In both cases, the break point was obtained at the same D-biotin concentration (4.8 pmoles) indicating that 5 µM FDP is sufficient for efficient catalysis. Another method to ensure sufficient catalysis can be to set the substrate concentration at 5-20 fold higher than the Km value (29).

Estimation of immobilized streptavidin/ NeutrAvidin™ concentration

The ratio of b-4-f (or biotin) to free, unbound streptavidin/NeutrAvidin™ is 4:1 (18). The ratio will be smaller for immobilized streptavidin/NeutrAvidin™ because one or more biotin binding sites per streptavidin/NeutrAvidin™ tetramer may be blocked during immobilization (11). There are approximately 3 active biotin-binding sites per bead (11), which may vary depending on the coupling chemistry selected and the conditions used for the reactions. The value can be used to estimate the total concentration of immobilized streptavidin/NeutrAvidin™ on the surface of the solid support.

The estimated streptavidin/NeutrAvidin™ concentrations (assuming stoichiometric binding of 1:3) on different solid supports were, streptavidin coated magnetic beads (2.8 µm) ≈ 2 x 10^{-18} moles of streptavidin/bead, NeutrAvidin™ coated magnetic beads (2.8 µm) ≈ 1 x 10^{-18} moles of
NeutrAvidin\textsuperscript{TM}/bead, non-magnetic streptavidin polystyrene blue particles (0.31 µm) \( \approx 3 \times 10^{21} \) moles of streptavidin/particle and 96 well streptavidin coated micro-titer plates \( \approx 3 \times 10^{11} \) moles of streptavidin/well.

**Advantages of biotin-4-fluorescein method**

Due to its unique quenching properties, the b-4-f method is advantageous over many other fluorimetric techniques. For instance, in the case of streptavidin plates, using biotinylated fluorescein to determine the biotin binding capacity needs several additional steps such as washing, incubations, heating, cooling, spinning etc. (9), which makes the procedure time consuming, complex and sometimes less precise. The b-4-f method is very simple and straightforward with an optimized incubation time of only 5 min. The method does not require calibration for individual assays, and the b-4-f stock solution needs to be standardized only once per year when stored at -70°C (14). An experiment with blue colored streptavidin polystyrene blue particles demonstrates that the method can also be applied to various streptavidin/NeutrAvidin\textsuperscript{TM} coated solid supports with background fluorescence.

The b-4-f method can be used as a standard protocol for comparing a wide range of streptavidin/NeutrAvidin\textsuperscript{TM} immobilization techniques including but not limited to magnetic, non-magnetic, colored, colorless, spherical, plane or particulate types of solid supports to study the degree of immobilization.

**Conclusion**

A standard fluorimetric assay was developed to determine the BBC of a variety of streptavidin/NeutrAvidin\textsuperscript{TM} coated solid supports. The method was tested on four different solid supports - streptavidin magnetic beads (2.8 µm), NeutrAvidin\textsuperscript{TM} magnetic beads (2.8 µm), non-magnetic streptavidin polystyrene blue particles (0.31 µm) and 96 well streptavidin micro-titer
plates. BBC using this method were found to be $4 \times 10^6$ biotin/bead, $2 \times 10^6$ biotin/bead, $6 \times 10^3$ biotin/particle and $5 \times 10^{13}$ biotin/well, respectively. It is a rapid and sensitive method with an optimized incubation time of 5 min for each of the solid supports used.

References


17. **Invitrogen Corporation (Dynal Biotech)**, Technical notes: Dynabeads® M-280 Tosyl-activated; Dynabeads® M-280 Streptavidin.

18. **Sigma-Aldrich**: Certificate of Analysis (Lot#124K1368)


21. **Spherotech Inc.** Technical notes.

22. **Piercenet.** Technical notes: Streptavidin high binding capacity (HBC) coated plates.


Chapter 3

A fluorimetric assay to determine the total number of biotinylated ligands bound to solid supports coated with streptavidin/ NeutrAvidin™
Introduction

Introducing streptavidin-coated solid phases to immunoassays was reported by Suter and Butler in 1986 (1, 2). Techniques that use immobilized streptavidin have many advantages over other immobilization techniques such as - a uniform distribution of biotinylated ligands on solid supports, stronger ligand binding interactions, the preservation of ligand binding activity, high surface coverage, etc. (2). Various biotinylated ligands are used for immunoassay, protein purification, cell isolation, nucleic acid detection/purification and other tasks. For the success of all of the above and to ensure low detection limits and higher sensitivity, it is critical to maintain an optimum surface coverage of the solid supports with biotinylated ligands. Four commonly used means of surface coverage detection are - radioactive, enzyme-linked, fluorescent, and chemiluminescent immunoassays (3, 4).

The general approach to determine the ligand binding capacity is to use commonly available biotinylated ligands as sensitive markers, which can be detected by means depending on the type of marker selected (3). By knowing the molecular weight of the sensitive marker, an estimate is made regarding the surface coverage of the streptavidin with respect to a ligand of similar size and weight (3). However these methods do not consider one or more parameters on which binding efficiency of ligands may depend on – for example, the geometric arrangements of streptavidin/NeutrAvidin™ on solid supports; steric hindrances due to streptavidin, biotinylated ligand and/or markers; attractive or repulsive forces due to charges on the ligands, surfaces and markers; ligand-ligand interactions, ligand-streptavidin interactions and others.

A method has been developed to determine the ligand-binding capacities of various streptavidin/NeutrAvidin™ coated solid supports using biotin-4-fluorescein (b-4-f). The
method is based on the exceptional properties of b-4-f to lose its fluorescence [84-88% (5)] on streptavidin/NeutrAvidin™ binding. The concept of this method is illustrated in Figure 1.

On non-cumulatively titration of immobilized streptavidin with biotinylated antibodies, the antibodies anchor on the biotin binding sites of streptavidin (6). When b-4-f will be added on this antibody-coated streptavidin, the quenching of b-4-f in each well will depend on the number of vacant biotin binding sites on streptavidin. The first well (control) does not contain any biotinylated antibody hence all the available biotin binding sites will be occupied by b-4-f giving maximum quenching, and therefore the lowest fluorescence. Fluorescence will increase linearly as the concentration of biotinylated antibodies is increased. A stage will be reached when all the accessible biotin binding sites of streptavidin become completely saturated with biotinylated antibodies. At and beyond this point, little or no b-4-f will bind to the beads due to the lack of vacant biotin binding sites, thereby giving maximum fluorescence. Hence when a b-4-f signal is plotted against the biotinylated antibody concentration, a linear increase in fluorescence is observed followed by a plateau as shown in Figure 1. The ligand concentration at the asymptote intersection is critical in determining the streptavidin/NeutrAvidin™ binding capacity for a given ligand.

Materials and methods

Reagents

Immunopure® NeutrAvidin™ (biotin binding protein) and ‘streptavidin high binding capacity coated plate’ were from Pierce (Rockford, IL, USA). Biotin-4-fluorescein and fluorescein diphosphate (FDP) were from Invitrogen™, Molecular Probes™ (Eugene, OR, USA). Streptavidin-coated M-280 Dynabeads® [~6-7 x 10⁸ beads/ml, 2.8 µm diameter] and tosyl-activated M-280 Dynabeads® [~ 2 x 10⁹ beads/ml, 2.8 µm diameter] were from Invitrogen™
Figure 1: Determination of ligand binding capacities of streptavidin coated solid supports using biotin-4-fluorescein
(Carlsbad, CA, USA). Bovine serum albumin (BSA) fraction V powder (biotech grade), sodium azide, sodium chloride (ACS grade), magnesium chloride (MgCl$_2$.6H$_2$O, ACS grade), Tris (hydroxymethyl) aminomethane (THAM, laboratory grade), micro-centrifuge tubes (1.5 ml) and borosilicate culture tubes (12 x 75 mm) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). White/clear round bottom 96-well micro-titer plates were obtained from Fisher Scientific (Pittsburgh, PA, USA). Glycine (99.5%) and potassium phosphate monobasic (ACS grade) were acquired from Matheson Coleman Bell (Norwood, OH, USA). Potassium phosphate dibasic was from J. T. Baker Chemical Co. (Baker Analyzed Reagent; Phillipsburg, NJ, USA). The neodymium-iron-boron rare earth magnets were obtained from Radio Shack (Fort Worth, TX, USA). Non-magnetic Spherotech™ streptavidin-coated polystyrene blue particle [1.0% (w/v), 0.31 µm diameter] was from Spherotech Inc (Libertyville, IL, USA). Black polystyrene assay plates (96 well, flat bottom) were from Corning Inc. (Corning, NY, USA). Biotinylated donkey anti-mouse IgG [H+L] (β-d-α-m-IgG) and alkaline phosphatase conjugated mouse IgG [H+L] (m-IgG-ALP) were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Tween 20 was from Aldrich Chemical (Milwaukee, WI, USA). All chemicals were used without further purification.

**Apparatus**

**Optical microscope:** An Olympus BX-40 microscope with a Hitachi HV-C20 camera and Scion CG-7 frame grabber software on a Macintosh G3 platform were used to count beads (7). The obtained Images were processed with Scion Image Software (7).

**Luminescence spectrometer:** A Perkin Elmer LS 50B luminescence spectrometer was used as a fluorescence plate reader.
**Other apparatus** - Incubator (Wedco Inc.: Silver Spring, MD, USA), micro-centrifuge with a fixed angle rotor [Heraeus Sepatech (Abbott)], micro-titer plate shaker with orbital movement (Ika Works Inc.: Wilmington, NC, USA), Diode array UV-vis spectrophotometer (Hewlett Packard 8452A) and Neubauer hemocytometer (Fisher Scientific, Pittsburg, PA).

**Buffers**

Three aqueous buffer solutions were used. **PBS reaction buffer**, pH 7.4 [PBS-R, 0.044 M KH$_2$PO$_4$, 0.056 M K$_2$HPO$_4$, 0.1 M NaCl, 1% (w/v) BSA, 0.02% (w/v) NaN$_3$, a drop of Tween 20]; **PBS conjugation buffer**, pH 7.4 [PBS-C, 0.044 M KH$_2$PO$_4$, 0.056 M K$_2$HPO$_4$, 0.1 M NaCl, 0.02% (w/v) NaN$_3$] and **Tris buffer** pH 9.0: [0.1 M THAM, 5 mM MgCl$_2$.6H$_2$O, 10 mM glycine, 0.02 % (w/v) NaN$_3$].

**Experimental**

**3.1.1 Preparation of NeutrAvidin$^{TM}$ coated beads**

The method for preparing the NeutrAvidin$^{TM}$ coated beads was adapted from that described in Dynabeads Technical Notes (8) and by Farrell (9) and briefly described in Section 2.1.1.

**3.2.1 Determination of ligand binding capacity of ‘streptavidin high binding capacity coated plate’ using biotin-4-fluorescein**

β-d-α-m-IgG (0 – 4 µg/well) in PBS-R was added to the wells of a ‘Streptavidin high binding capacity coated plate’ and the total volume in each well was maintained at 120 µl using PBS-R. This solution was incubated for 5 min on a plate shaker at room temperature. Then, antibodies were aspirated and 90 µl of Tris buffer was added to each well. Finally, b-4-f (5.57 x 10$^{-6}$ M, 30 µl) was added, and solution was further incubated for 5 min. After incubation, b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).
Several optimization steps were done: Detecting for three different incubation periods - 5, 15 and 30 min, optimized the b-4-f incubation time. The b-4-f concentration was optimized by doing experiments at 1:0.5, 1:1 and 1:4 times the molar ratio of available biotin binding sites to b-4-f concentration. Two different incubation periods – 5 and 30 min were examined to optimize the antibody incubation time. The number of washing steps needed after antibody incubation was optimized in two separate experiments. In the first, 3 washings were done before adding b-4-f, and in the second, each well was aspirated but not washed with buffer. The precision of the method was confirmed by repeating the experiment three times.

3.2.2 Determination of ligand binding capacity of streptavidin/NeutrAvidin™ coated beads using biotin-4-fluorescein

Streptavidin/NeutrAvidin™ beads (stock) were diluted several fold using PBS-C buffer, and diluted beads were counted on a Neubauer hemocytometer under an optical microscope. The stock streptavidin/NeutrAvidin™ bead concentration was found to be 8.0 x 10^8 beads/ml.

Streptavidin/NeutrAvidin™ magnetic beads (8.0 x 10^8 beads/ml, 150 µl) were washed twice with PBS-R buffer using a magnet (for bead capture), and re-suspended in PBS-R (150 µl). β-d-α-m-IgG (0 - 1.6 µg/well) was added to each well containing washed streptavidin/NeutrAvidin™ beads (8.0 x 10^8 beads/ml, 10 µl). The total volume of bead-antibody suspension was maintained at 60 µl using PBS-R. This suspension was incubated for 60 min at room temperature on a plate shaker, washed (5 times) with Tris buffer (aided by a magnet) and finally re-suspended in Tris buffer (120 µl). Finally, a fixed volume (48 µl) of bead suspension from each well was transferred to fresh wells containing b-4-f (2.50 x10^{-6} M, 65 µl) and incubated for 5 min on a plate shaker at room temperature. b-4-f was excited at 494
nm (slit width 5 nm) and the emission was measured at 514 nm (slit width 5 nm). Detection was done in a white/clear polypropylene assay plate (96 well, round bottom) and black polystyrene assay plates (96 well, flat bottom).

A similar experiment was done with NeutrAvidin™ beads and the incubation time with b-4-f was optimized for 5, 15 and 30 min.

3.2.3 Determination of ligand binding capacity of streptavidin coated polystyrene blue particles using biotin-4-fluorescein

Streptavidin coated polystyrene blue particles (6.1 x10¹¹ particles/ml, 150 µl) were washed (twice) centrifugally (13,000 rpm, minimum RCF 13,400g, 5 min) with PBS buffer using a microcentrifuge with a fixed angle rotor (24-place x 1.5-2.0 ml) and finally re-suspended in PBS-R buffer (150 µl).

