Measuring microsphere binding capacity

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Assays to determine the binding capacity of streptavidin-coated magnetic microspheres can provide critical information about their suitability for IVD applications.

Magnetic microspheres have been used for some time as the solid phase for immunological tests and assays encompassing a number of formats (see Table I). A major advantage of microspheres over such solid supports as filters, tubes, wells, or large plastic beads, is their ease of separation from an aqueous phase. The magnetic character of the microspheres currently on the market varies, with a popular choice being microspheres that are superparamagnetic, meaning that they retain no magnetic character after being removed from a magnet.

Recently, several companies have begun to offer these magnetic microspheres preconjugated with some type of generic binding protein. A common choice is streptavidin (from *Streptomyces avidinii*, or produced recombinantly), which is similar to the avian egg protein avidin. Streptavidin interacts strongly with the molecule biotin, and so by biotinylating the ligand to be coupled to the microspheres, an attachment with a bond strength approaching that of a covalent bond (Ka = 10^{15}/M) can be achieved in a one-step chemical reaction. This reduces both the time involved in protein conjugation and the expense of wasted reagents.

An important parameter when choosing streptavidin-coated microspheres is their binding capacity. The general approach to determining the binding capacity of such microspheres is to conjugate a biotinylated ligand that will serve as a sensitive marker. Common markers are detected by chemiluminescence, enzymatic activity, radioactivity, and fluorescence. By knowing the molecular weight of this marker, a reliable estimate can be made regarding the capacity of the streptavidin-coated microspheres to bind a ligand of similar size and weight. Whether developing a binding-capacity assay or relying on the binding capacity reported by the manufacturer, the user should ensure that the reported percent of solids is accurate, since these assays are characterized in terms of the weight of bound ligand per unit weight of microspheres. Therefore, a preliminary gravimetric percent of solids determination is recommended. Finally, the magnetic character of the base particles will play an important role in the ease of handling of the microspheres. Therefore, the efficiency of magnetic separation for various types of base particles should be looked at as well.

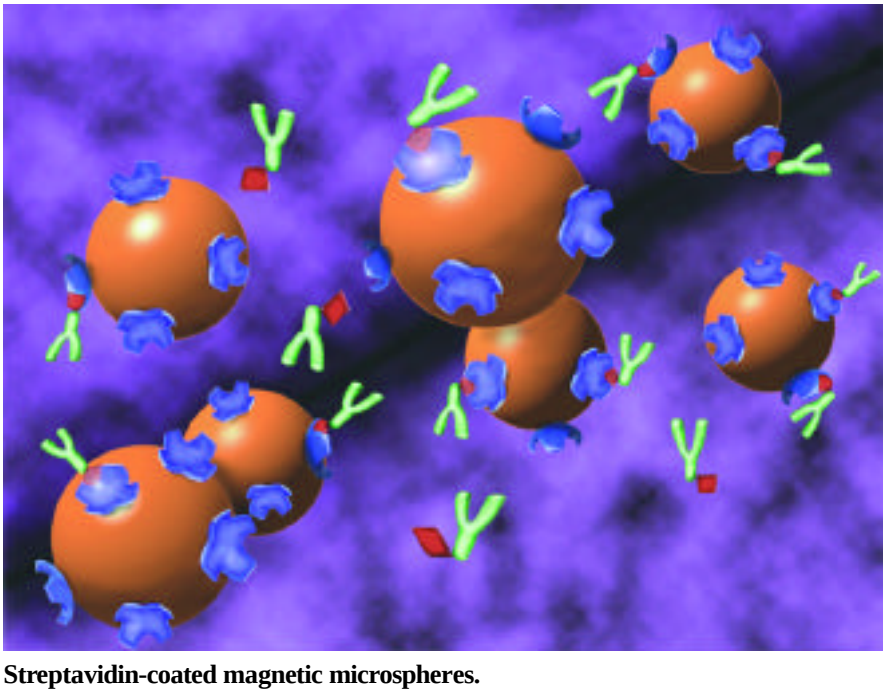

ILLUSTRATION BY OSCAR MEZA

Streptavidin-coated magnetic microspheres.

Possible Assay Strategies

The fundamental reason for designing a binding-capacity assay for this type of solid support is to simplify subsequent ligand attachment to the microspheres. The idea is to bind a marker of similar size and weight to the ligand that will be used for the final application.

