Abstract: Bioaffinity interactions have been, and continue to be, successfully adapted from nature for use in separation and detection applications. It has been previously reported that the magnetophoretic mobility of labeled cells show a saturation type phenomenon as a function of the concentration of the free antibody–magnetic nanoparticle conjugate which is consistent with other reports of antibody–fluorophore binding. Starting with the standard antibody–antigen relationship, a model was developed which takes into consideration multivalence interactions, and various attributes of flow cytometry (FCM) and cell tracking velocimetry (CTV) measurements to determine both the apparent dissociation constant and the antibody-binding capacity (ABC) of a cell. This model was then evaluated on peripheral blood lymphocytes (PBLs) labeled with anti CD3 antibodies conjugated to FITC, PE, or DM (magnetic nanoparticles). Reasonable agreements between the model and the experiments were obtained. In addition, estimates of the limitation of the number of magnetic nanoparticles that can bind to a cell as a result of steric hinderance was consistent with measured values of magnetophoretic mobility. Finally, a scale-up model was proposed and tested which predicts the amount of antibody conjugates needed to achieve a given level of saturation as the total number of cells reaches $10^{10}$, the number of cells needed for certain clinical applications, such as T-cell depletions for mismatched bone marrow transplants.

Keywords: magnetic cell separation; magnetic nanoparticles; dissociation constant; antibody affinity

INTRODUCTION

Bioaffinity interactions are successfully adapted from nature for use in separation and detection applications that range from affinity chromatography to detection technology for human diagnostics, to the most recent applications in biowarfare detection (Polanowski et al., 2003; Safar et al., 2002; Zhou et al., 2002). A fundamental measure of the performance of a bioaffinity interaction is the strength of the non-covalent interactions between the receptor–ligand (i.e., single antibody-antigen or multiple ligand in the case of avidin-biotin) referred to as the affinity of the interaction (Goldsbey et al., 2000). When antibodies are used to bind to cells, typically, complex antigens are present with multiple repeating antigenic determinants resulting in multiple binding sites for the antibody. The presence of these multiple binding sites can have a cooperative effect on the binding of the antibody to the cell. In addition, the typically used immunoglobulin G (IgG) is a dimer, with each monomer having a single binding site for the antigenic determinants. Consequently, the strength of such multiple interactions is referred to as avidity of the antibody–antigen interaction (Goldsbey et al., 2000).

The binding constants of the bioaffinity interactions substantially define the performance of affinity-based separation and diagnostic/detection systems. Consequently, modifications in the magnitude of these parameters can have significant effects on the performance and economics of the technology (Comella et al., 2001; McCloskey et al., 2003b). Over the years, a number of labels have been used to covalently link to antibodies or other affinity molecules to assist in the detection of binding; examples of these labels include radioactive atoms, fluorescent molecules, gold nanoparticles, magnetic nano and microparticles (Benedict et al., 1997; Bobrovnik, 2003; Friguet et al., 1993). Magnetic particles are typically made of magnetically susceptible materials embedded in a polymer material (Safarik and Safarikova, 1999). Despite the similar structure, the commercially available magnetic particles differ significantly in size: from the size of a cell, on the order of 4.5 microns (i.e., Dynabeads® M-500 particles, Dynal AS, Oslo, Norway), to a molecular size not much bigger than an antibody, on the
order of 50–100 nanometers, (MACS™ particles, Miltenyi Biotech, Bergisch Gladbach, Germany). Finally, non-commercial, research-based particles include magnetoferritin, which is on the order of 12 nanometers (Zborowski et al., 1996).

While magnetic micrometer sized particles have been in use for over 20 years, the more recent advent of magnetic nanoparticles (one of the more popular is the 50–100 nm MACS™ particles (Miltenyi Biotech)) has significantly increased the popularity of immunologically based magnetic separations (Buck et al., 2003; Despres et al., 2000; Hu et al., 2003; Thiel et al., 1998). A number of advantages exist for the use of magnetic nanoparticles over magnetic microparticles, not the least of which is the colloidal behavior of antibody–nanoparticle conjugates. While the very small size of the magnetic nanoparticles requires a significant number of antibody–nanoparticle conjugates to be bound before a separation can be accomplished (unlike the binding of a single, magnetic microbead), it has been demonstrated that over specific ranges, the magnetic force that is imparted on cells labeled with these antibody–nanoparticle conjugates (when the cells are placed in a magnetic energy gradient) is proportional to the antibody-binding capacity (ABC), of the cell (McCloskey et al., 2000).

This experimentally observed proportionality between the imparted magnetophoretic mobility, m, and the cells’ ABC presents the possibility of separating cells not simply in a binary mode of operation but also based on the surface expression level of the cell (Chalmers et al., 1998; McCloskey et al., 2003a). In addition, it was observed that the magnetophoretic mobility of immunomagnetically labeled cells is a saturation type function of the concentration of the antibody–nanoparticle conjugates used to label the cells (Chosy et al., 2003; Comella et al., 2001). While a saturation type relationship is theoretically expected, based on typical antibody–antigen interactions, the scale-up of immunomagnetic cell separations for clinical applications may require a considerable amount of antibody which can be financially limiting. Significant experimental evidence has demonstrated that the performance of both the commercial MACS systems as well as the flow through technology being developed in our laboratories is a clear function of the magnetophoretic mobility of the labeled cell (Chosy et al., 2003; Comella et al., 2001; McCloskey et al., 2003a).

This manuscript focuses on the application of a Langmuir-binding model to explain and quantify the binding and binding constants of antibody–antigen and biotin–streptavidin (SA) interactions when one of the binding entities is covalently bound to either a fluorescence dye or a magnetic nanoparticle. Finally, using these relationships, a scale-up model is proposed to guide the development of protocols for large volume, clinical samples.

**THEORY**

If one assumes monovalent binding, at equilibrium the interaction of a receptor–ligand complex can be expressed by:

\[
R + \frac{k_a}{k_d} RL
\]

where \(k_a\) and \(k_d\) are the rate constants for the association and dissociation reaction, and [R], [L], and [RL] are the concentrations of free receptors, free ligands, and receptor–ligand complexes at equilibrium, respectively. Two constants are widely used to characterize the strength of this interaction: an equilibrium dissociation constant, \(K_D\), and an equilibrium association constant, \(K_A\), given by Equation 2:

\[
K_D = \frac{k_d}{k_a} = \frac{[R][L]}{[RL]} = \frac{1}{K_A}
\]

It is generally reported that \(K_D\) is a quantitative indicator of the stability of receptor–ligand interactions, with low values representing stable (strong) interactions and high values representing weak interactions (Midelfort et al., 2004). Published values of \(K_D\) can range from \(10^{-15}\) for Avidin-Biotin to \(10^{-7}\) to \(10^{-11}\) for antibody antigen binding (Garcia et al., 1999). It should be noted that the dissociation constant, \(K_D\), defined in Equation 2, is based on the concept of thermodynamic equilibrium for species suspended in a homogeneous solution (Goldberg and Djavadi-Ohanian, 1993).