β-d-α-m-IgG (0 - 10 µg/well) was added to each well containing washed streptavidin polystyrene blue particles (9.15 x 10⁹ particles/tube, 15 µl). The total volume in each well was maintained at 90 µl using PBS-R. Particles were incubated for 60 min at room temperature on a plate shaker and washed (5 times) with Tris buffer by centrifuging between each washing step (13000 rpm, 3-5 min). Particles were finally re-suspended in Tris buffer (90 µl). A small volume of the particles (2 µl) in each tube was transferred to a micro-titer plate containing b-4-f (1.42 x 10⁻⁶ M, 3.5 µl) and Tris buffer (85 µl). This suspension was incubated for 5 min on a plate shaker. Immediately after incubation, the b-4-f was excited at 494 nm (slit width 10 nm) and emission was measured at 514nm (slit width 10 nm).

3.3.1 Determination of ligand binding capacities of ‘Streptavidin high binding capacity coated plate’ using antibody-enzyme conjugate (m-IgG-ALP)
β-d-α-m-IgG (0 - 0.7 μg/well) was added to the wells of a ‘Streptavidin high binding capacity coated plate’ and the total volume in each well was maintained at 120 μl using PBS-R. This antibody solution was incubated for 60 min on a plate shaker at room temperature. The β-d-α-m-IgG solution was then aspirated and the wells were washed with PBS-R buffer (5 times). To each washed well, m-IgG-ALP (25 μg/ml, 120 μL) was added and the solution was incubated for 60 min at room temperature. After incubation, these wells were again washed with PBS-R buffer (3 times) and Tris buffer (twice). Finally, FDP substrate (200 μM, 120 μl) was added to each washed well. FDP was excited at 494 nm (slit width 2.5 nm) and the emission measured at 514 nm (slit width 2.5 nm). The experiment was repeated to establish the reproducibility of the method.

3.3.2 Determination of ligand binding capacity of streptavidin and NeutrAvidin™ coated beads using antibody-enzyme conjugate (m-IgG-ALP)

This method was modified from that used by Farrell (8). In short, streptavidin/NeutrAvidin™ magnetic beads (8 x 10^8 beads/ml, 70 μl) were washed twice with PBS-R buffer aided by a magnet (for bead capture), and re-suspended in PBS-R (70 μl). β-d-α-m-IgG (0 – 0.45 μg/well) was added to each well containing washed streptavidin/NeutrAvidin™ beads (8.0 x 10^8 beads/ml, 2.5 μl) giving final bead concentration as 2.0 x 10^6 beads/well. The total volume was maintained at 15 μl with PBS-R. The plate was sealed with Parafilm™ and the beads incubated at room temperature for 60 min on a plate shaker. After incubation, the beads were washed with PBS-R (5 times) with the help of a magnet. Washed streptavidin/NeutrAvidin™ beads were then re-suspended in m-IgG-ALP in PBS-R (145 μg/ml, 15 μl). The mixture was again incubated for 60 min at room temperature on a plate shaker. After incubation, the beads were washed with PBS-R (3 times) and Tris buffer (twice) and finally re-suspended in Tris buffer (30
µl). A fixed volume of bead suspension (5 µl) from each well was added to fresh wells containing FDP substrate (20 µM, 90 µl). The FDP was excited at 490 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).

### 3.3.3 Determination of ligand binding capacities of streptavidin polystyrene blue particles using antibody-enzyme conjugate (m-IgG-ALP)

Streptavidin polystyrene blue particles (6.1 x 10^{11} particles/ml, 200 µl) were washed twice with PBS-R buffer by centrifugation (13,000 rpm, 5 min), and re-suspended in 200 µl of the same buffer.

β-d-α-m-IgG (0 - 10 µg/well) was added to each well, which contained washed particles (9.1 x 10^9 particles/tube, 15 µl). The total volume in each well was maintained at 90 µl using PBS-R buffer. The suspension was incubated for 60 min at room temperature on a plate shaker and washed centrifugally (13,000 rpm, 5 min) with PBS-R buffer (5 times). Particles were finally re-suspended in 90 µL of Tris buffer. A fixed volume (45 µl) of particle suspension from each well was added to fresh wells containing m-IgG-ALP in PBS-R (350 µg/ml, 45 µl). This suspension was incubated for 60 min at room temperature on a plate shaker. After incubation, particles were washed with PBS-R (3 times) and Tris buffer (twice), and finally re-suspended in 45 µl of Tris buffer.

**Detection:** Some optimization of particle and FDP concentration was done. Particles were diluted 1:20 times and 3 µl of diluted particles from each well were transferred to fresh wells containing FDP substrate (20 µM, 90 µl). FDP was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).

In order to optimize the conjugate concentration, the same experiment was repeated with m-IgG-ALP in PBS-R (500 µg/ml, 45 µl) using a slit width of 2.5 nm (instead of 5 nm).
3.4.1 Calibration curve for determination of unknown biotinylated ligand concentration using biotin-4-fluorescein (determination of detection limit and linear range)

This experiment is the same as the one described in Section 3.2.1, except that more data points were taken here for lower and higher concentrations in order to determine the linear range. A washing step and a longer incubation period with the primary antibody was used to lower the detection limit.

ß-d-α-m-IgG (0 – 2.4 µg/well) in PBS-R was added to the wells of a ‘Streptavidin high binding capacity coated plate’ and the total volume in each well was maintained at 120 µl using PBS-R buffer. This solution was incubated for 15 min on a plate shaker at room temperature. Then, antibodies were aspirated, washed with Tris buffer (once) and re-suspended in 98 µL of Tris buffer. Finally, b-4-f (5.57 x 10^{-6} M, 22 µl) was added, and solution was further incubated for 5 min on a plate shaker. After incubation, b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).

Results and Discussion

3.1.1 Preparation of NeutrAvidin™ coated beads

NeutrAvidin™ is a deglycosylated avidin derivative in which surface charges are neutralized, resulting in exceptionally low non-specific binding (9, 11). Tosyl activated beads provide reactive sulphonyl esters that can physically and covalently bind to primary amino groups of NeutrAvidin™ (13). No further activation is needed and the rate of reaction increases at higher temperature and pH (9). A schematic for NeutrAvidin™ bead preparation is in Figure 4 of Section 2.1.1.
3.2.1 Determination of ligand binding capacity of ‘streptavidin high binding capacity coated plate’ using biotin-4-fluorescein

The objective of this section was to apply b-4-f method for the determination of antibody binding capacity of ‘streptavidin high binding capacity coated plate’. Comparison of results obtained from b-4-f method with other previously established method would validate the proposed method to quantitate the ligand capacity of various streptavidin or NeutrAvidin™ coated plates.

In a standard assay, streptavidin-coated wells were titrated with biotinylated antibodies, and then b-4-f was added. As the concentration of biotinylated antibodies was increased linearly, fewer biotin binding sites were available for b-4-f, resulting in a linear increase in fluorescence (due to unquenched, free b-4-f) with increased antibody concentration. An initial linear trend (first 4 data points: \( R^2 = 0.9660 \)) seen in Figure 2 is attributed to this phenomenon. At and beyond the saturation point, biotinylated antibodies occupied all/most of the active biotin-binding sites of immobilized streptavidin; hence little b-4-f was bound giving a plateau (last 6 date points: \( R^2 = 0.8598 \)) of maximum fluorescence.

Regression lines before (first 4 data points: \( m = 62.89 \)) and after saturation (last 6 data points: \( m = 3.4604 \)) indicated significantly different fluorescence intensities since the ratio of their slopes (\( m \)) was about 18. The concentration of biotinylated ligand at the saturation point was found by extrapolating each end of the hyperbolic curves by drawing a tangent as shown in Figure 2. The intersection of the two asymptotes gave an antibody-binding capacity of 0.77 \( \mu g/well \). The experiment was repeated 3 times to obtain the uncertainty of the determination and to support the precision of the method.
Figure 2: Determination of biotinylated antibody capacity for 'streptavidin high binding capacity coated plate': β-d-α-m-IgG (0 - 4 µg/well) was added linearly to the streptavidin wells (total volume: 120 µl). After sufficient incubation and washings, b-4-f (5.57 x 10^-6 M, 30 µl) was added and incubated for 5 min. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). The concentration of biotinylated ligand at the saturation point was found by extrapolating each end of the hyperbolic curves by drawing tangents. The intersection of the two asymptotes represents the saturation point. For clarity, while drawing tangents, β-d-α-m-IgG concentration up to 1.8 µg/well (instead of 0 – 4 µg/well) was plotted. The complete saturation curve (0 - 4) µg/well can be seen in the smaller graph.
The concentration of b-4-f was optimized for the assay. The total biotin binding capacity of the plate as per the manufacturer’s website (Piercenet) is 125 pmoles/well. Three independent experiments were done with 1:0.5, 1:1 and 1:4 molar ratio of (total biotin binding sites of immobilized streptavidin on well): (moles of b-4-f added). Background fluorescence (‘0’ concentration of antibody) was much higher for the molar ratio 1:4 (fluorescence: 410 a.u.), as compared to the molar ratios 1:1 (fluorescence: 65 a.u.) and 1:0.5 (fluorescence: 24 a.u.). For comparison purpose and better data representation, fluorescence signal was normalized for the molar ratios of 1:1 and 1:0.5, by subtracting all the data points with a fixed value (388 for the molar ratio 1:4 and 42 for the molar ratio 1:1). The subtraction was done so that all the three graphs start from same point.

The molar ratio 1:4 gave inconsistent data with larger error bars (Figure 3). The saturation point for the molar ratios 1:1 and 1:0.5 were determined as an intersection of tangents to the hyperbola curve. The saturation point in both cases was achieved approximately at the same antibody concentration (0.75 µg/well for 1:1 molar ratio and 0.86 µg/well for 1:0.5 molar ratio). More data point towards the saturation point may decrease/eliminate the small discrepancies in result. Fluorescence intensities were comparatively lower when the molar ratio was 1:0.5. Hence 1:1 is the optimized molar ratio of (available biotin binding sites): (moles of b-4-f added).

Antibody capacity (0.77 µg/well) was approximately same at 5, 15 and 30 min of b-4-f incubation (Figure 4); and 5 and 30 min of biotinylated antibody incubation (Figure 5). Hence 5 min was considered as the optimal incubation time for b-4-f and biotinylated antibody.
Figure 3: Optimization of biotin-4-fluorescein concentration for streptavidin coated plate:

β-d-α-m-IgG (0 – 4.2 µg/well) was added linearly to each well (total volume 120 µl) followed by addition of b-4-f (5.57 x 10^{-6} M, 30 µl). Three experiments were done using molar ratios of total biotin binding sites: b-4-f at 1:0.5, 1:1 and 1:4. For the molar ratios 1:1 and 1:0.5, subtracting all the data points with a fixed value (388 for the molar ratio 1:4 and 42 for the molar ratio 1:1), normalized the fluorescent signal. The concentration of biotinylated ligand at the saturation point was found by extrapolating each end of the hyperbolic curves by drawing tangents. The intersection of the two asymptotes represents the saturation point. For clarity, while drawing tangents, β-d-α-m-IgG concentrations up to 1.6 µg/well (instead of 0 – 4.2 µg/well) were plotted. The complete saturation curve (0 – 4.2) µg/well can be seen in the smaller graph. Some error bars are smaller than the symbol heights, and are not clearly visible.
Figure 4: Optimization of biotin-4-fluorescein incubation time for streptavidin coated plate: β-d-α-m-IgG (0 – 2.5 µg/well) was added linearly to each well (total volume 120 µl), then b-4-f was added (5.57 x 10^{-6} M, 30 µl) and incubated for 5, 15 and 30 min. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Some error bars are smaller than the symbol heights, and are not clearly visible.
Figure 5: Optimization of primary antibody incubation time for streptavidin coated plate: β-d-α-m-IgG (0 – 3.0 µg/well) was added linearly to each well (total volume 120 µl). Antibodies were incubated for 5 and 30 min, and then b-4-f was added (5.57 x 10⁻⁶ M, 30 µl). b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Some error bars are smaller than the symbol heights, and are not clearly visible.
Identical results were obtained when each well was washed 3 times after primary antibody incubation verses no wash (Figure 6). Therefore it is not necessary to wash unbound antibodies off the plate from each well.

For many data points in the above figures, error bars are smaller than the symbol heights, thus they are not clearly visible.

3.2.2 Determination of ligand binding capacity of streptavidin/NeutrAvidin™ coated beads using biotin-4-fluorescein

The objective of this section was to apply b-4-f method to determine the ligand binding capacity of streptavidin and NeutrAvidin™ coated magnetic beads. Biotinylated antibodies were used as a representational ligand. Detection was done for commercially available streptavidin bead (2.8 µm, magnetic) and laboratory made NeutrAvidin™ beads (2.8 µm, magnetic). Similar technique can be extended to various types (magnetic or non-magnetic) of streptavidin and NeutrAvidin™ coated microspheres.