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Company Name	System Name	Sandwich Assay	Competitive Assay	Label Type	Generic Solid Phase
Bayer	Immuno 1	Yes	Yes	Enzyme	Yes
Biotrol	Biotrol 7000 and 8000 Magia 8000	Yes	Yes	Enzyme	No
Roche (Boehringer Mannheim)	Elecsys	Yes	Yes	Electrochemiluminescent	Yes
Chiron	ACS:180 Plus	Yes	Yes	Chemiluminescent	No
Dade (Syva)	Aca plus	Yes	No	Enzyme	No
Johnson & Johnson	Amerlex M	Yes	No	Radioactive	No
Quest (Nichols Diagnostics)	Advantage	Yes	Yes	Chemiluminescent	Yes
Beckman-Coulter (Sanofi)	Access	Yes	Yes	Chemiluminescent	Yes
Dade (Syva)	Vista	Yes	Yes	Enzyme	Yes
Tosoh Medics	AIA-600, 1200, 1200DX	Yes	Yes	Fluorescent	No
Serono	Maia, SR1, Maiaclone, Serozyme	Yes	Yes	Radioactive and enzyme	Yes

Table I. Commercial magnetic particle-based assay systems.

Using this number as a guideline for the ultimate coupling reaction removes later guesswork regarding proper reagent usage. This capacity for increased efficiency in reagent usage is one of the reasons that the biotin-streptavidin coupling strategy is becoming more popular than conventional covalent coupling protocols in immunoassay and molecular biology applications.

In developing an appropriate assay strategy, both the means of detection and utility for various sized ligands should be considered. Table II lists four widely used detection methods, as well as common markers for each. The corresponding reaction schemes are illustrated in Figure 1.

Four commonly used means of detection, in the order of least to most sensitive, are radioactive (RIA), enzyme-linked (EIA), fluorescent (FIA), and chemiluminescent (CLIA) immunoassays.[1] While immunoassays based on fluorescence are quite sensitive, they can be problematic as well. Although our initial work involved this type of assay, we no longer use it because the equipment necessary for optimal detection (a fluorimeter) is not readily available. Also, difficulty working with FITC-labeled biotin, a common fluorescent tag, made it prohibitive for use as a primary binding-capacity assay. These difficulties included the fact that the FITC-labeled biotin was labile, and that it was difficult to dissolve in an aqueous suspension. For these reasons, this approach is omitted from the following assay descriptions.

Radioactive Assay: Tritiated Biotin

The earliest types of streptavidin binding-capacity assays used ^{125}I-, ^{14}C-, or ^{3}H-labeled biotin as tracers. Because of the simplicity of these assays, they are still widely used today. Radioactivity has some distinct advantages over other means of detection, as it causes only very minor changes to the structure of the labeled antigen (a tritiated biotin has the same molecular size as nonradioactive biotin), is easy to quantify, and is simple to detect.[2] This makes the use of these materials very convenient for the study of binding reactions of small molecules. Additionally, a large biotinylated molecule can be radioactively labeled to easily determine the binding capacity of streptavidin-coated microspheres for higher molecular weight ligands, such as immunoglobulins. Before deciding to use an apparently simple radioactive assay as the primary biotin binding-capacity assay, however, users should give consideration to such complicating factors as the low specific activity, even of ^{125}I-labeled molecules; the labile nature of some radioactively modified molecules; the regulatory pressures and constraints involved in using such tests; and the need for specialized detection equipment.

There are two commonly used approaches to this type of assay. The first is to incubate varying amounts of microspheres with an excess of radioactively labeled biotin to calculate binding capacity. Perhaps the more common approach is to incubate varying amounts of radioactive biotin with a constant amount of microspheres to generate a Scatchard plot. A version of the Scatchard plot is