In contrast, when an antibody, or the ligand for a specific antibody, is bound to a solid surface or support (such as cells or ELISA plate), the assumption of freely suspended antibodies, ligands, and antibody–ligand conjugates is not valid. Consequently, techniques such as the classical Scatchard analysis are not necessarily valid. This observation is well documented by Bobrovnik (2003) and Underwood (1993).

Given the added complexity of such heterogeneous phase systems, such as the binding of an antibody to the antigen on a cell surface or on an ELISA plate, a typical approach is to use the term “apparent binding constants,” which may not have values close to the true binding constants obtained in suspension. This apparent binding constant takes into consideration the potential of antibody valence, steric hindrance, or other non-ideal effects (Goldberg and Djavadi-Ohanian, 1993). In order to determine this apparent binding constant, the following model was constructed and an experimental analysis was conducted.

**Binding Model**

Taking the antibody–antigen interaction as an example, when an antibody conjugate binds to a cell, at least five scenarios can occur: (A) monovalent binding, (B) homogeneous bivalent binding, (C) multiple antibodies binding to a single antigen, (D) heterogeneous bivalent binding, and (E) cross-linked binding. Figure 1 presents examples of each of these five cases. This complexity is partially the result of the possibility of multiple binding epitopes per single antigen on the cell surface. This complexity of multiple epitopes per
antigen was well summarized by Davis et al. (1998), and will be discussed in more detail below.

With respect to the five scenarios presented in Figure 1, in the following discussion we will only consider scenarios A, B, and C. The dissociation constant for scenario A is given by:

\[
K_{D1} = \frac{[\text{Ag}][\text{Ab}]}{[\text{Ag} \cdot \text{Ab}]} \tag{3}
\]

and potentially for scenario B and C:

\[
K_{D2} = \frac{[\text{Ag}][\text{Ag} \cdot \text{Ab}]}{[\text{Ag} \cdot \text{Ab}]} \quad \text{and} \quad K_{D3} = \frac{[\text{Ab}][\text{Ag} \cdot \text{Ab}]}{[\text{Ab} - \text{Ag}]} \tag{4}
\]

It should be noted that \([\text{Ag}],[\text{Ab}],\) and \([\text{Ag} \cdot \text{Ab}]\) represent the concentrations of free antigens, free antibodies, and bound antibody–antigen complexes.

A mass balance can be written for scenario A for both the antibody and the antigen:
\[ [\text{Ag}]_{\text{Total}} = [\text{Ag}] + [\text{Ag} \cdot \text{Ab}] \quad (5) \]

If one were to substitute Equation 3 into Equation 5 and solve for \([\text{Ag} \cdot \text{Ab}]\), one would obtain:

\[ [\text{Ag} \cdot \text{Ab}] = \frac{[\text{Ab}][\text{Ag}]_{\text{Total}}}{K_D^1 + [\text{Ab}]} \quad (6) \]

Finally, dividing both sides of Equation 6 by \([\text{Ag}]_{\text{Total}}\), one obtains the classical form of the Langmuir Isotherm:

\[ \theta = \frac{[\text{Ab}]}{K_D^1 + [\text{Ab}]} \quad (7) \]

where \(\theta\) corresponds to the fraction of the total surface antigen sites bound with antibody.

For scenarios B and C, the situation becomes more complex. For scenario B:

\[ [\text{Ag}]_{\text{Total}} = [\text{Ag}] + [\text{Ag} \cdot \text{Ab}] + 2[\text{Ag} \cdot \text{Ab} \cdot \text{Ag}] \quad (8) \]

and for scenario C:

\[ [\text{Ag}]_{\text{Total}} = [\text{Ag}] + [\text{Ag} \cdot \text{Ab}] + [\text{Ab} \cdot \text{Ab} \cdot \text{Ag}] \quad (9) \]

When one uses either a flow cytometer (FCM) or cell tracking velocimeter (CTV) to quantify the fluorescence intensity (FI) or the magnetophoretic mobility of a cell labeled with an antibody–fluorochrome conjugate, or an antibody–magnetic nanoparticle conjugate, respectively, one is not able to distinguish between \([\text{Ag} \cdot \text{Ab}]\) and \([\text{Ag} \cdot \text{Ab} \cdot \text{Ag}]\). In contrast, in the case of \([\text{Ab} \cdot \text{Ag} \cdot \text{Ab}]\), if the number of Ag is known, in an ideal case, the maximum number of antibodies determined by FCM or CTV would be double that of Ag. Consequently, the term [complex] is introduced for scenario B:

\[ [\text{complex}] = [\text{Ag} \cdot \text{Ab}] + [\text{Ag} \cdot \text{Ab} \cdot \text{Ag}] \quad (10) \]

and for scenario C:

\[ [\text{complex}] = [\text{Ag} \cdot \text{Ab}] + 2[\text{Ab} \cdot \text{Ag} \cdot \text{Ab}] \quad (11) \]

Next, the concept of valence, \(\lambda\), is introduced for scenario B:

\[ \lambda = \frac{[\text{Ag} \cdot \text{Ab}] + [\text{Ag} \cdot \text{Ab} \cdot \text{Ag}]}{[\text{Ag} \cdot \text{Ab}] + 2[\text{Ag} \cdot \text{Ab} \cdot \text{Ag}]} \quad (12) \]

and for scenario C:

\[ \lambda = \frac{[\text{Ag} \cdot \text{Ab}] + 2[\text{Ab} \cdot \text{Ag} \cdot \text{Ab}]}{[\text{Ag} \cdot \text{Ab}] + [\text{Ab} \cdot \text{Ag} \cdot \text{Ab}]} \quad (13) \]

Inspection of Equations 12 and 13 indicates that if all the antibodies bind to the cell in a monovalent nature (Scenario A), the concentration of \([\text{Ag} \cdot \text{Ab} \cdot \text{Ag}]\) or \([\text{Ab} \cdot \text{Ag} \cdot \text{Ab}]\) is 0 and the valence is 1. In contrast, if all the antibody binding is of the homogeneous, bivalent nature (Scenario B), then \([\text{Ag} \cdot \text{Ab}]\) is 0 and the valence is 0.5, while for Scenario C if all of the antigen binding is bivalent, the valence is 2.

There is experimental precedent for the concepts represented in Scenarios A and B, with respect to Langmuir binding behavior, and the corresponding valence term, \(\lambda\) (Chosy et al., 2003; Davis et al., 1998; Siiman and Burshteyn, 2000). In the study conducted by Davis et al. (1998), it was experimentally demonstrated that either Scenario A, B, or a combination of A and B can occur depending on the specific antibody clone targeting the CD4 receptor and whether the receptor is on a cell or a polystyrene bead.