In the case of streptavidin-coated beads, antibody saturation curve was achieved at 0.77 µg/well (Figure 7). The number of antibodies per bead was calculated using the equation I-

\[
\text{antibody concentration at saturation point} \times \frac{1}{\text{antibody/bead}} = \frac{1}{\text{antibody molecular weight}} \times \frac{1}{\text{number of beads in each well}} \times \text{Avogadro’s no.}
\]

where, antibody amount at saturation point = 0.77 µg/well (Figure 7), number of beads/well = 8 x 10^6, antibody molecular weight ≈150 kDa (13, 14) and Avogadro’s number = 6.022 x 10^{23} molecules/mole.
Figure 6: Optimization of washing steps (after antibody incubation) for streptavidin coated plate: In two separate experiments, β-d-α-m-IgG (0 – 3 µg/well) was added linearly to each well (total volume 120 µl). After incubation, antibodies in experiment 1 were washed 3 times with buffer; excess antibodies were not washed away in experiment 2. Then b-4-f (5.57 x 10^{-6} M, 30 µl) was added. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).
Figure 7: Determination of biotinylated antibody capacity for streptavidin coated beads, using biotin-4-fluorescein: β-d-α-m-IgG in PBS buffer (0 – 1.6 µg antibodies/well) was added linearly to each well containing washed streptavidin beads (8.0 x 10⁸ beads/ml, 10 µl; total volume 60 µl), incubated (60 min) and washed (5 times) with Tris buffer. Beads were finally re-suspended in Tris buffer (120 µl). A fixed volume (48 µl) of bead solution from each well was transferred to fresh wells containing b-4-f (2.50 x10⁻⁶ M, 65 µl) and incubated for 5 min on a plate shaker at room temperature. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). The concentration of biotinylated ligand at the saturation point was found by extrapolating each end of the hyperbolic curves by drawing a tangent. The intersection of the two asymptotes represents the saturation point. Some error bars are smaller than the symbol heights, and are not clearly visible.
Using this formula, the antibody-binding capacity for a M-280 streptavidin beads was estimated to be $4 \times 10^5$ antibodies/bead.

Detection was done in a white/clear polypropylene assay plate (96 well, round bottom) and a black polystyrene assay plate (96 well, flat bottom), to compare the signals obtained on the two different types of plates. The assay sensitivity for the white/clear polypropylene plates (96 well, round bottom) was much higher than the black polystyrene plates (96 well, flat bottom) as shown in Figure 8. The higher sensitivity of the white/clear plate can be attributed to the nature of the material used for the two different plates since polypropylene plates have a lower background fluorescence and hence better detection limits than polystyrene plates (15).

The saturation concentration for the M-280 NeutrAvidin™ beads was 0.48 µg/well (Figure 9), from which an antibody-binding capacity was calculated as $2 \times 10^5$ antibodies/bead. Identical results were obtained when NeutrAvidin™ beads were incubated for 5, 15 and 30 min with b-4-f (Figure 9). Hence 5 min was considered as the optimum incubation period.

3.2.3 Determination of ligand binding capacity of streptavidin coated polystyrene blue particles using b-4-f

The objective of this section was to validate the application of b-4-f method in case of non-magnetic, particulate type of solid supports. Another objective was to determine the method efficiency in case of colored matrices. The streptavidin coated polystyrene blue particles was used as a representational solid supports. These are non-magnetic, small size particles with average diameter of 0.31 µm. Due to presence of dye, particles are blue in color. Method may be extended to various types (magnetic or non-magnetic) of streptavidin and NeutrAvidin™ coated particles with colored matrices.
Figure 8: Antibody saturation curve for streptavidin coated beads using biotin-4-fluorescein: comparison of two different micro-titer plates: The experiment is similar to that described in Figure 7. The results from the two micro-titer plates - white/clear polypropylene assay plate (96 well, round bottom) and black polystyrene assay plate (96 well, flat bottom) were compared for assay sensitivity.
Figure 9: Determination of biotinylated antibody capacity and optimization of biotin-4-fluorescein incubation time for M-280 NeutrAvidin™ coated beads: β-d-α-m-IgG in PBS-R buffer (0 – 1.6 µg antibodies/well) was added linearly to each well containing washed NeutrAvidin™ beads (8.0 x 10^8 beads/ml, 10 µl; total volume: 60 µl using PBS-R buffer), incubated (60 min) and washed (5 times) with Tris buffer. Beads were finally re-suspended in Tris buffer (120 µl). A fixed volume (48 µl) of bead suspension from each well was transferred to fresh wells containing b-4-f (2.50 x 10^-6 M, 65 µl) and incubated for 5, 15 and 30 min on a plate shaker at room temperature. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).
Streptavidin polystyrene blue particles were not counted under the microscope because of their small diameters (0.31 µm). They were calculated using the equation-II in Section 2.2.2, provided by Spherotech Inc. (10). The calculated concentration of stock streptavidin polystyrene blue particles was 6.1 x 10^{11} particles/ml.

The presence of blue dye in the streptavidin polystyrene blue particles interfered with the fluorescence signal, and hence optimization of the particle concentration was necessary for detection. Since these are non-magnetic particles, centrifugation was required during each washing step. The streptavidin polystyrene particles saturation concentration occurred at 1.5 µg/well, with each well holding 9.15 x 10^9 particles (Figure 10). Hence using the equation-I in Section 3.2.2, the number of antibodies was calculated as 1 x 10^3 antibodies/particles.

3.3.1 Determination of ligand binding capacity of ‘Streptavidin high binding capacity coated plate’ using antibody-enzyme conjugate (m-IgG-ALP)

This section illustrates the techniques to validate the b-4-f method used for detection of ligand binding capacity of ‘Streptavidin high binding capacity coated plate’. Antibody-enzyme conjugate (m-IgG-ALP) was used for detection purpose. Due to the high streptavidin-biotin affinity [dissociation constant Kd = 5 x 10^{-15} M (16-18)], antibodies were immobilized on the solid support when β-d-α-m-IgG was added to streptavidin. Conjugated m-IgG-ALP has a high affinity for β-d-α-m-IgG. Hence, as the concentration of β-d-α-m-IgG was increased linearly in each well, the concentration of m-IgG-ALP attached to immobilized streptavidin also increased proportionally. Enzyme ALP catalyzed the hydrolysis of the colorless, non-fluorescent substrate fluorescein diphosphate (FDP) to strongly fluorescent fluorescein (19). The slope of the signal due to FDP hydrolysis was plotted against β-d-α-m-IgG concentration. Tangents
Figure 10: Determination of biotinylated antibody capacity for streptavidin polystyrene blue particles, using biotin-4-fluorescein: β-d-α-m-IgG (0 - 10 µg/well) was added to each well containing washed streptavidin polystyrene blue particles (9.15 x 10^9 particles/tube, 15 µl; total volume: 90 µl). After incubation and washing steps, the particles were re-suspended in Tris buffer (90 µl). A small volume of particles (2 µl) from each tube was transferred to a micro-titer plate containing b-4-f (1.42 x 10^-6 M, 3.5 µl) and Tris buffer (85 µl). Immediately after 5 min incubation, b-4-f was excited at 494 nm (slit width 10 nm) and the emission was measured at 514 nm (slit width 10 nm). The concentration of biotinylated ligand at the saturation point was found by extrapolating each end of the hyperbolic curves by drawing a tangent. The intersection of the two asymptotes represents the saturation point. For clarity, while drawing tangents, β-d-α-m-IgG concentration up to 5 µg/well (instead of 0 – 10 µg/well) was plotted. The complete saturation curve (0 – 10 µg/well) can be seen in the smaller graph. Some error bars are smaller than the symbol heights, and are not clearly visible.
were drawn at the hyperbolic curve and the intersection of asymptote was used to find the antibody-binding capacity of each immobilized streptavidin/NeutrAvidin™ solid support as shown in Figure 11.

To re-confirm the reproducibility of the method, a second experiment was done from which error bars were plotted. Using m-IgG-ALP, the streptavidin plate capacity was estimated to be 0.27 µg/well (Figure 11). The streptavidin plate capacity using the b-4-f method was 0.77 µg/well (Figure 2). Unequal steric hindrance attributable to the large difference in marker size [b-4-f M W: 644.69 Da (9); calf intestinal alkaline phosphatase 140 kDa (20, 21), IgG-MW: ~150 kDa (13)] may contribute significantly to the difference in sensitivities of the two methods used.

3.3.2 Determination of ligand binding capacity of streptavidin and NeutrAvidin™ coated beads using antibody-enzyme conjugate (m-IgG-ALP)

The objective of this section was to determine the biotinylated ligand capacity of magnetic streptavidin/NeutrAvidin™ coated beads (2.8 µm) using antibody-enzyme conjugate method. The results obtained by this technique were used to validate b-4-f method applied for similar solid support (streptavidin/NeutrAvidin™ coated beads) and biotinylated ligands (antibodies).

In this method, 145 µg/ml of m-IgG-ALP was used as a secondary antibody to saturate streptavidin/NeutrAvidin™ beads in each well (2.0 x 10⁶ beads/well). It was proved previously by Farrell (8) that 45 µg/ml of m-IgG-ALP is sufficient to completely saturate the same concentration of streptavidin/NeutrAvidin™ beads. Hence the plateau obtained for streptavidin/NeutrAvidin™ saturation curves were not due to a limiting concentration of antibody conjugate, but was a result of the beads being saturated by biotinylated antibodies.
Figure 11: Determination of ligand binding capacity of ‘Streptavidin high binding capacity coated plate’ using antibody-enzyme conjugate (m-IgG-ALP): β-d-α-m-IgG (0 - 0.7 µg/well) was added linearly to streptavidin wells (total volume: 120 µl). After sufficient incubation and washings, m-IgG-ALP (25 µg/ml, 120 µl) was added to each well. Beads were incubated and washed followed by addition of FDP substrate (200 µM, 120 µl). FDP was excited at 494 nm (slit width 2.5 nm) and emission was measured at 514 nm (slit width 2.5 nm). In order to re-confirm the reproducibility of the method, two different experiments were done from which error bars were plotted.
For NeutrAvidin™ beads, in order to study the reaction velocity of alkaline phosphatase, the fluorescence signal due to FDP hydrolysis was plotted against time (Figure 12). It was observed that as the concentration of biotinylated antibodies increased linearly (~ 0 - 0.18 µg/well), there was a proportional increase in the fluorescence signal. After a specific antibody concentration (~ 0.23 µg/well), all biotin-binding sites were filled with biotinylated antibodies resulting in a plateau of maximum fluorescence (~ 0.23- 0.45 µg/well). All the data points are not shown in the figure for clarity, but they all followed the same trend. A similar trend was observed for streptavidin-coated beads.

NeutrAvidin™ beads were saturated at 0.22 µg/well (Figure 13) whereas streptavidin beads were saturated at 0.20 µg/well (Figure 14). The antibody - binding capacity of both streptavidin/NeutrAvidin™ beads using this method was estimated to be 4 x 10^5 antibodies/bead. Using the b-4-f method, the estimated antibody - binding capacity of streptavidin beads was 4 x 10^5 antibodies/bead and that for NeutrAvidin™ beads was 2 x 10^5 antibodies/bead. The value obtained by Farrell (8) for streptavidin beads was 4 x 10^5 antibodies/bead and that for NeutrAvidin™ bead was 2 x 10^5 antibodies/bead.

3.3.3 Determination of ligand binding capacities of streptavidin polystyrene blue particles (0.31 µM), using antibody-enzyme conjugate (m-IgG-ALP)

The objective of this section was to validate the results obtained from b-4-f method (Section 3.2.3) to detect the biotinylated antibody capacity of non-magnetic (0.31 µm) streptavidin coated polystyrene blue particles. m-IgG-ALP was used for detection. Two experiments were done using 350 µg/ml and 500 µg/ml of conjugate. In order to determine the reaction velocity of alkaline phosphatase, fluorescence signal due to FDP hydrolysis were plotted against time.
Figure 12: Reaction velocity of alkaline phosphatase for the determination of ligand binding capacity of NeutrAvidin™ coated beads, using antibody-enzyme conjugate (m-IgG-ALP): β-d-α-m-IgG (0 – 0.45 µg/well) was added to NeutrAvidin™ beads (2.0 x 10^6 beads/well, 2.5 µl) with total volume of 15 µl. After incubation and washing, m-IgG-ALP in PBS-R (145 µg/ml, 15 µl) was added followed by further incubation and washings. Fixed volumes of beads (5 µl) from each well were added to fresh wells containing FDP substrate (20 µM, 90 µl). FDP was excited at 490 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Plotting a trendlines for each antibody concentration (using excel) depicted the trend of the data.
Figure 13: Determination of ligand binding capacity of NeutrAvidin™ coated beads (2.8 μm) using antibody-enzyme conjugate (m-IgG-ALP): The slope of the signal due to FDP hydrolysis (Figure 12) was plotted against the concentration of β-d-α-m-IgG. At the intersection of the asymptote, the antibody concentration was 0.22 μg/well from which the ligand-binding capacity was determined as 4 x 10⁵ antibodies/bead.
Figure 14: Determination of ligand binding capacity of streptavidin coated beads (2.8 µm) using antibody-enzyme conjugate (m-IgG-ALP): Slope of signal due to FDP hydrolysis was plotted against the concentration of β-d-α-m-IgG. At the intersection of the asymptotes the antibody concentration was 0.2 µg/well from which the ligand-binding capacity was determined as 4 x 10^5 antibodies/bead.
The slope of the fluorescence signal was further plotted against the concentration of antibodies added in each well to get a hyperbolic curve (Figs 15 and 16). The concentration of biotinylated ligand at the saturation point was found by extrapolating each end of the hyperbolic curves by drawing a tangent as shown in Figs 15 and 16. The intersection of the asymptote gave approximately the same concentration of antibody per well [350 µg/ml conjugate: 3 µg/well (Figure 15); and 500 µg/ml conjugate: 2.13 µg/well (Figure 16)]. This confirmed that the saturation points obtained in Figs 15 and 16 were not due to limited conjugate concentration, but was caused by complete saturation of particles with biotinylated antibodies, and that 350 µg/ml of conjugate was sufficient to saturate all the biotinylated antibodies immobilized on the particle surface. The ligand binding capacity using both 350 and 500 µg/ml of conjugate was 1 x 10³ antibodies/particle. Using the b-4-f method, the ligand-binding capacity was 1 x 10³ antibodies/particle.