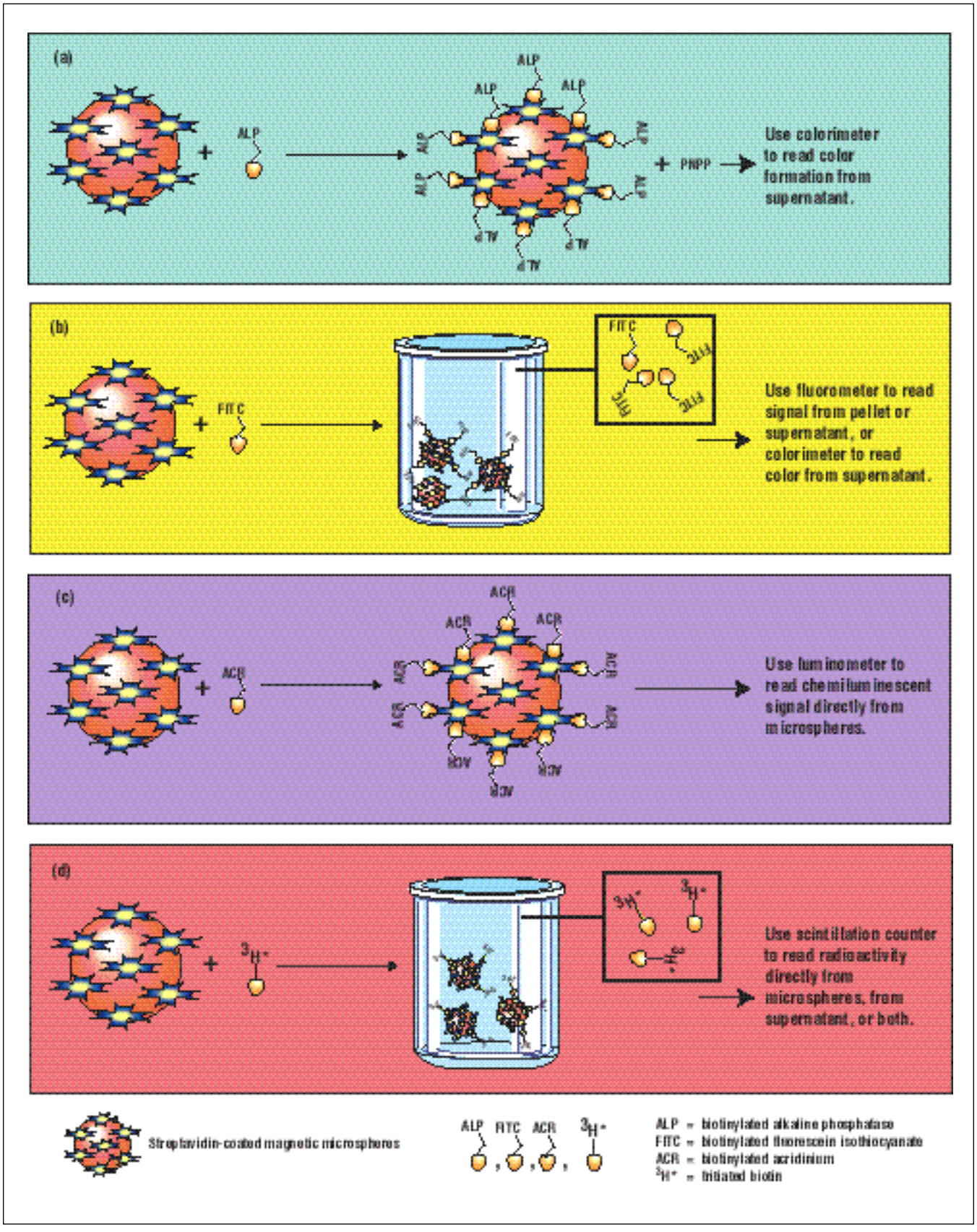

Figure 1. Basic binding-capacity assay strategies: (a) large-molecule enzymatic assay using biotinylated alkaline phosphatase with a molecular weight (MW) of approximately 140,000; (b) small-molecule fluorescent assay using biotinylated fluorescein isothiocyanate (MW ~633); (c) small-molecule chemiluminescent assay using biotinylated acridinium (MW ~877); and (d) pure biotin radioactive assay using tritiated biotin (MW ~247).