Figure 2A–D are plots of some of the data from Davis et al. (1998). Specifically, the reported FI as a function of the concentration of the antibody clone (nM), or a Fab fragment of the clone used to label either a cell suspension or recombinant CD4-poly styrene conjugates, rCD4s beads, is presented.

While the actual data points in this figure are from the publication of Davis et al. (1998), the lines were developed for this present publication using a non-linear, hyperbola, single rectangular, two-parameter model (i.e., this is a model of the form of Equation 6). It is assumed that the equilibrium concentration of free antibody is not significantly different from the total antibody concentration. The validity of this assumption depends on the relative abundance of antigen sites (proportional to number of cells present) and antibodies added. This will be addressed later with regard to the experiments reported in this work. Using this model, the value of “\(a\)” corresponds to the maximum FI and “\(b\)” corresponds to \(K_D^1\) of the antibody. Consistent with the analysis by Davis et al., using other experimental, calibration techniques, the Fab fragment of Leu 3a bound twice as many antigen sites (FI of 3,330 vs. 1,670) on the cells as did the divalent form. In addition, the dissociation constant, \(K_D^1\), is significantly different; \(8.97 \times 10^{-10} \text{M}\) and \(1.33 \times 10^{-10} \text{M}\) for Fab and mAb, respectively, indicating a 6.7-fold difference in binding affinity between the monovalent and bivalent form of the antibody. In contrast, the Fab fragment of L120 only bound 1.5 times as many antigen sites (FI of 3,030 vs. 2,020); however, as with the Leu 3a clone, \(K_D^1\) is significantly different; \(1.0 \times 10^{-8}\) and \(7.6 \times 10^{-9}\) for Fab and mAb, respectively, indicating a 1.3-fold difference in binding affinity.

Of equal interest are the studies of the binding of these same four mAb and Fab molecules to polystyrene beads to which recombinant CD4 molecules were conjugated. Unlike the cells, there is very little difference between the total number of bivalent and Fab molecules binding to the rCD4 beads indicating only monovalent binding. Consistent with this monovalent binding is the similarity in binding affinities (\(K_D\) values in Fig. 2) which are very similar (ranging from 2.5 to \(7.4 \times 10^{-9} \text{nM}\)). Davis et al. (1998) further suggest that the difference in valence and \(K_D\) values when binding to a lymphocyte in contrast to a CD4-conjugated polystyrene
bead is a potential function of the availability of the epitopes of the target antigen in the cell membrane and the fluid mobility of the antigen within the cell membrane. They also noted that they did not observe any effect of the concentration of the surface density of the antigen on the monovalent versus bivalent binding.

These two examples demonstrate the variability of the valence as well as the dissociation constant on a variety of factors which can not be predicted from first principles. Consequently, it may be shown that Equation 6 should be modified for the case represented in Scenarios B and C to be:

\[
\frac{1}{2} \frac{\text{Ag}}{C_1} \frac{\text{Ab}}{C_1} + \frac{1}{2} \frac{\text{Ag}}{C_1} \frac{\text{Ab}}{C_1} = \frac{1}{2} \frac{\text{Ab}}{C_1} \frac{l}{a} K_D + \frac{1}{2} \frac{\text{Ab}}{C_1} \frac{1}{b}
\]

(14)

and in a normalized form:

\[
\theta = \frac{[\text{Ab}]^l}{x K_D + [\text{Ab}]^l} \tag{15}
\]

where \( \lambda \), as defined above, is the valence of the antibody binding and the term \( x \) is an experimentally determined constant. It should be noted that most likely, \( x \) is a function of \( \lambda \) as well as the availability of the specific epitopes in cell membrane and membrane fluidity.

**Measurement of Receptor–Ligand Interactions**

In addition to determining, qualitatively, the presence of a surface marker on a cell, flow cytometry (FCM) can be used to determine the binding affinity between cellular receptors and fluorescently labeled ligands (Benedict et al., 1997) as well as quantification of the number of antigens on the surface of a cell. The amount of ligand receptor complexes
formed per cell can be quantified using the measured FI of the cell. According to Schwartz et al. (1996), the FCM’s response to fluorescence signal (FI) can be described by:

\[ FI = e \cdot \log_{10}[Ab \cdot Ag] + FI_{bg} \]  \hspace{1cm} (16)

if logarithmic amplification is used (typical of instruments produced in the past), or

\[ FI = e \cdot [Ab \cdot Ag] + FI_{bg} \]  \hspace{1cm} (17)

if linear amplification is used (a practical choice on most new instruments sold currently). In these relationships, FI is the fluorescence intensity of a labeled cell at a certain wavelength, in arbitrary units, and \( e \) is the conversion factor between FI and the amount of antibody–antigen complexes formed per cell. \( e \) is a function of a number of variables including the instrument setting, the laser intensity, the photomultiplier voltage, as well as intrinsic characteristics of the fluorescent dyes. \( FI_{bg} \) is the background FI of unlabeled cells which is often referred to as cellular autofluorescence. Autofluorescence is a strong function of the excitation wavelength, the detection wavelength, the type of cell as well as the source of the cells. This autofluorescence is most pronounced in the emission wavelength of two of the more commonly used fluorescent dyes, fluorescein isothiocyanate (FITC), and phycoerythrin (PE) (Viksman et al., 1994).

As an alternative to fluorescent labels, the number of antibody–antigen complexes formed can also be quantified by magnetophoretic mobility measurements if the antibody is conjugated to magnetic nanoparticles. Magnetophoresis is the motion of particles in a viscous fluid under the influence of a magnetic energy gradient. Previous work in our laboratories has demonstrated that a relationship can be written, and experimentally verified over a specific range of conditions, for the magnetophoretic mobility, \( m \), of an immunomagnetically labeled cell or micro particle (McCloskey et al., 2003b):

\[ m = \frac{ABCn_2\Delta \chi_{nano}V_{nano}}{3\pi D_c \eta} = \frac{ABCn_2 \phi_{nano}}{3\pi D_c \eta} = \beta ABC \phi_{nano} \]  \hspace{1cm} (18)

\[ ABC = n_1 \theta_1 \lambda_1 \]  \hspace{1cm} (19)

\[ \beta = \frac{n_2}{3\pi D_c \eta} \]  \hspace{1cm} (20)

where ABC is the antibody-binding capacity, \( n_2 \) is the number of magnetic nanoparticles conjugated to the antibody, \( \phi_{nano} \) is the field interaction parameter of the magnetic nanoparticle which is the product of the difference in volumetric magnetic susceptibility of the nanoparticle and the suspending fluid, \( \Delta \chi \), and the volume of the particle, \( V_{nano} \). Further, \( D_c \) is the diameter of the cell, and \( \eta \) is the viscosity of the suspending fluid. ABC can be further defined as the product of \( n_1 \), the number of antigen sites per cell, \( \theta_1 \), the fraction of the antigen sites on the cell surface bound (degree of saturation), and \( \lambda_1 \), the valence of the antibody as defined previously.