3.4.1 Calibration curve for determination of unknown biotinylated ligand concentration using biotin-4-fluorescein (determination of detection limit and linear range)

The detection of unknown concentration of biotinylated ligand can be done if the calibration curve of the same ligand is available with its linear range and detection limit. The experiment was done to determine the calibration range and detection limit of biotinylated antibodies.

A calibration curve (0 – 2.4 µg/well) was plotted for β-d-α-m-IgG using ‘Streptavidin high binding capacity coated plate’, as shown in Figure 17. The calibration range was 0.07 – 1.2 µg/well (4 - 65 nM) of antibody, assuming antibody molecular weight as 150 kDa (13, 14). It is a sensitive (0.09-1.08 µg/well: m=45.07) method with LOD of 0.5 nM [LOD = 3.3 x S.D. of blank (0.62) / Slope of calibration curve (45.07), (22)] and a linear range (0.09-1.08 µg/well: R²=0.9277).
Figure 15: Antibody saturation curve for streptavidin polystyrene blue using antibody-enzyme conjugate (m-IgG-ALP: 350 µg/ml conjugate): Slope of the signal due to FDP hydrolysis was plotted against the concentration of β-d-α-m-IgG. At the intersection of the asymptotes, the antibody concentration was 3 µg/well from which the ligand-binding capacity was estimated as $2 \times 10^5$ antibodies/particle.
Figure 16: Antibody saturation curve for streptavidin polystyrene blue particles using antibody-enzyme conjugate (m-IgG-ALP: 500 µg/ml conjugate): Slope of signal due to FDP hydrolysis was plotted against the concentration of β-d-α-m-IgG. At the intersection of asymptote, antibody concentration was 2.13 µg/well from which ligand-binding capacity was estimated as $1 \times 10^3$ antibodies/particle.
Figure 17: Calibration curve for determination of unknown biotinylated ligand concentration using biotin-4-fluorescein (determination of detection limit and linear range): β-d-α-m-IgG (0 – 2.4 µg/well) was linearly added to each well of ‘Streptavidin high binding capacity coated plate’ (total volume: 120 µl) followed by incubation (15 min) and washing (1 time). Tris buffer (98 µl) and b-4-f (5.57 x 10⁻⁶ M, 22 µl) was added followed by incubation (5 min). b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). The antibody concentration (µg/well) was first converted to their log values (exponents of 10) and then plotted on a linear axis against fluorescence signal.
Thus this novel method can be used to determine the concentration of various biotinylated ligands immobilized to various streptavidin/NeutrAvidin™ coated solid supports.

**Advantages of biotin-4-fluorescein method**

The b-4-f method can be used as a standard method to determine a wide range of the ligand capacities of various streptavidin/NeutrAvidin™ solid supports. The method can also be used to determine the unknown concentration of biotinylated ligands using a calibration curve as shown in Section 3.4.1.

One of the preparative steps in many sandwich immunoassays is to optimize the concentration of capture antibody needed for the solid support. Using a secondary antibody-enzyme conjugate (specific to the capture antibody) for such detection is tedious and time consuming, especially in the case of non-magnetic supports due to several washing steps. Since b-4-f binds directly to the solid support, and not through the biotinylated ligand, the presence of unbound ligand does not affect the assay efficiency. Hence the number of washing steps can be minimized without compromising the assay efficiency, which is very advantageous for the laboratories dealing with such detections on a daily basis. The method can also be useful in the case of capture antibodies for which specific secondary antibody-enzyme conjugate is not available. Also, the same b-4-f marker can be used for a wide variety of biotinylated antibodies without worrying about the specificity of the secondary antibody-enzyme conjugate for each biotinylated antibody, making the method economical. The method is also advantageous over techniques using enzyme markers since enzyme markers are unstable and require longer incubation times, and several optimization steps. In short, the detection of ligand-binding capacity of streptavidin/NeutrAvidin™ coated solid supports using the b-4-f method is quick, convenient and economical.
**Biotin binding capacity vs. ligand binding capacity of streptavidin and NeutrAvidin™ coated solid supports**

Biotin binding capacity significantly depends on the size and shape of the solid support used. For example: streptavidin forms monolayer on polystyrene particles (size > 200 nm) and an approximate bilayer on smaller particles (23). This trend not only affects the orientation of streptavidin on solid support (influencing biotin binding capacity), but also influences the ligand binding capacity of the solid supports (23) since ligand binding to streptavidin/NeutrAvidin™ of different orientations will experience different levels of steric hindrances and accessibility issues.

If biotin-binding capacity of each solid support is considered as 100%, then ligand-binding capacity can be determined in terms of % surface coverage using equation II-

\[
\% \text{ surface coverage} = \frac{\text{Number of biotinylated antibodies per bead}}{\text{Number of biotin binding sites per bead}} \times 100
\]

M280 streptavidin and NeutrAvidin™ beads have same diameter (2.8 µm) and shows same ligand binding capacity with surface coverage of 10%. Surface coverage increases to 17% in case of smaller sized streptavidin coated polystyrene blue particles (0.31 µM). This trend is consistent with that found in literature, which can be due to high steric hindrance and/or lack or accessibility of biotinylated ligands in case of spheres of larger size (23). As there is increase in diameter of bead, surface area to volume ratio (SA/V) decreases (since SA/V = 3/radius) resulting in lesser surface area accessibility for ligand immobilization giving higher steric hindrance and lower binding capacity, as seen in Table 1.
<table>
<thead>
<tr>
<th>Solid supports</th>
<th>b-4-f method</th>
<th>Surface area/Volume ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of biotin binding sites</td>
<td>Number of ligand binding sites</td>
</tr>
<tr>
<td>Streptavidin bead (2.8 µm)</td>
<td>4 x 10⁶</td>
<td>4 x 10⁵</td>
</tr>
<tr>
<td>NeutrAvidin™ bead (2.8 µm)</td>
<td>2 x 10⁶</td>
<td>2 x 10⁵</td>
</tr>
<tr>
<td>Streptavidin particle (0.31 µm)</td>
<td>6 x 10³</td>
<td>1 x 10³</td>
</tr>
<tr>
<td>Streptavidin plate (per well)</td>
<td>5 x 10¹³</td>
<td>3 x 10¹²</td>
</tr>
</tbody>
</table>

Table 1: Biotin binding capacity vs. ligand binding capacity of streptavidin and NeutrAvidin™ coated solid supports
Spheres have lowest SA/V ratio as compared to solids of other geometries. Lower SA/V ratio of 96 well micro-titer plates may increase the steric hindrance as compared to microspheres (24), giving lower surface coverage of 6 %, as seen in Table 2.

% Surface coverage will also depend on the size of the biotinylated ligands used and is expected to decrease as the size of the ligand increases due to higher steric hindrance (23).

**Conclusion**

A new fluorimetric method has been developed to determine the binding capacities of a variety of streptavidin/NeutrAvidin™ coated solid supports for a wide range of biotinylated ligands. The method was tested on four different solid supports – streptavidin/NeutrAvidin™ coated beads (2.8 µm), streptavidin polystyrene blue particles (0.31 µm) and streptavidin coated 96 well micro-titer plate. The results are shown in Table 2. Streptavidin (2.8 µm) beads showed 15fold decrease, while 0.31 µm streptavidin particles showed a 4fold decrease in biotinylated antibodies than their respective biotin binding capacities published by manufacturers. In the case of streptavidin coated polystyrene plates, this decrease was ~ 25fold. The method can also be used to determine the unknown concentration of biotinylated ligand in a solution by plotting a calibration graph using standards for the same ligand. When such a calibration graph was plotted using the ‘streptavidin high binding capacity coated plate’ as a solid support and biotinylated antibody as a model ligand, a linear range of 0.07 – 1.2 µg/well (4 nM - 65 nM) and a detection limit of 0.5 nM were obtained.
<table>
<thead>
<tr>
<th>Solid Supports</th>
<th>Biotin-4-fluorescein method</th>
<th>Biotinylated alkaline phosphatase method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin bead (2.8 µm, magnetic)</td>
<td>$4 \times 10^5$ antibodies/bead</td>
<td>$4 \times 10^5$ antibodies/bead</td>
</tr>
<tr>
<td>NeutrAvidin™ bead (2.8 µm, magnetic)</td>
<td>$2 \times 10^5$ antibodies/bead</td>
<td>$4 \times 10^5$ antibodies/bead</td>
</tr>
<tr>
<td>Streptavidin particle (0.31 µm, non-magnetic)</td>
<td>$1 \times 10^3$ antibodies/particle</td>
<td>$1 \times 10^3$ antibodies/particle</td>
</tr>
<tr>
<td>Streptavidin plate</td>
<td>$3 \times 10^{12}$ antibodies/well</td>
<td>$1 \times 10^{12}$ antibodies/well</td>
</tr>
</tbody>
</table>

Table 2: Ligand binding capacities of solid support
References


10. **Spherotech Inc.,** Technical notes.


Chapter 4

Application of PAMAM dendrimers for NeutrAvidin™ coated solid supports
Introduction

Detecting biological agents in water is of extreme importance for maintaining health and sustaining life. The importance of having a reliable method with higher sensitivity to detect biological agents becomes more prominent during emergencies such as natural calamities, bioterrorism and water borne diseases. Even a very small concentration of microorganisms can be deadly, such that a glass of water with 10 organisms of *E. coli* O157:H7 would be lethal (1, 2). Due to the huge volume and continuous flow of water, there is a significant dilution of a bio-agent. Hence there is always a need for developing methods that can further reduce the detection limits of antigens and/or give higher assay sensitivity. The focus of this research is to use dendrimers to increase the sensitivity of immunoassay for detecting microorganisms in water.

The sandwich immunoassay has been previously done in our laboratory using a streptavidin-coated paramagnetic bead as a capture bead, and calibration curves for four different categories of bio-agents MS2 bacteriophage (virus), *Escherichia coli* (bacterium), ovalbumin (toxin) and *B. anthracis* (spores) were established (3). For all such immunoassays, capture beads play a crucial role in the process. The efficiency and capacity of the capture beads can be raised by increasing the number of antibodies bound per bead, and also by increasing the flexibility of the antibody (by reducing steric hindrance), which is usually achieved by using polymer linkers (Figure 1) such as higher generation dendrimers. The polymers may increase the number of ligands bound per unit surface area of the solid support by providing greater conformational freedom, resulting in more effective capturing of the analyte. Also, they increase the effective diameter of the capture bead providing higher surface area/volume ratios. Hence an increase in sensitivity is expected while detecting biological agents of different sizes.
Figure 1: Polymer linkers for the immobilization of biotinylated ligands
The efficiency of many assays such as immunoassay, protein purification, DNA detection, cell isolation etc., is directly proportional to the degree of labeling i.e. the number of capture ligands immobilized per unit area of the solid support (4). In this research, the effect of dendrimers of different generations was studied with respect to the quantity (number) and efficiency of biotinylated ligands immobilized on NeutrAvidin™ coated solid supports. Biotinylated antibody was used as a model system and 2.8 µm NeutrAvidin™ coated magnetic beads as a representational solid support. Similar techniques can be used on a wide variety of other immobilized ligands and solid supports.

PAMAM dendrimers, generations G5.5 and G6.5, were covalently linked to 2.8 µm tosyl beads, and NeutrAvidin™ was covalently attached to the carboxyl terminal of the dendrimer using EDC-sNHS coupling. Plain NeutrAvidin™ coated beads and commercially available streptavidin-coated beads (both without the dendrimer linker), were used as controls. All beads were finally coated with biotinylated antibodies. Moles of antibodies bound per bead for each generation of dendrimer were compared with plain beads. Detection was done by fluorescence. Finally, dendrimer-NeutrAvidin™ beads and plain NeutrAvidin™ beads (both coated with antibodies) were used as capture beads for the immunoassay and the sensitivity of the assay was compared for two different sets of beads.

**Materials and methods**

**Reagents**

Immunopure® NeutrAvidin™ was from Pierce (Rockford, IL, USA). biotin-4-fluorescein (b-4-f) and fluorescein diphosphate (FDP) were obtained from Invitrogen™, Molecular Probes™ (Eugene, OR, USA). Streptavidin-coated M-280 Dynabeads® [~6-7 x 10^8 beads/ml, 2.8 µm diameter] and tosyl-activated M-280 Dynabeads® [~2 x 10^9 beads/ml, 2.8 µm diameter] were
from Invitrogen Corporation (Carlsbad, CA, USA). Bovine serum albumin (BSA) fraction V powder (biotech grade), sodium azide, sodium chloride (ACS grade), magnesium chloride (MgCl$_2$.6H$_2$O, ACS grade), Tris (hydroxymethyl) aminomethane (THAM, laboratory grade), microcentrifuge tubes (1.5 ml) and borosilicate culture tubes (12 x 75 mm) were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Glycine (99.5%), boric acid and potassium phosphate monobasic (ACS grade) were from Matheson Coleman Bell (Norwood, OH, USA). Potassium phosphate dibasic was from J. T. Baker Chemical Co. (Baker Analyzed Reagent; Phillipsburg, NJ, USA). The neodymium-iron-boron rare earth magnets were obtained from Radio Shack (Fort Worth, TX, USA). Non-magnetic Spherotech™ streptavidin-coated polystyrene blue particles [1.0% (w/v), 0.31 µm diameter] were acquired from Spherotech Inc (Libertyville, IL, USA). Black polystyrene assay plates (96 well, flat bottom) were from Corning Inc. (Corning, NY, USA). Tween 20, ethylene diamine (EDA) (99+), G5.5 and G6.5 PAMAM dendrimers (5 wt.% in methanol) were from Aldrich Chemical (Milwaukee, WI, USA). 2-mercaptoethanol was from Sigma (St. Louis, MO, USA). Biotin-SP-AffiniPure donkey anti-mouse IgG (H+L) (β-d-α-m-IgG), alkaline phosphatase conjugated rat anti-mouse IgG (H+L) (ALP-r-α-m-IgG), biotin-SP-ChromPure mouse IgG (whole molecule) (β-m-IgG) and alkaline phosphatase conjugated mouse IgG (H+L) (m-IgG-ALP) were from Jackson ImmunoResearch Lab. Inc. (West Grove, PA, USA). (2(N-morpholino) ethanesulfonic acid) (MES) was from Calbiochem-Behring Corp. (La Jolla, CA, USA). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (s-NHS) were from Pierce (Rockford, IL, USA). All chemicals were used without further purification.