Detection Method	*Common Markers*	*Advantages*	*Disadvantages*
Fluorescence	FITC	Small-molecule assay Easy to perform Can be quantitated either fluorimetrically or spectrophotometrically	FITC label is labile FITC-biotin is difficult to dissolve in an aqueous suspension Indirect assay (results are calculated from dye left in solution rather than directly from particle)
Radioactivity	Tritiated biotin ^{14}C-labeled biotin ^{125}I-labeled biotin Radiolabeled, biotinylated immunoglobulins	Direct small- or large-molecule assay	By itself, not a measure of real-world ligand binding (unless first conjugated to appropriate molecular weight ligand) Problems caused by disposal of radioactive reagents
Enzymatic (colorimetric)	Alkaline phosphatase Horseradish peroxidase	Direct assay for large molecular weight ligands Substrate addition noncritical High signal levels	Reading signal "on the fly" Amplification of background Nonspecific binding
Chemiluminescence	Acridinium esters Alkaline phosphatase Horseradish peroxidase	Rapid turnaround time High signal-to-noise ratio	Limited availability of commercial reagents Stickiness of acridinium necessitates extensive blocking steps

Table II. Characteristics of different binding capacity assay formats for streptavidin-coated magnetic microspheres.

the typical approach for quantifying the number of receptors (i.e., binding sites) on a cell surface, and thus can be modified for quantifiying the number of binding sites on a microsphere surface (see Figure 2). Our approach to this assay was to incubate varying amounts of streptavidin-coated magnetic microspheres with a constant amount of tritiated biotin, always in excess of the stoichiometrically calculated number of biotin binding sites on the microspheres. The microspheres were then washed, and the radioactivity was determined directly from the microspheres by scintillation counting. The actual binding capacity is a conversion of the fraction of counts per minute of bound biotin divided by the counts per minute of total biotin added. For more accurate measurement of tritiated biotin activity, a check can be run to detect the counts per minute of the supernatant, ensuring that this count, plus that for the microspheres, adds up to the counts per minute for the original volume of tritiated biotin.

When measuring the binding capacity of our streptavidin-coated magnetic microspheres radioactively, we encountered some interesting considerations. We found that a broader size distribution of base microspheres, such as are offered by several leading suppliers of magnetic particles, can lead to inaccurate and low binding-capacity values. It is believed that this is a result of "fines," or smaller particles that are not pulled to the magnet in the same amount of time as the main population of microspheres. These fines could theoretically bind biotin yet not be detected by scintillation counting of the microsphere pellet, thereby lowering the apparent binding capacity. One way around this source of error would be to use a more powerful magnet with the capacity to pull microspheres that have a lower amount of iron oxide, as is the case with fines.

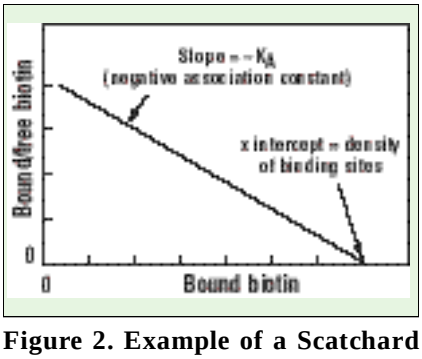

Figure 2. Example of a Scatchard plot, typically used to quantify the number of receptors on a cell surface.

Enzymatic Activity Assay: Biotinylated Alkaline Phosphatase

As regulatory issues involving radioactive markers have become more prevalent, interest has grown in using other means of detection for binding-capacity assays. A main focus in assay development has been the use of enzyme labels. While several enzymes can be used as markers, one of the most common, because of its widespread commercial availability, is alkaline phosphatase. Reasons for the popularity of this type of binding assay include its simplicity, the strong signal given by the enzymatic reaction with a substrate, and the fact that the size and molecular weight of alkaline phosphatase mirror those of many commonly attached ligands, such as immunoglobulins. By developing a standard curve based on the absorbance of varying concentrations of enzyme per constant concentration of substrate, precise quantitation of binding

can be derived simply by colorimetric detection using a spectrophotometer.[3,4]

For streptavidin-coated magnetic microspheres, this assay method involves first making serial dilutions of biotinylated alkaline phosphatase (B-ALP) and reacting these with a constant concentration of substrate, in this case paranitrophenyl phosphate (PNPP). The concentration of substrate used will determine the reaction kinetics, so some optimization will be required to find a suitable concentration that allows sufficient time after addition for accurate spectrophotometric measurement. Once this concentration has been determined, the serially diluted B-ALP is reacted with the substrate, and the absorbance readings at 405 nm are used to generate a standard curve. The proper dilutions for the B-ALP are determined such that they will fall within the range of linearity for absorbance measurements on the spectrophotometer that is used.

Once these variables have been optimized, the last variable to consider is the concentration of microspheres to be used in the actual assay. As the B-ALP conjugated microsphere concentration increases, so does the amount of signal given off by the ALP-PNPP interaction. Therefore, a microsphere concentration must be established such that the signal is within the limits established by the standard curve.