Equation 18 neglects the contribution of the unlabeled cell to the magnetophoretic mobility (the intrinsic magnetophoretic mobility), since it is assumed that cells have a very small or negligible intrinsic magnetophoretic mobility. While it is true for most types of cells suspended in buffer (mainly phosphate buffered saline (PBS)), it has been reported that red blood cells show different intrinsic magnetic properties as a result of the oxygenation state of hemoglobin (Zborowski et al., 2003). In addition, Moore et al. (2004) successfully used paramagnetic salts to modify the magnetic property of the suspending buffer to obtain higher separation resolution, in which case, this assumption is no longer true. To account for the contribution of unlabeled cells to the magnetophoretic mobility, Zhang et al. (2005) modified Equation 18 in a manner analogous to fluorescence labeling:

\[ m = (\beta \cdot \phi_{nano})ABC + m_{bg} \]  \hspace{1cm} (21)

where the intrinsic magnetophoretic mobility of a cell is given by:

\[ m_{bg} = \frac{\Delta \chi_{cell} V_{cell}}{3\pi \eta D_c} \]  \hspace{1cm} (22)

Unlike FI measurements, however, measurements of \( m \) are reported in specific units, such as \( \text{mm}^3/T\cdot\text{A} \cdot \text{s} \). Therefore, if the product \( \beta \cdot \phi_{nano} \) and \( m_{bg} \) are known, Equation 21 indicates that the measurement of \( m \) allows one to determine the value of ABC.

In summary, the analogy between FI and magnetophoretic mobility, \( m \), allows one to estimate the equilibrium dissociation constant of an antibody–magnetic nanoparticle conjugate in a manner analogous to the use of FI measurements from FCM. The remainder of this manuscript will present the experimental procedures used to prepare, conduct, and analyze the labeling studies of antibody–fluorophore and antibody–magnetic nanoparticle conjugates and corresponding procedure used to estimate the equilibrium dissociation constants. Finally, the implication of this constant on the scale-up of a process for clinical applications will be presented and discussed.

**MATERIALS AND METHODS**

**Preparation of Peripheral Human Blood**

Buffy coat prepared from a 500 mL sample of blood drawn from a healthy donor was purchased from the American Red Cross (Columbus, OH) (Approved by OSU IRB). Theseuffy coats were diluted with Hanks balanced salt solution (HBSS) (IRB Biosciences, Lenexa, KS) at the ratio of 1:2. Diluted blood (22.5 mL) was carefully layered over 17.5 mL of Accu-Prep lymphocytes Ficoll (1.077 g/mL) density
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Magnetic Labeling of Biotinylated Microspheres

Two types of commercial, magnetic nanoparticles bound to SA were used: MACSTM particles (Catalog number 481-01/02; Miltenyi Biotec, Auburn, CA) and BD IMagTM-SA particles (Catalog number 551308, BD Pharmingen). SA is coated on the outer surface of both nanoparticles, which consist of an iron oxide core coated with a polysaccharide. The MACS beads are reported by the manufacturer to be 50 nm in diameter. The BD IMagTM particles are reported by the manufacturer to be on the order of 200 nm and by Zhang et al. (2005) to be 231 nm in diameter. The SA-MACS particles are supplied in a suspension of 0.1% gelatin with 0.05% NaN3, and the BD SA nanoparticles are suspended in aqueous buffered solution containing 0.09% NaN3, 0.5% BSA, and 2 mM EDTA.

Magnetic Labeling of PBLs

BD IMagTM mouse anti-human CD3 (clone # HIT3a) nanoparticle conjugates, anti-CD3-DM, were used to label PBLs. PBLs were suspended in PBS at the concentration of 1.0 \times 10^6 cells per 100 \mu L. Cells were incubated with an appropriate amount of antibodies at 4°C for 30 min to ensure that equilibrium was attained. Cells were then washed with PBS, and centrifuged at 350g, 4°C for 5 min. Supernatants were carefully taken without disturbing pellets for further analysis. Pellets were then resuspended at the concentration of 0.5 \times 10^6 cells per milliliter of labeling buffer to ensure that no significant cell–cell interaction is involved (Zhang et al., 2005). The measurement of magnetophoretic mobility was performed using CTV.

Cell Fluorescence Intensity Measurements

The measurement of FI was performed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA), and the data were analyzed using WinMDI version 2.8. This FCM uses logarithmic amplification as described by Equation 16. Quantification of the FI signals were obtained through the use of QuantiBRITE PE beads (BD Biosciences, Catalog # 340495) following the manufactures protocol. Each package of QuantiBRITE PE beads contains a lyophilized pellet of four subpopulations of beads, each subpopulation has different amount of PE molecules conjugated to the bead surface.

Magnetophoretic Mobility Measurements

Magnetophoretic mobility measurements were performed on an instrument referred to as a CTV, discussed previously (Chalmers et al., 1999; Zhang et al., 2005). Briefly, CTV measures the magnetically induced movement of cells or particles, on a cell-by-cell basis, in a well-defined magnetic field. Movements of cells or particles in this area are captured and recorded using an inverted microscope and a 30 Hz Cohu CCD 4915 camera (Cohu Electronics, San Diego, CA). The images are digitized into a two dimensional matrix of 640 by

gradient (Accurate Chemical and Scientific Corp., Westbury, NY) in a 50 mL conical tube (Fisher Scientific, Pittsburgh, PA), and centrifuged at 400g and room temperature with brake off for 30 min. After centrifugation, the mononuclear cell layer was carefully removed from the interface of plasma and Ficoll layer, washed several times by centrifugation at 350g, 4°C for 5 min with a solution consisting of Dulbecco’s phosphate buffered saline (DPBS modified; JRH Biosciences), 2 mM ethylene-diamine tetraacetic acid (EDTA; Sigma, St. Louis, MO), and 0.5% fraction V bovine serum albumin (BSA; Sigma). Pellets were then resuspended in labeling buffer, and the cell concentration was determined using a Coulter® Multisizer II (Coulter Electronics Limited, Luton, UK). The cells were resuspended in medium, RPMI-1640 (ATCC, Manassas, VA), and 10% Fetal bovine serum (FBS; JRH Biosciences), and cultured in T-75 flasks (Fisher Scientific) at 37°C, 5% CO₂ to deplete adherent monocytes. After 48 h of culture, the medium was carefully removed from the flasks, and Ficoll density separation was performed again to further enrich for the lymphocytes as previously described. After centrifugation, the lymphocyte layer was carefully removed and washed in labeling buffer two times. The resulting pellet was then resuspended in labeling buffer and analyzed on the Coulter® Multisizer II for the purity and concentration of lymphocytes, which are subsequently referred to as peripheral blood lymphocytes (PBLs).