**Optical microscope:** Streptavidin/NeutrAvidin™ beads were counted on an Olympus BX-40 microscope equipped with a Hitachi HV-C20 camera using Scion CG-7 frame grabber software.
Scion Image Software was used for image processing (5).  

**Luminescence spectrometer:** A Perkin Elmer LS 50B luminescence spectrometer was used as a fluorescence plate reader.

Other apparatuses used were: Incubator (Wedco Inc., Silver Spring, MD, USA), micro-centrifuge with a fixed angle rotor [Heraeus Sepatech (Abbott)], micro-titer plate shaker with orbital movement (Ika Works Inc, Wilmington, NC, USA), diode array UV-vis spectrophotometer (Hewlett Packard 8452A) and Neubauer hemocytometer (Fisher Scientific, Pittsburg, PA, USA).

**Buffers**

Five buffers were prepared: **PBS conjugation buffer**, pH 7.4: [PBS-C, 0.044 M KH$_2$PO$_4$, 0.056 M K$_2$HPO$_4$, 0.1 M NaCl, 0.5 % v/v Tween 20, 0.02% (w/v) NaN$_3$]; **Tris buffer**, pH 9.0: [0.1 M THAM, 5 mM MgCl$_2$.6H$_2$O, 10 mM glycine, 0.02 % (w/v) NaN$_3$]; **Borate buffer**, pH 9.5 (0.1 M boric acid); **MES buffer**, pH 6.5 (0.1 M MES, 0.5 M NaCl); **PBS buffer**, pH 7.4 (0.044 M KH$_2$PO$_4$, 0.056 M K$_2$HPO$_4$, 0.1 M NaCl, 1 w/v % BSA, 0.02 w/v % NaN$_3$, 0.5 v/v % Tween 20).

**Experimental**

6.1.1 Preparation of NeutrAvidin™ coated beads

The method for preparing NeutrAvidin™ coated beads was adapted from that described in Dynabeads Technical Notes and by Farrell (6, 7) and briefly described in Section 2.1.1.

6.1.2 Preparation of G5.5 and G6.5 dendrimer-NeutrAvidin™ coated beads

Dendrimer coated beads were prepared using carbodiimide coupling (8, 6). The procedure was adapted from that used by Farrell (6). It is a four-step reaction as shown in Figure 2.
Figure 2: Schematic for preparing dendrimer coated bead: (Step-I)- Tosyl beads on treatment with EDA formed beads with amino terminal group, and tosyl group as a side product. (Step-II)- G5.5 and G6.5 dendrimers were activated using carbodiimide-coupling procedure. (Step-III)- Products of the first two steps were mixed to form dendrimer-coated beads. (Step-IV)- Dendrimer coated bead was activated with EDC and sNHS. Finally NeutrAvidin™ was added to the activated beads to form dendrimer-NeutrAvidin™ beads.
Step 1: **Conversion of tosyl group to amino group**

The experiment was repeated twice. Tosyl beads (67 µl, ~ 2 x 10^9 beads/ml) were washed with borate buffer (3 times). A magnet was used to retain the beads while washing. After washing, the beads were re-suspended in borate buffer (1 ml) and ethylene diamine (EDA, 50 µl) was added. The reaction mixture was incubated overnight at 37 °C on a plate shaker. After incubation, the beads were washed several times with MES buffer and re-suspended in MES buffer (200 µl).

Step 2: **Activation of dendrimer**

Methanol solvent (200 µl) in G6.5 dendrimers (5 wt. % in methanol) was evaporated using N₂ gas, and 10 mg of EDC in 500 µl MES (30 second incubation) and 4 mg sNHS in 500 µl MES (10 min incubation) were added. Activation was terminated using β- mercaptoethanol (2 µl). The G5.5 dendrimer (5 wt. % in methanol) was activated similarly.

Step 3: **Preparation of dendrimer-coated beads**

Amino terminal beads (200 µl, step I) were added to activated G6.5 and G5.5 dendrimer solution (1 ml, step II) in two separate culture tubes. The mixture was incubated overnight at room temperature on a plate shaker, washed (5 times) with MES buffer and re-suspend in same buffer (200 µl).

Step 4: **Preparation of dendrimer-NeutrAvidin™ coated bead**

Carboxylic groups of dendrimer-beads were further activated using EDC-sNHS coupling as described in step 2. NeutrAvidin™ (2 mg in 500 µl MES) was added to these activated beads. The suspension was incubated overnight at room temperature on a plate shaker. After incubation, beads were washed several times with PBS buffer and re-suspended in 200 µl of PBS buffer (final bead concentration: ~ 6.7 x 10^8 beads/ml).
6.2.1 Determining the antibody capacity for plain and dendrimer-coated beads

The method was adapted from a procedure used by Farrell (6). Saturation studies were done for NeutrAvidin™, commercially available streptavidin, G5.5 dendrimer-NeutrAvidin™ and G6.5 dendrimer–NeutrAvidin™ coated beads (2.8 µm). The procedure for determining the streptavidin/NeutrAvidin™ bead capacity for biotinylated antibodies is detailed in Section 3.3.2.

First, G5.5 dendrimer-NeutrAvidin™ magnetic beads (8 x 10^8 beads/ml, 70 µl,) were washed twice with PBS-R buffer with the help of a magnet (used for bead capture) and re-suspended in PBS-R (70 µl). Then, β-d-α-m-IgG (0 – 10.5 µg/well) was added to each well containing washed beads (8.0 x 10^8 beads/ml, 5 µl) and the total volume in each well was maintained as 30 µl using PBS-R. Parafilm™ was used to seal the plate and the beads were incubated at room temperature for 60 min on a plate shaker. Beads were then washed with PBS-R buffer (5 times), aided by a magnet, and re-suspended in m-IgG-ALP in PBS-R (250 µg/ml, 30 µl). The mixture was again incubated for 60 min at room temperature on a plate shaker. After incubation, the beads were washed with PBS-R (thrice) and Tris buffer (twice), and finally re-suspended in Tris buffer (30 µl). A fixed volume of beads (5 µl) from each well was added to the fresh wells containing FDP substrate (20 µM, 90 µl). FDP was excited at 490 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).

A similar experiment was done with G6.5 dendrimer beads except that different concentrations of β-d-α-m-IgG (0.3–13.5 µg/well) and m-IgG-ALP (900 µg/ml), 30 µl) were added.

6.3.1 Biotin-4-fluorescein assay to determine the total number of biotin binding sites per bead
Commercially available streptavidin beads (2.8 µm), NeutrAvidin™ beads (2.8 µm) and G5.5, G6.5 dendrimer-NeutrAvidin™ beads were titrated with b-4-f. Titrations for streptavidin and NeutrAvidin™ beads are detailed in Section 2.2.1.

G5.5 and G6.5 dendrimer-NeutrAvidin™ beads (8.0 x 10^8 beads/ml, 200 µl) were washed with Tris buffer (twice) with the help of a magnet (for capture) and re-suspended in the same buffer (200 µl). The concentration of b-4-f was verified by UV-vis absorption at 494 nm. At 494 nm and pH 9.0, the molar extinction coefficient of b-4-f is 73,200 M⁻¹ cm⁻¹ (9).

b-4-f in each well (3.5 x 10^-6 M, 15 µl) was non-cumulatively titrated with G5.5 beads (8 x 10^8 beads/ml, 2 µl – 8 µl) in a black 96 well polystyrene micro-titer plate and the total volume in each well was adjusted to 100 µl using Tris buffer containing 0.1 % BSA. Suspensions were sealed with Parafilm™ and incubated on a plate shaker for 30 min followed by fluorescence measurements. b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). A similar experiment was done with G6.5 dendrimer-NeutrAvidin™ beads (concentration: 8 x 10^8 beads/ml, 0.5 µl – 9 µl).

The concentrations of G5.5 and G6.5 beads were further verified by diluting 10 µl of working standards many-fold using PBS buffer pH 7.0 and counting beads on an optical microscope using the Neubauer hemocytometer. The calculated concentration of working standards for both G5.5 and G6.5 beads using the above method was 8.0 x 10^8 beads/ml.

6.4.1 A sandwich immunoassay to compare the G6.5 dendrimer-NeutrAvidin™ and plain NeutrAvidin™ bead

Preparation of the artificial micro-organism (antigen), Bugbead
The preparation of Bugbead is detailed elsewhere (10). In short, 0.31 µm streptavidin polystyrene blue particles (2 x 10⁹ particles/ml, 300 µl) were incubated with β-m-IgG (1 mg/ml, 18 µl) and PBS buffer (1482 µl) for 10 min at room temperature, on a vortex. The particles were washed (five times) centrifugally (13,000 rpm, minimum RCF 13,400g, 2 min) with PBS buffer (1.5 ml) using a micro-centrifuge with a fixed angle rotor (24-place x 1.5-2 ml) and finally re-suspended in PBS buffer (300 µl).

**Preparation of the G6.5 dendrimer-NeutrAvidin™ capture beads**

G6.5 dendrimer-NeutrAvidin™ beads (8 x 10⁸ particles/ml, 30 µl) were incubated on a vortex with β-d-α-m-IgG (700 µg/ml, 67.5 µl) and PBS buffer (82.5 µl) for 10 min at room temperature. Beads were washed (five times) with PBS buffer (200 µl), aided by a magnet, and finally re-suspended in PBS buffer (30 µl).

**Preparation of plain NeutrAvidin™ capture beads (without dendrimer)**

NeutrAvidin™ beads (2.8 µm; 8 x 10⁸ particles/ml, 20 µl) were incubated with β-d-α-m-IgG (700 µg/ml, 2 µl) and PBS buffer (98 µl) for 30 min at room temperature on a vortex. Beads were washed (five times) with PBS buffer, aided by a magnet, and finally re-suspended in PBS buffer (20 µl).

**Immunoassay**

An experiment was carried out in culture tubes. Bugbeads were added (2 x 10⁸ particles/ml, 0 - 25 µl) to G6.5 dendrimer-NeutrAvidin™ capture beads (8 x 10⁸ beads/ml, 2.5 µl), and the total volume in each tube was maintained at 30 µl with PBS buffer. The mixture was incubated for 1 hour at room temperature. After incubation, the particles were washed with PBS buffer (5
times), ALP-r-α-m-IgG (60 µg/ml, 30 µl) was added to these washed beads and the suspension was incubated for 1 hour. Then, beads were again washed (5 times) with PBS buffer and re-suspended in PBS buffer (30 µl). A similar experiment was done using plain NeutrAvidin™ beads (without dendrimers). Beads (5 µl) from each well were added to fresh wells containing FDP substrate (10 µM, 90 µl). FDP was excited at 490 nm (slit width 5 nm) and the emission was measured at 514 nm (slit width 5 nm).

**Results and discussion**

6.2.1 *Determination of the antibody capacity for dendrimer-coated and plain beads*

The aim of this research was to study the effect of dendrimers on the ligand binding capacity of the solid support. To study this effect, it was crucial to compare the ligand-binding capacity of dendrimer-NeutrAvidin™ coated beads with that of plain NeutrAvidin™ beads (control). Also, the NeutrAvidin™ beads were made in our laboratory, and hence a comparison of the antibody capacity of commercially available streptavidin beads of similar size (2.8 µm) with that of lab-made NeutrAvidin™ beads should give some indication of the success of our NeutrAvidin™ bead preparation.

Antibody saturation plots for plain streptavidin and NeutrAvidin™-coated beads are shown in Section 3.3.2. Both immobilized streptavidin and NeutrAvidin™ bind to biotin with <1:4 ratio (11), and beads are of equal diameter (2.8 µm). Both beads carried an equal number of antibodies (4 x 10⁵ antibodies/bead), supporting the success of the NeutrAvidin™ immobilization reaction.

Saturation curves for G5.5 and G6.5 dendrimer-NeutrAvidin™ beads were plotted using non-linear regression (least squares fit) with data analysis software GraphPad Prism v. 5.00 (San...
Diego, California, USA) for Mac OSX. Michaelis-Menten equation was used for fitting, which is similar in form to that of Langmuir equation.

As per the software, the saturation point for G5.5 dendrimer was at 1.2 µg/well (Figure 3). The number of antibody molecules/bead was calculated using the equation-I –

\[
\text{Antibody/bead} = \frac{1}{\frac{\text{Antibody concentration at saturation point}}{\text{Number of beads in each well}}} \times \frac{1}{\text{Avogadro’s no.}} \times \frac{1}{\text{Molecular weight of antibody}}
\]

where,

Number of beads in each well = 4 x 10^6,

Molecular weight of antibody = ~150 kDa (12),

Avogadro’s number = 6.022 x 10^{23} molecules/mole.

The bead capacity of G5.5 dendrimer-NeutrAvidin™ beads was 1.2 x 10^6 antibodies/bead.

The saturation point for G 6.5 dendrimer was (3.5 µg/well) (Figure 4). The capacity of G6.5 dendrimer-NeutrAvidin™ beads was found to be 3.5 x 10^6 antibodies/bead. G0.5 and 2.5 dendrimer-NeutrAvidin™ beads saturated at 0.015 and 0.37 µg/well (4 x 10^6 beads/well), respectively, with corresponding bead capacity of 0.2 x 10^5 and 4.0 x 10^5 antibodies/bead (6).