Biotinylated Microspheres

ProActive® biotin-coated microspheres (catalog number CP10N; Bangs Laboratories, Fishers, IN) are reportedly uniformly sized polystyrene microspheres, 5.1 \mu m in diameter, with a standard deviation of 0.1 \mu m. These microspheres come suspended in 100 mM Borate (pH 8.15), 0.1% BSA, 0.05% Tween 20, 10 mM EDTA, and 0.1% NaN3. These microspheres come supplied in a suspension of 0.1% gelatin with 0.05% NaN3, and the BD SA nanoparticles are suspended in aqueous buffered solution containing 0.09% NaN3, 0.5% BSA, and 2 mM EDTA.

Fluorescent and Magnetic Labeling of PBLs

Mouse anti-human CD3 antibodies (clone # HIT3a) conjugated to PE, FITC, or BD IMagTM magnetic nanoparticles from BD Biosciences Pharmingen (San Jose, CA) were used to label the PBL. Specifically, the anti-CD3-PE and anti-CD3-FITC antibodies (Cat # 555340, Lot # 000054407 and Cat # 555339, Lot # M076161, respectively), were reported by the manufacturer to have antibody concentrations of 1.5 \mu g/mL and 50 \mu g/mL, respectively. The anti-CD3-Imag nanoparticles (Lot number 000056357) did not report a protein concentration. Measurements of this concentration were performed in this study and reported below.

PBLs were suspended in PBS at the concentration of 1.0 \times 10^6 cells per 100 \mu L. Cells were incubated with appropriate amount of antibodies at 4°C for 15 min to ensure that the binding equilibrium was reached. Cells were washed with PBS, by centrifugation at 350g, 4°C for 5 min. Pellets were then resuspended at the concentration of 1.0 \times 10^6 cells per milliliter of fixing solution, 2% paraformaldehyde (Mays Chemical, Indianapolis, IN) in PBS.
480 pixels by a µTech image grabbing board (MuTech Corp., Billerica, MA). As a result of continuing improvements, this system is currently semi-automated and an analysis of over 1,000 cells or particles can be performed within 20 min.

Detection of Free Antibody Concentration in Suspension

As reported above, in commercial anti-human CD3 magnetic particle samples, such as BD IMag™ anti-human CD3 particles (anti-CD3-DM), another protein, usually gelatin or BSA is added to the suspension to stabilize the antibody coated on the particle surface. In an attempt to measure the actual concentration of the antibody–nanoparticle conjugate, or SA–nanoparticle conjugate, in equilibrium with the antibody–nanoparticle conjugate which is bound, gel filtration was performed on the equilibrium supernatant before a total protein assay was performed. Two types of gel filtration columns were used: PD-10 column (Amersham Biosciences, Piscataway, NJ) for the smaller, SA-MACS™ particles and a Sepharose™ CL-4B column for the relatively larger BD IMag™ particles.

Figure 3. Dot plots of forward and side light scattering for peripheral blood lymphocytes obtained from Ficoll density gradient (A), after 24 h culture (B), after 48 h culture (C), and Ficoll density gradient after 48 h culture (D). In (A), the gated region R1 corresponds to viable lymphocytes, region R2 monocytes and macrophages, region R3 is cell debris, and region R4 contains dead cells. Note progressive increase in density in R1 region and subsequent decrease in other three regions (gates).
The actual operation of the gel column consisted of introducing 700 μL PBS buffer to the CL-4B or PD-10 column. Next, 350 μL of the sample to be analyzed was added, and then 5,950 μL of PBS. A total of 20, sequential, 350 μL eluant samples were collected. Each eluant was then analyzed for protein concentration using Micro BCA total protein assay (Pierce Biotechnology, Rockford, IL) and Spectra MAX 250 (Molecular Devices, Sunnyvale, CA) in duplex following the manufacturer’s recommendation.

Curve Fitting of Data

Two approaches were used to analyze the binding data to determine the binding constants: traditional double reciprocal plots and the use of the Single Rectangular 2 Parameter Hyperbola model:

\[ y = \frac{ax}{b + x} \]  

with the SigmaPlot 9.0 software (SPSS, Inc., Chicago, IL). This best fit algorithm assumes each data point is equally weighted unlike the double reciprocal plot (the 2nd approach), which places disproportional weight on the low concentration data points. In both cases, the concentration of free antibody is assumed to be not significantly different from the total concentration of added antibody. The validity of this assumption is addressed later.

RESULTS

The experimental results of this study will be presented in the following order: (1) demonstration of the performance of the purification process of lymphocytes which were subsequently used in binding studies, (2) representative elution profile and calibration curve used in the determination of antibody concentration, (3) saturation curves of the various antigen–antibody combinations, and (4) experimental and theoretical implications of the equilibrium dissociation constants.

Enrichment of Lymphocytes From Mononuclear Layer Obtained After Ficoll Density Separation

To obtain accurate measurements of the binding constants of specific receptors on lymphocytes, it is highly desirable to have a pure cell suspension. While the buffy coat purchased from the Red Cross contains enriched leukocytes (relative to whole blood), there is still significant contamination of cell debris, erythrocytes, and monocytes. To further enrich for lymphocytes, Accu-prep, Ficoll density gradient separations as well as 48 h monocyte depletion cultures were performed on buffy coats.

Figures 3 and 4 present FCM and Coulter Counter analysis of a typical blood sample at the various stages of enrichment, respectively, and Table I summarizes the results. Figure 3 presents the progression in the increase in purity: Ficoll after obtaining from the Red Cross (Fig. 3A), 24 h of culture (Fig. 3B), 48 h of culture (Fig. 3C), and after a final Ficoll separation (Fig. 3D). A similar progression in purity is shown in Figure 4, from the initial Ficoll after obtaining from the Red Cross (red line), 24 h of culture (green line), and 48 h of culture (blue line). Quantitatively, as presented in Table I, FCM and Coulter Counter analysis presents similar increases in purity from 13–16% to 78–80%.

Histograms of Lymphocytes Labeled With FITC, PE, and Magnetic Nanoparticles

Figure 5A and B present histograms of the FI of purified lymphocytes labeled with anti-CD3-FITC and
anti-CD3-PE-conjugated antibodies at initial concentrations of 1.1 μg/mL and 1.2 μg/mL, respectively. Figure 5C and D are a linear and log histogram presentation of the magnetophoretic mobility of purified lymphocytes labeled with anti-CD3-DM conjugates at an initial concentration of 24 μg/mL. Note the similarity in the percent positive populations for the three methods of labeling (Figure 5A–C).

**Determination of Equilibrium Antibody-Label Conjugate Concentrations**

Figure 6A is a calibration curve for the MicroBCA protein assay in the form of absorbance at 562 nm as a function of concentration of the provided protein standards. A representative elution curve of anti-CD3-DM sample separated on a CL-4B column is presented in Figure 6B. Each solid data point corresponds to an eluant of 350 μL and is plotted in the form of the absorbance of the eluant at 562 nm as a function of total volume eluted from the column. Two peaks are visible, a small peak which corresponds to antibody–nanoparticle conjugate at 4,500–5,500 μL and a much larger peak at 7,900 μL, which corresponds to the BSA. This larger BSA peak was confirmed by the addition of only BSA (solid symbols) to the column in a separate elution experiment. As further confirmation that the antibody–nanoparticle conjugates elute at 4,500–5,500 μL, a yellow band, corresponding to the magnetic particles, can be observed moving through the column at this position. This yellow tint has been observed with a number of different magnetic nanoparticle colloids.

The actual protein concentration of each eluent was determined from the calibration curve presented in Figure 6A. The total protein eluting in a specific peak was approximated using the linear trapezoidal rule. Using this technique, the concentration of the antibodies in the anti-CD3-IMag conjugate reagent (Lot number 0000056357) was 67 μg/mL. To test the recovery of proteins after eluting from Sepharose CL-4B column, 50 μL of albumin standard (2,000 μg/mL) was eluted through the gel column yielding a recovery of 99%.

**Table I.** Percentage of lymphocytes in peripheral blood cells after culture and Ficoll separation determined by Coulter counter and flow cytometry.

<table>
<thead>
<tr>
<th>Percentage of lymphocytes (%)</th>
<th>Coulter counter</th>
<th>Flow cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>After Ficoll</td>
<td>15.8</td>
<td>13.8</td>
</tr>
<tr>
<td>24 h culture</td>
<td>18.7</td>
<td>15.1</td>
</tr>
<tr>
<td>48 h culture</td>
<td>26.1</td>
<td>25.0</td>
</tr>
<tr>
<td>48 h culture &amp; Ficoll</td>
<td>77.8</td>
<td>80.3</td>
</tr>
</tbody>
</table>

**Figure 5.** Histograms of peripheral blood lymphocytes labeled with mouse anti-human CD3 antibody conjugated to FITC (A), PE (B), magnetic nanoparticles in linear scale (C), and magnetic nanoparticles in logarithmic scale (D). In histograms (A–C), two peaks are identified as CD3 negative and CD3 positive. In (D), only one peak is present, since negative values of mobility are not plotted on a log scale.
Development of Saturation Curves for Affinity Interactions

Since the FCM instrument used in this study uses logarithmic amplification circuits, for each set of labeling studies, the FI for each data point was normalized by dividing by the highest FI in that set of experiments. Such a normalization procedure results in a relationship analogous to Equation 7 and allows experiments on different days to be compared.

Figure 7A and B contain data from two different studies of the binding of anti-CD3-FITC to purified, human lymphocytes; Figure 7A is a plot of normalized FI as a function of initial antibody concentration and Figure 7B is a classic double reciprocal plot of the same data. The two different symbols correspond to two different sets of experiments conducted on different days with different cell samples. Figure 7C and D contain data from three sets of independent experiments, similar to Figure 7A and B, except that anti-CD3-PE antibodies were used.

Figure 7E and F present a saturation curve and double reciprocal plot of the magnetophoretic mobility of lymphocytes labeled with anti-CD3-IMag conjugates. Specifically, Figure 8A presents the histogram of PE fluorescent intensity for each of the four calibration particles from one of the two calibration studies. Figure 8B presents the linear relationship of mean FI to the mean number of PE molecules per bead (as reported by the manufacturer), and Figure 8C presents the mean number of PE molecules per cell as a function of initial concentration of the labeling antibody. A final note is that an assumption (from information from the manufacturer) was made in these calculations that one PE molecule is conjugated to one antibody.

Binding of SA-MACS and SA-IMag to Biotinylated Microspheres

Figure 9A–D present the normalized mobility of the biotinylated microspheres labeled with different amounts of SA-MACS particles and SA-IMag particles. Analysis was carried out in a similar manner to the anti-CD3-MACS and IMag-binding studies.

DISCUSSION

Model Fitting of the Binding Data

Both visual inspections (Figs. 7A, C, and E, and 9A and C) and quantitative measure ($R^2$ value) indicate that the Langmuir isotherm and the two-parameter Hyperbolic model, Equations 7 and 23, fit the data reasonably well. The double reciprocal plot of the data, Figures 7B, D, and F, 9B and D, does not fit the data as well for all but the data in Figure 7E and F based on both visual as well as...
Figure 7. Saturation curve and double reciprocal plot for peripheral blood labeled with BD anti human CD3-FITC, (A) and (B), BD anti human CD3-PE, (C) and (D), and BD IMag™ anti human CD3 beads-DM, (E) and (F).
As discussed previously, the reported values of the dissociation constant of the antibody–antigen interactions can range over four orders of magnitude ($10^{-5} - 10^{-11}$ M) and the values reported in this study fall within that range. An interesting observation is that when one compares the dissociation constant for the three antibody conjugates, Ab-FITC, Ab-PE, and Ab-DM, (note all three antibodies are the same clone, HIT3a) a trend of increasing values of $aK_D$ is obtained with increasing size of the conjugated “tag” (i.e., smallest to largest: FITC:PE:DM). While this data is insufficient to make any firm conclusions, one can speculate that the size of the tag conjugated to an antibody decreases the antigen–antibody affinity. Further evidence of this speculation is given by Siiman and Burshteyn (2000) who reported that the dissociation constant for a different antibody clone...
(relative to this study) binding to CD3 receptors on human lymphocytes ranged from $1.1 \times 10^{-10}$ to $1.9 \times 10^{-11}$ M and this constant increased approximately 10-fold ($1.5-2.5 \times 10^{-9}$) when the fluorescent molecule RD1 was conjugated and approximately 100-fold ($4.5-5.5 \times 10^{-9}$) when FITC was conjugated to the antibody.

Table II. Apparent dissociation constant for the various conjugates in $\mu$g/mL and $M$.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>$\alpha K_D (\mu g/$mL$)$</th>
<th>$\alpha K_D (M)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-FITC</td>
<td>0.35</td>
<td>$2.3 \times 10^{-9}$</td>
</tr>
<tr>
<td>Ab-PE</td>
<td>0.70</td>
<td>$4.7 \times 10^{-9}$</td>
</tr>
<tr>
<td>Ab-DM</td>
<td>4.0</td>
<td>$2.7 \times 10^{-8}$</td>
</tr>
<tr>
<td>SA-MACS</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>SA-DM</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

Estimation of ABC for Anti-CD3-PE on PBLs

Visual inspections of Figures 7C and 8C (QuantiBRITE studies) indicate that saturation of the CD3 antigen on the PBLs was not reached. Also, comparison of Equation 7 with the two-parameter Hyperbolic model, Equation 23, suggests that if either the fluorescence signal or magnetophoretic mobility, is normalized to the maximum, saturated value in a set of experiments, the least squares regression of the data returns an “$a$” constant equal to 1. Clearly, a value of 1.7 is far from saturation.