Plain streptavidin, NeutrAvidin™ beads and dendrimer - coated beads (G0.5 - G6.5) were plotted against total number of antibody molecules/bead in the bar graph shown in Figure 5. G0.5 dendrimer had the lowest number of antibodies/bead (0.2 x 10^5) (6). Plain streptavidin/NeutrAvidin™ beads and G2.5 dendrimer-NeutrAvidin™ beads had nearly the
Figure 3: Antibody saturation curve to determine the capacity of G5.5 dendrimer-NeutrAvidin™ bead: β-d-α-m-IgG (0 –10.5 µg/well) was added to culture tubes containing washed beads (8.0 x 10⁸ beads/ml, 5 µl). Total volume was maintained as 30 µl using PBS-R. After several incubation and washing steps, beads were re-suspended in m-IgG-ALP in PBS-R (250 µg/ml, 30 µl). Beads were incubated further, washed and re-suspended in 30 µl of Tris buffer. Beads (5 µl) from each well were added to fresh wells containing FDP substrate (20 µM, 90 µl). FDP was excited at 490 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Saturation curve was fitted using non-linear regression (least squares fit) with data analysis software GraphPad Prism v. 5.00 (San Diego, California, USA) for Mac OSX. Michaelis-Menten equation was used for fitting, which is similar in form to that of Langmuir equation.
Figure 4: Antibody saturation curve to determine the capacity of G6.5 dendrimer-NeutrAvidin® bead: β-d-α-m-IgG (0.3 –13.5  µg/well) was added to culture tubes containing washed beads (8.0 x 10^8 beads/ml, 5 µl). Total volume was maintained at 30 µl using PBS-R. After several incubation and washing steps, the beads were re-suspended in m-IgG-ALP in PBS-R (900 µg/ml, 30 µl). Beads were incubated further, washed and re-suspended in 30 µl of Tris buffer. Beads (5 µl) from each well were added to fresh wells containing FDP substrate (20 µM, 90 µl). FDP was excited at 490 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Saturation curve was fitted using non-linear regression (least squares fit) with data analysis software GraphPad Prism v. 5.00 (San Diego, California, USA) for Mac OSX. Michaelis-Menten equation was used for fitting, which is similar in form to that of Langmuir equation.
Figure 5: Antibody capacity studies for streptavidin, NeutrAvidin™, G0.5, G2.5, G5.5 and G6.5 dendrimer-NeutrAvidin™ beads, summary (*6): Each set of beads was titrated with various concentrations of β-d-α-m-IgG [Streptavidin/NeutrAvidin™: (0 – 0.45 µg/well), G0.5 dendrimer-NeutrAvidin™ (0.003 - 0.09 µg/well), G2.5 dendrimer-NeutrAvidin™: (0.03 – 1.35 µg/well), G5.5 dendrimer- NeutrAvidin™: (0 –10.5 µg/well), G6.5 dendrimer-NeutrAvidin™: (0.3 –13.5 µg/well)], followed by addition of m-IgG-ALP [Streptavidin/NeutrAvidin™: (2.2 µg/well), G0.5 dendrimer-NeutrAvidin™: (0.9 µg/well), G2.5 dendrimer-NeutrAvidin™: (1.5 µg/well), G5.5 dendrimer-NeutrAvidin™: (7.5 µg/well) , G6.5 dendrimer-NeutrAvidin™: (27 µg/well)]. In all cases, FDP was excited at 490 nm and emission was measured at 514 nm.
same number of antibodies/bead (4 x 10^5). G6.5 dendrimer-NeutrAvidin™ bead showed the highest number of antibodies/bead (3.5 x 10^6). This trend may be observed due to the fact that PAMAM dendrimers at lower generations (G = 1-2) have an open, asymmetric disk-like structure that may produce high steric hindrance for NeutrAvidin™ binding; whereas at here higher generations (G ≥3), they form densely packed spherical shapes (13, 14) providing a larger three dimensional space for NeutrAvidin™ (consequently for antibody) binding as shown in the Figure 6.

6.3.1 Biotin-4-fluorescein assay to determine the total biotin binding sites of bead

It is an important step to compare the total biotin binding sites (i.e. NeutrAvidin™ incorporation) for dendrimer coated beads verses plain beads (without dendrimers). This will help to determine if the higher binding capacities of dendrimer beads are only due to a higher number of NeutrAvidin™ (i.e. greater surface area) or the presence of dendrimer increases conformational freedom of the antibodies and decreases steric hindrance, resulting in higher antibody concentration.

A b-4-f assay was used to determine the number of active biotin binding sites for 2.8 µm streptavidin beads (Section 2.2.1), 2.8 µm NeutrAvidin™ beads (Section 2.2.1), G5.5 dendrimer-NeutrAvidin™ beads and G6.5 dendrimer-NeutrAvidin™ beads. In each case, two regression lines coinciding at stoichiometric equivalence points were plotted to get b-4-f titration curve. The break point of two regression lines was at 4.44 x 10^6 beads (Figure 7) for G5.5 dendrimer-NeutrAvidin™ beads and at 3.6 x 10^6 beads (Figure 8) for G6.5 dendrimer-NeutrAvidin™ beads, when 5.25 x 10^{11} moles of b-4-f were used for titration. The active biotin binding capacity of each set of beads was calculated using the formula in Section 2.2.1 and is depicted in

119
Figure 6: Dendrimer-NeutrAvidin™ beads (left) vs. plain NeutrAvidin™ beads (middle) [Bead diameter: 2.8 µm, PAMAM dendrimer diameter range: 1.5-14.5 nm (15), NeutrAvidin™ dimensions: 4.5 x 4.5 x 5.8 nm (16, 17), antibody dimensions: 14.2 nm x 8.5 nm x 3.8 nm (18, 19)]
Figure 7: Determination of biotin binding capacity for G5.5 dendrimer-NeutrAvidin™ coated beads, using biotin-4-fluorescein: b-4-f in each well (3.5 x 10⁻⁶ M, 15 µl) was non-cumulatively titrated with G5.5 beads (8 x 10⁸ beads/ml, 2–8 µl) in a black 96 well polystyrene micro-titer plate (total volume: 100 µl). After incubation, b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Two regression lines coinciding at stoichiometric equivalence points were plotted to get titration curve.
Figure 8: Determination of biotin binding capacity for G6.5 dendrimer-NeutrAvidin™ coated beads, using biotin-4-fluorescein: b-4-f in each well (3.5 x 10⁶ M, 15 µl) was non-cumulatively titrated with G5.5 beads (8 x 10⁸ beads/ml, 0.5-9 µl) in a black 96 well polystyrene micro-titer plate (total volume: 100 µl). After incubation, b-4-f was excited at 494 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm). Two regression lines coinciding at stoichiometric equivalence points were plotted to get titration curve.
Table 1. It was observed that dendrimer-NeutrAvidin™ beads had more biotin binding sites than plain streptavidin and NeutrAvidin™ beads (controls). Also, the number of biotin-binding sites for G6.5 beads was higher than for the G5.5 beads.

The effect of the dendrimer on bead capacity was studied by comparing the estimated surface coverage of antibodies on beads (Table 2) using the equation-II-

\[
\text{Surface coverage} \% = \frac{\text{Number of biotinylated antibodies per bead}}{\text{Number of biotin binding sites per bead}} \times 100 \quad \text{(II)}
\]

Streptavidin and NeutrAvidin™ beads had the low surface coverage (~10% and 20%, respectively). G6.5 dendrimer had ~40% coverage. This indicates that increase in number of capture antibodies for G6.5 dendrimers (Section 6.2.1) was mainly due to the presence of dendrimers and partly due to more NeutrAvidin™ being on the bead surface.

6.4.1 A sandwich immunoassay to compare G6.5 dendrimer-NeutrAvidin™ beads and plain NeutrAvidin™ beads

Incorporating the dendrimer to NeutrAvidin™ bead results in an increased antibody concentration per unit surface area as demonstrated in Section 6.2.1. The possible influence of the dendrimer was examined by comparing the efficiency of a sandwich immunoassay with two different capture beads i.e. dendrimer-NeutrAvidin™ beads vs. plain NeutrAvidin™ beads. G6.5 dendrimer beads were chosen for this assay due to their highest antibody binding capacity (3.5 x 10^6 antibodies/bead) compared to the other tested dendrimer beads. A lab-made plain NeutrAvidin™ bead without dendrimer was used as a control.
<table>
<thead>
<tr>
<th>Active biotin binding sites</th>
<th>Beads (control)</th>
<th>Dendrimer-NeutrAvidin&lt;sup&gt;TM&lt;/sup&gt; beads</th>
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</thead>
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<tr>
<td></td>
<td>Streptavidin</td>
<td>NeutrAvidin&lt;sup&gt;TM&lt;/sup&gt;</td>
</tr>
<tr>
<td>Molecules (b-4-f/bead)</td>
<td>$4 \times 10^6$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>Moles (Moles of b-4-f/bead)</td>
<td>$6 \times 10^{-18}$</td>
<td>$4 \times 10^{-18}$</td>
</tr>
</tbody>
</table>

Table 1: Biotin binding capacities for streptavidin, NeutrAvidin<sup>TM</sup> and dendrimer-NeutrAvidin<sup>TM</sup> coated solid supports using the biotin-4-fluorescein method
<table>
<thead>
<tr>
<th>Active Biotin Binding sites</th>
<th>Beads (control)</th>
<th>Dendrimer-NeutrAvidin\textsuperscript{TM} beads</th>
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</thead>
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<tr>
<td></td>
<td>Streptavidin</td>
<td>NeutrAvidin\textsuperscript{TM}</td>
</tr>
<tr>
<td>Molecules (b-4-f/bead)</td>
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<td>$2 \times 10^6$</td>
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<tr>
<td>Antibody capacity (antibody/bead)</td>
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<td>Estimated surface coverage</td>
<td>10%</td>
<td>20%</td>
</tr>
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</table>

Table 2: Estimation of surface coverage for streptavidin, NeutrAvidin\textsuperscript{TM} and G6.5 dendrimer-NeutrAvidin\textsuperscript{TM} coated solid supports using the biotin-4-fluorescein method
A sandwich immunoassay was developed using an artificial microorganism (Bugbead) as an antigen and G6.5 dendrimer-NeutrAvidin™ capture bead as shown in Figure 9. The Bugbead concentration range used for the assay was 0-5.58 x 10⁷ Bugbeads/ml. As the Bugbead concentration was increased, there was an increase in fluorescence signal due to higher catalysis of substrate. Reaction velocity of enzyme catalysis was plotted for each concentration of Bugbead. Such a plot for G6.5 dendrimer NeutrAvidin™ bead is illustrated in Figure 10. The chart for slope of reaction velocity against Bugbead concentration for both G6.5 dendrimer-NeutrAvidin™ bead and plain NeutrAvidin™ bead was plotted (Figure 11). The slopes’ equations for sensitivity of the assay was \( y = 1 \times 10^{-7} x + 0.1621 \) using G6.5 dendrimer beads and \( y = 2 \times 10^{-8} x + 0.036 \) using plain NeutrAvidin™ beads (Figure 12). Hence G6.5 dendrimer-NeutrAvidin™ beads show 5 times more assay sensitivity than plain NeutrAvidin™ beads.

**Summary**

Incorporation of higher generation dendrimer to a NeutrAvidin™ coated bead resulted in increase in both biotin and ligand (antibody) binding efficiency of the bead. When evaluated with plain NeutrAvidin™ beads, biotin binding sites for G6.5 dendrimer-NeutrAvidin™ beads was 4.5 times higher (G6.5 dendrimer-NeutrAvidin™ beads: 9 x 10⁶ antibody/bead, plain NeutrAvidin™ beads: 2 x 10⁶ antibody/bead) as compared to 10 times higher ligand binding capacity (G6.5 dendrimer-NeutrAvidin™ beads: 4 x 10⁶ antibody/bead, plain NeutrAvidin™ beads: 4 x 10⁵ antibody/bead). Furthermore, antibodies occupied only 20% of the total biotin binding sites (surface coverage) for plain NeutrAvidin™ beads as compared to 40% of the total biotin binding sites for G6.5 dendrimer-NeutrAvidin™ beads. This proves that increase in ligand binding capacity of dendrimer-NeutrAvidin™ bead is not only due to higher number of
Figure 9: A sandwich immunoassay to compare dendrimer–NeutrAvidin™ beads and plain NeutrAvidin™ beads
Figure 10: Reaction velocity of alkaline phosphatase for sandwich immunoassay using G6.5 dendrimer-NeutrAvidin™ capture beads: Bugbeads (2 x 10⁸ particles/ml, 0-25 µl) were added to G6.5 dendrimer-NeutrAvidin™ capture beads (8 x 10⁸ beads/ml, 2.5 µl) with total volume of 30 µl. After incubation and washing, ALP-r-α-m-IgG (60 µg/ml, 30 µl) was added. Mixture was further incubated, washed and re-suspended in 30 µl of PBS. Fixed volumes of beads (5 µl) from each well were added to fresh wells containing FDP substrate (10 µM, 90 µl). FDP was excited at 490 nm (slit width 5 nm) and emission was measured at 514 nm (slit width 5 nm).
Figure 11: Chart representing reaction velocity vs. Bugbead concentration for sandwich immunoassay using G6.5 dendrimer–NeutrAvidin™ beads and NeutrAvidin™ beads as solid support. Bugbead concentration range was 0–5.7 x 10^7 Bugbeads/ml.
Figure 12: Plot of signal vs. Bugbead concentration for sandwich immunoassay using G6.5 dendrimer–NeutrAvidin™ beads and NeutrAvidin™ beads as solid supports. The Bugbead concentration range was 0 – 5.7 x 10^7 Bugbeads/ml.
NeutrAvidin™ attached (higher surface area) but also to change in orientation of NeutrAvidin™ on the bead surface (after dendrimer incorporation) providing more 3 dimensional space, conformational freedom and low steric hindrance.