The regression of the data using the Hyperbolic model in Figure 8C yielded an “$a$” value which corresponds to the actual number of PE molecules at saturation, which in this case is 58,700. This is consistent with the reported range in the number of CD3 antigens on a PBL from 52,000 to 177,000 (Davis et al., 1998). While this has not been reported,
it is highly likely that this large range is also the result of some studies which used a monovalent binding antibody while others used a bivalent binding antibody.

 Ideally, our experiments presented in Figures 7C and 8C should have been conducted over the full range of labeling concentrations; however, such experiments would be either prohibitively expensive or not possible with currently purchased reagents. Note the anti CD3-PE antibody conjugates purchased from BD and used in this study are supplied at a reported antibody concentration of 1.5 μg/mL. Therefore, to conduct a full saturation experiment would require the concentration of antibody conjugates prior to the studies.

**Estimation of ABC for Anti-CD3-DM on PBLs**

Complementary to the estimation of the ABC of the anti-CD3-PE on PBLs, one can estimate the ABC of anti-CD3-DM on PBLs from the results presented above as well as previously published values of the field interaction parameter, \( \phi \), of \( 91 \times 10^{-25} \) m\(^3\), for the DM nanoparticles (Zhang et al., 2005). Rearranging Equation 18, the ABC can be calculated from:

\[
ABC = \frac{m(3\pi Dn\eta)}{n_2\phi} \tag{24}
\]

With an experimentally determined cell diameter of 6.9 microns, a mobility, \( m \), at saturation of \( 2.4 \times 10^{-4} \) mm\(^2\)/V-s/kg, a viscosity, \( \eta \), of \( 9.6 \times 10^{-3} \) kg/m/s, and a value of \( n_2 \) equal to 1, the ABC has a value of approximately 1,600, which is significantly less than the 58,000 calculated with the anti-CD3-PE conjugates. As has been discussed previously (McCloskey et al., 2003b; Zhang et al., 2005), this significantly lower value is most probably the result of steric exclusion. A simple calculation of the space available on the surface of a 6.9 micron cell for closely packed, 230 nm spheres, indicates there is only enough room for approximately 2,100 spheres, further confirming the concept that the surface of the cells is completely covered with magnetic spheres. It should also be noted that for this study, the value of \( m_{bg} \) was assumed to be zero since the magnitude of the mean mobility of the labeled cells was two to three orders of magnitude greater than the reported value of \( m_{bg} \).

**Verification of the Assumption of the Equilibrium Antibody Concentration**

It was previously stated that it was assumed that the initial, labeling concentration and the final, equilibrium concentration of the anti-CD3-PE and anti-CD3-FITC antibodies was nearly the same. With the estimate of the number of PE molecules per cell as a function of initial antibody concentration (Fig. 8C), it is now possible to test his assumption by conducting an overall mass balance.

Specifically, assuming the molecular weight of an antibody to be 150,000, and that one PE molecule is conjugated to one antibody, a molecular balance is presented in Table III for one of the two experiments conducted with PBLs labeled with anti-CD3-PE conjugates and quantified with the QuantiBRITE particles (solid symbols in Fig. 8C). As can be observed, on average, the equilibrium concentration was calculated to be 10% lower than the initial concentration. Considering the difficulty in obtaining accurate antibody concentration at these concentrations, the assumption was reasonable.

**Implications of Model to Scale-Up of Immunological Labeling**

The good agreement between experimental data and Equation 6 and the estimates of the ABC of CD3 on PBLs has significant implications in the scale-up of immunomagnetic labeling for clinical applications. It has been reported that to perform an allogenic bone marrow transplant on an adult, one would need to have high-level depletion (> 4 log\(_{10}\)) of all of the CD3 positive PBLs (T cells) in a cell suspension of approximately \( 10^{10} \) cells. That is \( 10^4 \) times as many cells as were used in the studies in this publication. Also, as has been demonstrated previously, the performance of magnetic cell separation systems, in general, increase significantly as the magnetophoretic mobility of the labeled cells is increased (Comella et al., 2001; McCloskey et al., 2003a). Therefore, to obtain the highest level of depletion of

<table>
<thead>
<tr>
<th>Initial concentration of Ab (μg/mL)</th>
<th>Initial number of Ab molecules</th>
<th>Number of PE molecules bound per cell</th>
<th>Total number of PE molecules bound</th>
<th>Equilibrium number of Ab in solution</th>
<th>Equilibrium Ab concentration (μg/mL)</th>
<th>Percent decrease due to binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>2.00E+10</td>
<td>3.50E+03</td>
<td>2.63E+09</td>
<td>1.74E+10</td>
<td>0.043</td>
<td>13.1</td>
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<tr>
<td>0.14</td>
<td>5.60E+10</td>
<td>6.50E+03</td>
<td>4.88E+09</td>
<td>5.11E+10</td>
<td>0.128</td>
<td>8.7</td>
</tr>
<tr>
<td>0.21</td>
<td>8.40E+10</td>
<td>1.20E+04</td>
<td>9.00E+09</td>
<td>7.50E+10</td>
<td>0.188</td>
<td>10.7</td>
</tr>
<tr>
<td>0.31</td>
<td>1.24E+11</td>
<td>1.40E+03</td>
<td>1.05E+09</td>
<td>1.23E+11</td>
<td>0.307</td>
<td>0.8</td>
</tr>
<tr>
<td>0.5</td>
<td>2.00E+11</td>
<td>2.00E+04</td>
<td>1.50E+10</td>
<td>1.85E+11</td>
<td>0.463</td>
<td>7.5</td>
</tr>
<tr>
<td>0.675</td>
<td>2.70E+11</td>
<td>2.60E+04</td>
<td>1.95E+10</td>
<td>2.51E+11</td>
<td>0.626</td>
<td>7.2</td>
</tr>
<tr>
<td>0.85</td>
<td>3.40E+11</td>
<td>2.70E+04</td>
<td>2.03E+10</td>
<td>3.20E+11</td>
<td>0.799</td>
<td>6.0</td>
</tr>
<tr>
<td>1</td>
<td>4.00E+11</td>
<td>3.00E+04</td>
<td>2.25E+10</td>
<td>3.78E+11</td>
<td>0.944</td>
<td>5.6</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td>2.72E+09</td>
<td><strong>1.92E+11</strong></td>
<td><strong>0.604</strong></td>
<td><strong>7.5</strong></td>
</tr>
</tbody>
</table>
magnetically labeled cells, the highest level of magnetophoretic mobility (saturation of labeling) is desired. However, if not done properly, the costs of such depletions are prohibitively expensive with respect to routine, clinical applications.