Comparing the immunoassay sensitivity of G6.5 dendrimer-NeutrAvidin™ capture beads with plain NeutrAvidin™ capture beads tested the dendrimer influence on solid support. The comparison was based on the possibility that the dynamics of a higher generation dendrimer may provide higher antibody flexibility and less steric hindrance. Also, the multivalent effect of the higher generation dendrimer, especially in case of larger antigens, may result in higher avidity (by binding to many of its epitopes) and hence greater assay sensitivity. When such assay was done using ‘Bugbead’ as an antigen, dendrimer-NeutrAvidinTM coated beads gave 5fold increase in sensitivity as compared to plain beads. This method can be applied to various fields such as immunoassays, protein purifications etc. where success of the technique depends on the efficient streptavidin/NeutrAvidin™ immobilization (higher biotin binding efficiency), greater ligand binding efficiency or better assay sensitivity.

**Conclusion**

The effect of different dendrimers on the ligand binding capacity of 2.8 µm NeutrAvidin™ beads was studied. The number of antibodies per bead and the available biotin binding capacity increased from dendrimers of generations G0.5 to G6.5. Finally, G6.5 dendrimer-NeutrAvidin™ beads and plain NeutrAvidin™ beads (both coated with antibody) were used as capture beads for immunoassay and a 5fold increase in sensitivity was observed as compared to plain NeutrAvidin™ beads.
Future work

Streptavidin and NeutrAvidin™ beads are reproducible with respect to antibody and biotin binding capacity. The reproducibility of G0.5 - G5.5 dendrimer-NeutrAvidin™ beads was confirmed previously by Farrell (6). The reproducibility of G6.5 beads needs to be confirmed. Statistical analysis on sensitivities and comparisons of detection limits using two different capture beads needs to be done.

References


Chapter 5

Effect of monochloramine disinfectant on MS2 bacteriophage immunoassay
Introduction

Water plays a vital role in the existence of living beings. Polluted water jeopardizes all known forms of life. To sustain human life, drinking water is disinfected using thermal, chemical or physical methods (1). The chemical method is the most popular of all, and involves using for example, oxidizing disinfectants such as chlorine, monochloramine, ozone, or chlorine dioxide. Out of all the different forms of water disinfectants, chlorine is the most widely used, not only in United States (2), but throughout the world (3). Although chlorine has made an immense contribution in decreasing water borne diseases and improving water quality, its carcinogenic by-products have attracted researchers’ attention towards other methods.

In our laboratory, immunoassays were developed for simulants representing four different classes of microorganisms: ovalbumin (toxins), heat killed E. coli O157:H7 (bacteria), B. globigii and B. anthracis Sterne strain (spores), and MS2 bacteriophage (virus) (4). Also, the effects of various water components and disinfectants on assay sensitivity were studied (4). Chlorine and monochloramine had major effect on the sensitivity of MS2 bacteriophage and minor effects on B. globigii and ovalbumin (4). The effect of chlorine on the sensitivity of the MS2 immunoassay is reported in literature (5). Here, the research focuses mainly on the effect of monochloramine on the MS2 immunoassay.

Materials and methods

Reagents

Fluorescein diphosphate (FDP) was obtained from Invitrogen™, Molecular Probes™ (Eugene, OR, USA). Streptavidin-coated M-280 Dynabeads® [~6-7x10⁸ beads/ml, 2.8 µm diameter] were
from Invitrogen™ (Carlsbad, CA, USA). Bovine serum albumin (BSA) fraction V powder (biotech grade), sodium azide, magnesium chloride (MgCl$_2$.6H$_2$O, ACS grade), sodium chloride (ACS grade), ammonium chloride and borosilicate culture tubes (12 x 75 mm) were from Fisher Scientific (Fair Lawn, NJ, USA). White/clear round bottom 96-well micro-titer plates and platinum wire auxiliary electrodes were from Fisher Scientific (Pittsburgh, PA, USA). Black polystyrene assay plates (96 well, flat bottom) were from Corning Inc. (Corning, NY, USA). Tween 20 was from Aldrich Chemical (Milwaukee, WI, USA). Potassium phosphate monobasic (ACS grade) was from Matheson Coleman Bell (Norwood, OH, USA). The neodymium-iron-boron rare earth magnets were obtained from Radio Shack (Fort Worth, TX, USA). Sulfo-NHS-LC biotinylation kit was from Pierce (Rockford, IL, USA). The MS2 bacteriophage was from the US Army (Dugway Proving Ground, UT, USA) and the anti-MS2 IgG was from Tetracore (Rockville, MD, USA). The enzyme $\beta$-galactosidase was conjugated to anti-MS2 IgG by American Qualex (San Clemente, CA, USA). $p$-aminophenol-$\beta$-D-galactopyranoside (PAPG) was from Sigma (St. Louis, MO, USA). Sodium hypochlorite (4-6 w/v %), sodium phosphate dibasic, YM Centricons and potassium chloride were from Fisher Scientific (Fair Lawn, NJ, USA). Gold electrode (3 mm diameter) was from Bioanalytical Systems, Inc. (West Lafayette, IN, USA) and a saturated miniature Teflon Ag/AgCl reference electrode was from Cypress Systems, Inc. (Lawrence, KS, USA).

**Apparatus**

**Luminescence spectrometer:** All fluorescence measurements were done in 96 well plates using a Perkin Elmer LS 50B luminescence spectrometer as a fluorescence plate reader. Other apparatuses used were: incubator (Wedco Inc.: Silver Spring, MD, USA); micro-titer plate shaker with orbital movement (Ika Works Inc.: Wilmington, NC, USA); 100 BAS W RDE-2
amperometer (Bioanalytical Systems: West Lafayette, IN, USA) and HACH DR/890 colorimeter (HACH Company: Loveland, Colorado, USA).

Buffers

Three aqueous buffer were made: **PBS-R (reaction buffer)**, pH 7.3: (4.3 mM sodium phosphate dibasic, 1.4 mM potassium phosphate monobasic, 137 mM sodium chloride, 2.7 mM potassium chloride, 3 mM sodium azide, 1% (w/v) BSA, 0.5% (v/v) Tween 20); **PBS-D (detection buffer)**, pH 7.3: (4.3 mM sodium phosphate dibasic, 1.4 mM potassium phosphate monobasic, 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM magnesium chloride, 3 mM sodium azide), and **2x PBS buffer**, pH 7.4: (0.2 M sodium phosphate and 0.3 M sodium chloride).

Reagent preparation

1. **Preparation of biotinylated anti-MS2 IgG**: Sulfo-NHS-LC biotinylation kit was used to biotinylate anti-MS2 IgG as per the manufacturer’s (Pierce) instructions (6).

2. **Preparation of p-aminophenol-β-D-galactopyranoside solution (PAPG)**: PBS-R (5 ml) was deoxygenated for 30 min by passing N₂ gas. Fresh PAPG (5.3 mM) solution was made by adding PAPG (2.9 mg) to the deoxygenated PBS-R (2 ml) buffer. Solution was sealed with Parafilm™.

3. **Preparation of fluorescein di-β-D-galactopyranoside solution (FDG)**: FDG stock solution (20 mM, 15 µl) was diluted to 1.5 ml with PBS-D buffer to make 2 mM FDP working standard.

4. **Preparation of monochloramine solution**

   a) **Preparation of ammonium chloride solution (0.06 M)**: Ammonium chloride (2.0 g) was oven dried for 18 hours at 110 °C to evaporate water contents. The oven-dried ammonium
chloride (1.62 g) was dissolved in 500 ml of organic pure water. The mixture was stored in an amber colored bottle at 4°C.

**b) Preparation of NaOCl solution:** A chlorine stock solution was prepared by adding sodium hypochlorite solution (4-6 w/v %, 220 µl) to organic pure water (500 ml). The total concentration of chlorine was determined using the HACH meter (method 8167) as 17.9 mg/l.

**c) Preparation of monochloramine:** The chlorine stock solution (17.9 mg/l, 6.15 ml) was added to organic pure water (8.45 ml). NH₄Cl solution (0.06 M, 400 µl) was added drop-wise to the chlorine solution, with constant stirring. The beaker was sealed with Parafilm™ and the entire beaker covered with silver foil to avoid direct contact with light. The mixture was incubated for 90 min at room temperature. The monochloramine concentration was measured using HACH meter (Program 110) and found to be 9.2 mg/l, from which 8 mg/l, 4 mg/l and 2 mg/l solutions of monochloramine were prepared by diluting the stock with organic pure water.

**Experimental**

**5.1.1 A standard curve for MS2 bacteriophage**

The method was adapted from that used by Thomas et al. (7). The experiment was done in four steps:

**A) Preparation of capture beads**

In a culture tube, 2.8 µm magnetic streptavidin coated beads (6.7 x 10⁸ beads/ml, 130 µL) were washed twice with PBS-R with the help of a magnet (used for bead capture) and re-suspended in PBS-R (130 µL). The bead suspension was then incubated with biotinylated anti MS2 antibody (6.7 mg/ml, 1.6 µl) and sealed using Parafilm™. The mixture was incubated for 30 min on a slow vortex at room temperature. After incubation, the mixture was washed thrice
with PBS-R, aided by a magnet, and re-suspended in PBS-R (130 µl). Finally this suspension of antibody bead complex (10 µL) was added to the fresh wells of a micro-titer plate.

**B) Preparation of MS2 standard solutions**

MS2 standards were prepared in disposable culture tubes using 1 mg/ml of MS2 stock solution as follows-

<table>
<thead>
<tr>
<th>Solution numbers</th>
<th>PBS-R (µL)</th>
<th>MS2</th>
<th>Final conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>992</td>
<td>8 µL of stock (1 mg/ml)</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100 µL of solution 1</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>100 µL of solution 2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>100 µL of solution 3</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>100 µL of solution 4</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>100 µL of solution 5</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>100 µL of solution 6</td>
<td>0.125</td>
</tr>
<tr>
<td>8</td>
<td>200</td>
<td>- - -</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**C) The reaction of MS2 bacteriophage (antigen) with bead-antibody complex**

MS2 aliquots (10 µL) of concentrations between 0 µg/ml – 8 µg/ml (from step-II) were incubated with 10 µL each of the bead-antibody complex (from step-I) for 25 min, in a 96-well micro-titer plate. In each well, the bead suspension was washed twice with PBS-R and re-suspended in PBS-R (10 µL).
D) The reaction of bead-antibody-MS2 complex with labeled secondary antibody

To the suspended bead complex in each well (from step ‘C’), an aliquot of anti-MS2 antibody-beta galactosidase (250 µg/ml, 15µl) was added and the mixture was incubated for 30 min at room temperature, on a slow vortex. After incubation, the beads were washed with PBS-R (twice), PBS-D (four times) and finally re-suspended in PBS-D (30 µl).

Electrochemical detection

A 100 BAS W RDE-2 amperometer was used for hydrodynamic amperometric (current is measured with time) detection of MS2 immunoassay. A microscope glass plate was covered with Parafilm™ to provide a hydrophobic surface, and Ag/AgCl (reference electrode) and Pt wires (auxiliary electrodes) were mounted over it. The gold rotating disk electrode (3 mm diameter, working electrode) was polished with 0.3 nm alumina powder paste. Electrodes were positioned in such a way that the Ag/AgCl wire and Pt wire were beneath the gold rotating disk electrode as shown in Figure 1. Bead-antibody-MS2 complex (5 µl) were added to PAPG (5.3 mM, 30 µl), with the total volume of the micro-drop therefore as 35 µl. Single potential timebase technique was used for detection, with RDE spin rate of 3000 rpm, initial potential of 290 mV and sensitivity of 100 nA.

Fluorescence detection: Fluorescence detection was done using the fluorescence plate reader. Bead-antibody-MS2 complex (5 µl) were added to FDP (2mM, 90 µl) in a black 96 well micro-titer plate. FDP was excited at 485 nm (slit width 5 nm) and emission was measured at 538 nm (slit width 5 nm).
Figure 1: A rotating disk electrode (RDE) setup for hydrodynamic amperometric detection of the MS2 immunoassay: A micro-drop volume of 35 µl (5 µl bead-antibody-MS2 complex, 30 µl of 5.3 mM PAPG) was added on the Parafilm™ coated hydrophobic surface of the glass slide. Ag/AgCl (reference electrode), Pt wire (auxiliary electrode) and gold rotating disk (working electrode) was used for hydrodynamic amperometric detection of MS2 immunoassay. Single potential timebase technique was used for detection with RDE spin of 3000 rpm, initial potential of 290 mV and sensitivity of 100 nA.
5.1.2 Effect of monochloramine on MS2 immunoassay

MS2 bacteriophage (2 µg/ml) was treated with different concentrations of monochloramine (0 mg/l, 2 mg/l, 4 mg/l and 8 mg/l) in organic pure water. Reactions were done in glass vials to minimize/eliminate NSB. Each vial was sealed with Parafilm™ and covered with aluminum foil to avoid direct exposure to light. The solution was incubated for 7 d in the dark with occasional stirring. In order to maintain the ionic strength of the solution, at the end of the seven days an equal volume of 2x PBS buffer was added to the MS2 bacteriophage containing monochloramine, whereas 1x PBS was added to a standard sample (in PBS buffer containing 0 mg/ml of monochloramine). These incubated MS2 bacteriophage (2 µg/ml, 10 µl) samples were then added to the paramagnetic bead-antibody complex (6.8 x 10⁸ beads/ml, 10 µl) and the immunoassay continued as described in Section 5.1.1 (using fluorescence detection). A schematic diagram indicating the complete process is illustrated in Figure 2.

5.1.3 Effect of monochloramine components on MS2 immunoassay

A 4 mg/l monochloramine solution was prepared using ammonium chloride and sodium hypochlorite as described before (Chapter 5 : Methods and materials/reagent preparation). The free ammonia and free chlorine concentrations in 4 mg/l of monochloramine were measured using HACH meter (Program 46 and method 8167, respectively). A standard ammonium chloride sample was prepared using the same concentration of ammonium chloride in organic pure water, as that added to prepare 4 mg/l of monochloramine. The free ammonia concentration in standard ammonium chloride solution was measured using the HACH meter (Program 46). MS2 bacteriophage (2 µg/ml) was treated with the above monochloramine solutions (0, 4 mg/l)
Figure 2: A scheme to detect the effect of monochloramine on the MS2 immunoassay
and ammonium chloride solution. The reaction was carried out in a glass vial to minimize/eliminate NSB. All three-reaction mixtures were sealed with Parafilm\textsuperscript{TM}, covered with aluminum foil and incubated for 7 d. The immunoassay was carried out as described in Section 5.1.2.

**Results and discussion**

5.1.1 A standard curve for MS2 bacteriophage

Streptavidin-coated paramagnetic beads (2.8 µm diameter) were used as the solid support for the immunoassay and detection was done using fluorescence and electrochemical methods. The slopes of the reaction velocities were plotted against the concentration of MS2 bacteriophage as shown in Figure 3. Results are detailed in literature (4). The calibration curve was repeated in this research to confirm the reproducibility of the method and the quality of the reagents used. The electrochemical method had the linear equation: \( y = 1.644x + 1.645 \) with \( R^2 \) value of 0.99225 and the fluorescence method had the linear equation \( y = 1.659x + 0.3459 \) and \( R^2 \) value of 0.9904.

5.1.2 Effect of monochloramine on the MS2 immunoassay

A previous study was done to determine the concentration at which monochloramine (2 µg/ml) begins to interfere with the assay, when incubated with MS2 bacteriophage (2 µg/ml) for 1 h (4). The study was extended in this research to determine the effect of various monochloramine concentrations on MS2 bacteriophage (2 µg/ml), when incubated for 7 d.

The maximum EPA limit for a safe monochloramine concentration in drinking water is 4 mg/l (8, 7). Hence monochloramine concentrations selected for study were 0, 2, 4 and 8 mg/l.
Figure 3: Log-log calibration plot for MS2 immunoassay for the comparison of fluorescence and electrochemical detection methods: Streptavidin coated beads (2.8 µm; 6.7 x 10^8 beads/ml, 130 µL) were incubated with biotinylated anti MS2 antibody (6.7 mg/ml, 1.6 µl) for 30 min. After incubation, the beads were washed and 10 µL of beads from each wells were transferred to the fresh wells of a micro-titer plate containing MS2 aliquots (0 – 8 µg/ml, 10 µL). The mixture was incubated for 25 min, washed and re-suspended in 10 µL of PBS-R. Finally an aliquot of ‘anti-MS2 antibody conjugated to beta galactosidase’ (250 µg/ml, 15µl) was added to this suspension, incubated for 30 min, washed, and re-suspended in 30 µl of PBS-D. Detection was done by electrochemistry or fluorescence. Log-log plot was drawn for signal slope verses MS2 concentration.
An MS2 bacteriophage concentration of 2 µg/ml was selected because it is approximately at the middle of the calibration curve as shown in Section 5.1.1. The results in Figure 4 are reported as the ratio of interference reaction rate to the standard reaction rate in organic pure water. The ratios of reaction velocities were 0.92 ± 0.19 and 0.54 ± 0.07 and 0.57± 0.09 for MS2 in 2, 4 and 8 mg/l monochloramine (for 7 d incubation), respectively when compared against standard MS2 in organic pure water, run parallel to this assay. This proves that higher concentrations of monochloramine (4 and 8 mg/l) have negative effect on MS2 immunoassay, whereas signal is not affected significantly at lower concentration (2 mg/l).

5.1.3 Effect of monochloramine components on MS2 immunoassay

The effect of monochloramine on the MS2 immunoassay was examined in detail. The monochloramine sample contains free ammonia, ammonium chloride, and free chlorine. It is therefore important to separate these variables and determine whether the decrease in signal is only due to the monochloramine present in the solution, or if it is a combined or individual effect of the other components.

The monochloramine solution contained 11.5 mg/l of free ammonia (as nitrogen) and 0.2 mg/l of free chlorine when detected by the HACH meter. Also, the free ammonia concentration in NH$_4$Cl solution using the HACH meter was found to be 13.0 mg/l.

The results in Figure 5 are reported as the ratio of the interference reaction rate to the standard reaction rate in organic pure water. The ratios of reaction velocities were 0.59 ± 0.02 and 0.54 ± 0.07 for MS2 in 4 mg/l monochloramine and control ammonium chloride solution (for 7d incubation), respectively when compared against standard MS2 in organic pure water, run parallel to this assay. The effect of 0.2 mg/ml of chlorine on the MS2 (2 µg/ml)
Figure 4: The effect of various monochloramine concentrations on MS2 bacteriophage (7 d incubation): MS2 bacteriophage (2 µg/ml) was treated with different monochloramine concentrations (0, 2, 4 and 8 mg/l) and incubated for 7 d. After incubation, an equal volume of 2x PBS buffer was added to the MS2 bacteriophage in monochloramine and 1x PBS was added to standard sample (0 mg/ml of monochloramine). This modified MS2 bacteriophage (2 µg/ml, 10 µl) was added to the paramagnetic beads-antibody complex (6.8 x 10^8 beads/ml, 10 µl) and the immunoassay continued as described Figure 2.
Figure 5: Effect of free ammonia on MS2 assay: MS2 bacteriophage (2 µg/ml) was treated with ammonium chloride solution (13 mg/ml of ammonia), 0 mg/l (control) and 4 mg/l of monochloramine in organic pure water for 7 d, and the immunoassay proceeded as described in Figure 3.
immunoassay was shown previously in our laboratory to result in a reaction velocity ratio of 0.94 ± 0.07 (4), when compared to the standard (control) MS2 in organic pure water, treated in a similar manner. This indicates that a signal decrease in monochloramine can also be due to combined or individual effects of other residues present in the monochloramine solution, and variation in monochloramine preparation procedures (with different chlorine to nitrogen ratios) can affect reaction velocities to different extents.

The transfer of active chlorine from inorganic chloramine to amino acids or peptides is described in the literature (10). Other possible reasons for a decreased signal can be due to the modification of epitopes of the MS2 coat protein by forming organic chloramines on the epitopes of the protein. Epitope modification may decrease the affinity of the antibody for the antigen, thereby causing a decrease in signal.

**Conclusion**

The disinfecting agent monochloramine (4 and 8 mg/l) negatively affected MS2 (2 µg/ml) assay performance, when incubated for 7 d. In order to study this effect in detail, the effect of each variable of 4 mg/l of monochloramine (free ammonia, free chlorine and ammonium chloride) was separated. Incubation of MS2 bacteriophage (2 µg/ml) with free ammonia was found to affect the signal strength indicating that the signal decrease can be due to combined or individual effects of various residues present in monochloramine.

**References**


Chapter 6

Dissertation Summary
The first description of immunoassay dates back to the year 1959 (1). From that period till now, immunoassays have been constantly improvised and various different types of immunoassays, with numerous solid supports and antigens have been published (1). Heterogeneous immunoassay (requires solid support) is the most popular form of immunoassay due to low background, wide dynamic range and high specificity (2), resulting in higher sensitivity and lower detection limits. All of my projects dealt with heterogeneous immunoassays with streptavidin or NeutrAvidin™ coated solid supports.

While dealing with dendrimers (chapter 4) to immobilize NeutrAvidin™ on bead surface, I found the necessity for a method, which can quantitate the concentration of immobilized NeutrAvidin™. After getting inconsistence results with HABA assay (data not shown), first project was developed to fulfill the need for a reliable method, which can estimate the biotin-binding capacities of NeutrAvidin™ coated beads. The project was further extended to determine the biotin binding capacities of other solid supports such as streptavidin-coated particles (blue colored), micro-titer plates and beads. It was a simple, reproducible and economical method with optimized incubation time of only 5 min. A spectroscopic method for the measurement of biotin binding sites of immobilized avidin using HABA assay is published in the literature (3). NeutrAvidin™ is a deglycosylated avidin derivative with preserved biotin binding sites (4, 5). Due to the structural similarities of avidin with that of NeutrAvidin™ and streptavidin, b-4-f technique (till now used for of immobilized NeutrAvidin™ and streptavidin) may also be extended in future to determine the biotin binding capacities of immobilized avidin. Application of b-4-f method to detect free (unbound) avidin in the solution is already published by Kada et al. (6).
While working with immunoassays, it was observed that bead saturation study using secondary antibodies conjugated to enzyme was time consuming and required numerous optimization and washing steps. Washing steps were especially tedious in case of non-magnetic solid supports since they needed centrifugation and careful pipeting of supernatants (7) (to avoid loosing particles). Also, there were situations when secondary antibody conjugates were not available for given biotinylated antibodies. This method could not be generalized for any other biotinylated ligands, and stability of the enzyme was a concern. Moreover there is a continuous emergence of immobilization techniques and scientific evaluation of various immobilization techniques using different methods is tedious (8). Thus a novel method was developed using b-4-f, to detect the capacity of immobilized biotinylated ligands per unit surface area for a wide range of solid supports. This b-4-f method was successfully tested on - streptavidin and NeutrAvidin™ coated beads, streptavidin coated polystyrene blue particles and streptavidin coated mirco-titer plates. The method was also used to determine the unknown concentration of biotinylated ligand in solution, by plotting a calibration graph with standards for the same ligand using the ‘streptavidin high binding capacity coated plate’ as a solid support and biotinylated antibody as a model ligand. This gave a linear range of 0.07 – 1.2 µg/well (4 nM - 65 nM) and a detection limit of 0.5 nM.

Avidin is a streptavidin homologue exhibiting resemblance in structural properties with that of streptavidin (9, 10). Determination of ligand binding capacity of immobilized streptavidin and NeutrAvidin™ is shown in this project. Due to similar biotin binding properties of avidin with that of streptavidin (9, 10) and avidin derivative with preserved biotin binding sites - NeutrAvidin™ (4, 5); similar method may be applied to determine the ligand binding capacity of immobilized avidin.
While determining the linear range and detection limit for unknown biotinylated ligand using immobilized avidin, optimization with BSA or other blocking agent is needed since avidin suffers from higher non-specific binding due to the presence of carbohydrate and higher pI (10.5) than streptavidin (pl: 5-6), (11). Higher non-specific binding may increase the detection limit of the assay as compared to streptavidin as a solid support, when all the other conditions are kept constant.

In this project biotinylated antibodies are used as a model system. Since method is based on direct binding of b-4-f to free binding sites of immobilized streptavidin/NeutrAvidin™, same method may be applied to a wide variety of biotinylated ligands. Results may vary depending on the size of solid support, steric hindrance, size and chain length of biotinylated ligands etc. The effect of different sizes of biotinylated DNA on binding capacity of streptavidin coated solid support is shown by Huang et al. (12). It was found that binding capacity of DNA decreases with increase in DNA chain length (12).

The third project was to study the effect of PAMAM dendrimer on the binding capacity of a solid support for biotinylated ligands. This is an open-ended project and can be extended to various levels. For example, it will be exciting to do molecular simulation to visualize the three dimensional geometrical arrangement of NeutrAvidin™ and biotinylated antibodies on the dendrimer coated beads, or to study the effect of different sizes and shapes of antigens on the sensitivity and detection limits of dendrimer coated beads. The comparison of the sensitivities and detection limits of various generations of dendrimer-coated beads vs. plain beads would be another exciting project.
The fourth project was comparatively simple and was used to study the effect of various monochloramine concentrations on the immunoassay sensitivity of MS2 bacteriophage. Monochloramine concentrations of 4 and 8 µg/l on 7 d incubation with MS2 bacteriophage affected the sensitivity to a significant level. Also both the monochloramine components - free ammonia and free chlorine individually affected the signal strength indicating that the signal decrease can be due to combined or individual effects of various residues present in monochloramine. One of the possible reasons for signal decrease can be due to the transfer of active chlorine from inorganic chloramine to amino acids or peptides (13). Direct interactions of these ‘modified’ organic chloramines with that of substrates used for detection can be studied in future using fluorescence and electrochemistry to determine whether these interactions are resulting in signal decrease. Another possible reason for signal decrease may be that due to the monochloramine modified ‘epitope’ of the MS2, affinity of antibody for MS2 can decrease to a significant level. In order to test this possibility, SPR spectroscopy can be used to study the binding affinity of ‘modified’ (monochloramine treated) MS2 and antibody verses pure MS2 and antibody.

The application of first two projects is to use the b-4-f method as a standard protocol for comparing the wide range of streptavidin and NeutrAvidin™ immobilization techniques, determining biotin binding sites and total number of ligands per unit surface area of the solid support, and quantitating unknown concentration of biotinylated ligands. The streptavidin or NeutrAvidin™ solid supports to which method is applicable includes but not limited to magnetic, non-magnetic, colored, colorless, spherical, plane or particulate types. The relevance of dendrimer project may comprise the use of dendrimer as a polymer linker to increase the effective concentration and hence efficiency of biotinylated ligands on various solid supports.
useful for immunoassays, immunodiagnostics, protein purifications, etc. Monochloramine project is more of an academic oriented project, which is a step towards understanding the mechanism of decrease in immunoassay sensitivity on treatment of MS2 bacteriophage with oxidizing disinfectant Monochloramine.

References