The previous relationships can be rearranged to address this scale-up question. Specifically, Equation 15 can be rearranged to give:

\[
[\text{Ab}] = \frac{aK_{D1}}{1 - \frac{\theta}{\lambda}} \cdot \frac{\theta / \lambda}{1 - \theta / \lambda} \tag{25}
\]

where \([\text{Ab}]\) is in \(M\). It then follows that the total amount of antibodies needed is just:

\[
[\text{Ab}]_{\text{Total}} = \left(\frac{\theta / \lambda}{1 - \theta / \lambda} \cdot \frac{aK_{D1}}{1 - \theta / \lambda}\right) \cdot V_{\text{fluid}} + \left(\frac{\theta / \lambda \cdot \text{ABC} \cdot C}{N_A}\right) \cdot V_{\text{suspension}} \tag{26}
\]

where \([\text{Ab}]_{\text{Total}}\) is the total amount of antibody (in moles or grams if multiplied by the MW of the antibody), \(C\) is the concentration of cells in number of cells/L, and \(N_A\) is Avogadro’s number. The first term on the right hand side of Equation 26 is the equilibrium number of moles of antibodies in the solution around the cells and the second term on the right hand side is the number of moles of antibodies bound to the cell. Note that \(V_{\text{suspension}} = V_{\text{fluid}} + V_{\text{cells}}\).

In terms of degree of saturation, one obtains:

\[
\frac{\theta}{\lambda} = \left[\frac{[\text{Ab}]_{\text{Total}} + aK_{D1} + C'}{2C'}\right] - \sqrt{\frac{[\text{Ab}]_{\text{Total}} + aK_{D1} + C'}{2C'}} - 4\frac{[\text{Ab}]_{\text{Total}}C'}{N_A} \tag{27}
\]

Once the constant \(aK_{D1}\) and the ABC are determined for a specific system in small-scale experiments, predictions can be made on the total amount of antibody needed to achieve certain FI or magnetophoretic mobility or vice versa based on Equations 26 and 27.

To verify the proposed scale-up model shown above, the same conditions were used to label \(1 \times 10^6\), \(1 \times 10^7\), and \(2 \times 10^5\) similarly purified lymphocytes with the same anti CD3 antibodies we used in the previous study. The measured \(\theta\) is compared with the value predicted using Equation 27 and results from the study, as shown in Figure 10A. A reasonable agreement was found, which validates the scale-up model.

Finally, a theoretical predication can be made on the total amount of anti-CD3 antibody (FITC, PE, or DM conjugated) needed to achieve 90% saturation of the PBL when working with a total of \(10^{10}\) cells, Figure 10B. As can be clearly seen, the total amount of antibody needed drops significantly as the cell concentration increases. A more rapid drop of the total antibody needed begins at a cell concentration greater than \(1 \times 10^9\) cells/mL. Using a rough estimate of the volume of a lymphocyte (perfect sphere, mean diameter of 6.9 microns), a “solid” cell mass is achieved around \(5 \times 10^9\) cells/mL and at \(1 \times 10^9\) cells/mL approximately 50% of the cell suspension volume is cell mass. A correction for the volume of cell mass was used to create the lines in Figure 10B. The difference between the amount of antibody needed for the anti-CD3-PE (or FITC) versus the anti-CD3-DM is due to the lower antigen–antibody affinity of the latter.

Several observations can be made by inspecting Figure 10B. First, the importance of increasing the cell

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**Figure 10.** A: Comparison between theoretical (Equation 27) and measured fraction of saturation for a range of cell concentrations and the three immunological labels used in this study. B: The effect of cell concentration on the amount of antibodies needed to label \(10^{10}\) cells.
concentration (x-axis) to reduce the total amount of antibody conjugate that is needed. At first, this may seem counter intuitive; however, increasing the cell concentration lowers the total volume of the cell suspension for a given, total cell number. Since as was demonstrated in Table III, a majority of the antibody used to label a cell suspension remains in the suspension (is not bound to the cell), minimizing the total suspension volume lowers the total amount of antibody needed. This becomes very important as the total volume of cell suspension significantly increases as in the case of a clinical transplant. Second, the need for a significantly higher amount of antibody (higher concentration) is graphically demonstrated with the anti CD3-IMag conjugate. This is a pragmatic example of the effect that the decrease in the effective apparent dissociation constant has on labeling a large number of cells. Such a need for increased amount of antibody can more than double, potentially triple, the retail cost of reagents for large-scale separations. Consequently, there is a large motivation for improving the affinity of antibody–magnetic nanoparticle conjugates for large-scale separations.

**NOMENCLATURE**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>ABC</td>
<td>antibody-binding capacity (#/cell)</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>C</td>
<td>cell number concentration</td>
</tr>
<tr>
<td>Dc</td>
<td>cell diameter (m)</td>
</tr>
<tr>
<td>Fl</td>
<td>fluorescence intensity (unitless)</td>
</tr>
<tr>
<td>kq</td>
<td>rate constant for the association reaction (L/mol-s)</td>
</tr>
<tr>
<td>kd</td>
<td>rate constant for the dissociation reaction (s)</td>
</tr>
<tr>
<td>KD</td>
<td>equilibrium dissociation constant (mol/L)</td>
</tr>
<tr>
<td>KA</td>
<td>equilibrium association constant (L/mol)</td>
</tr>
<tr>
<td>L</td>
<td>ligand</td>
</tr>
<tr>
<td>m</td>
<td>magnetophoretic mobility (mm3-s/kg or mm3/T-A-s)</td>
</tr>
<tr>
<td>n1</td>
<td>number of antigens sites per cell (unitless)</td>
</tr>
<tr>
<td>n2</td>
<td>number of magnetic nanoparticles conjugated to an antibody (unitless)</td>
</tr>
<tr>
<td>R</td>
<td>receptor</td>
</tr>
<tr>
<td>V</td>
<td>volume (m³)</td>
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**Greek**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>α</td>
<td>experimentally determined constant that reflects the effects of cell membrane on KD</td>
</tr>
<tr>
<td>β</td>
<td>See Equation 20</td>
</tr>
<tr>
<td>ε</td>
<td>conversion factor between fluorescence intensity and number of fluorescent molecules</td>
</tr>
<tr>
<td>λ</td>
<td>valence of the antibody binding</td>
</tr>
<tr>
<td>θ</td>
<td>fraction of binding sites bound (unitless)</td>
</tr>
<tr>
<td>φ</td>
<td>particle-field interaction parameter (m³)</td>
</tr>
<tr>
<td>χ</td>
<td>volumetric magnetic susceptibility, SI units (unitless)</td>
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<tr>
<td>η</td>
<td>viscosity (kg/m-s)</td>
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<table>
<thead>
<tr>
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<tr>
<td>[ ]</td>
<td>concentration, M or µg/mL</td>
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<table>
<thead>
<tr>
<th>Subscripts</th>
<th>Definition</th>
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<td>bg</td>
<td>background</td>
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<tr>
<td>nano</td>
<td>nanoparticles</td>
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