An important precaution with this assay is the time allowed for substrate development (color formation). As ALP is allowed to react with PNPP, color continues to form until all of the substrate has been exhausted, giving the potential for a false high reading. Therefore, if a rate-dependent format is chosen, the time allowed for color development must be precisely controlled. Conversely, if an end-point reading is to be taken, the relative concentration of the ALP-conjugated microspheres must be controlled, such that allowing the reaction to go to completion will still give absorbance readings within the limits of the standard curve.

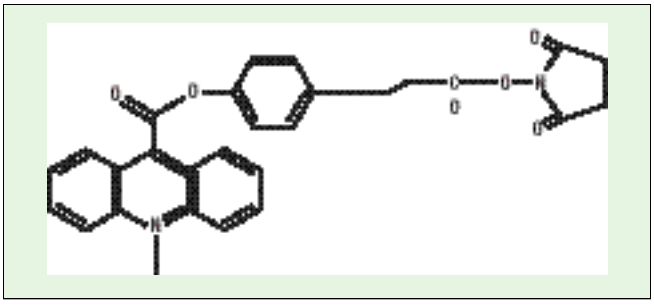

Figure 3. Acridinium C_2HNS ester (formula weight 632.55) can be biotinylated and used to develop a rapid and sensitive small-molecule biotin-binding assay.

Chemiluminescent Assay: Biotinylated Acridinium

Chemiluminescence is the chemical generation of visible light by a reaction, and as such does not use any light source. Thus the need for complicated and inefficient optical wavelength filtering systems is eliminated. Chemiluminescent systems fall into two classes. The first and easiest to develop uses enzymes to produce the chemiluminescent signal. Typically, either horseradish peroxidase or alkaline phosphatase is used, and the label is triggered by the addition of substrates that, under the influence of the enzyme system, give rise to a visible emission. This type of signal enhancement has enabled researchers to develop binding-capacity assays that are faster and more sensitive than any traditional colorimetric or radioactive assay.

Nonenzymatic direct chemiluminescent labels tend to have lower background signals than enzyme systems, and typically produce a signal very quickly.

The other chemiluminescent systems use a nonenzymatic direct chemiluminescent label. Direct labels tend to have lower background signals than enzyme systems, and typically produce a signal very quickly. With the acridinium ester system, after the immunological binding and subsequent wash step, the signal takes only 2 seconds to develop, compared with 30 minutes or longer for an enzyme-generated system (see Figure 3).

As luminometers used to detect chemiluminescence have become more common in today's laboratories, the interest in chemiluminescent binding assays for the quantitation of streptavidin-coated magnetic microspheres has increased. The advantage that chemiluminescence offers over colorimetric enzyme, fluorescent, or radioactive assays is enhanced sensitivity.[5] Of particular benefit when developing a binding-capacity assay specific to streptavidin-coated magnetic particles is that chemiluminescent and radioactive detection are the only formats that can be read in the presence of the microspheres without interference from the particles themselves.

This newer approach to developing a binding-capacity assay carries a disadvantage. Unlike the more conventional EIA, RIA, and FIA approaches, the commercial availability of well-characterized chemiluminescent esters that are able to be biotinylated is limited. Were this not the case, a direct binding-capacity assay could be run by incubating the chemiluminescent ester with the microspheres, measuring with a luminometer the relative light units (RLUs) emitted by a certain concentration of microspheres, and then converting this to actual binding based on the number of RLUs given off by one chemiluminescent molecule. As it is, a chemiluminescent ester can be biotinylated and purified. However, unless the concentration of acridinium in a given sample is known precisely, it is necessary to develop the assay using an indirect format.

With this obstacle in mind, such an assay was carried out by first flooding the microspheres with free biotin, washing, adding a constant concentration of biotinylated acridinium (B-ACR), and rewashing (see Figure 4). This ensured that,

based on stoichiometry derived from the titration for the number of groups on the base particle available for streptavidin binding, all of the binding sites on the streptavidin were occupied by free biotin. This was used as the "blank" on the luminometer, and any signal given off was attributed to nonspecific binding of the acridinium ester (a hydrophobic molecule) to the exposed surface of the hydrophobic microspheres. Optimization of the blockers used in the assay or more-vigorous washing steps are necessary to lower this value to near zero.

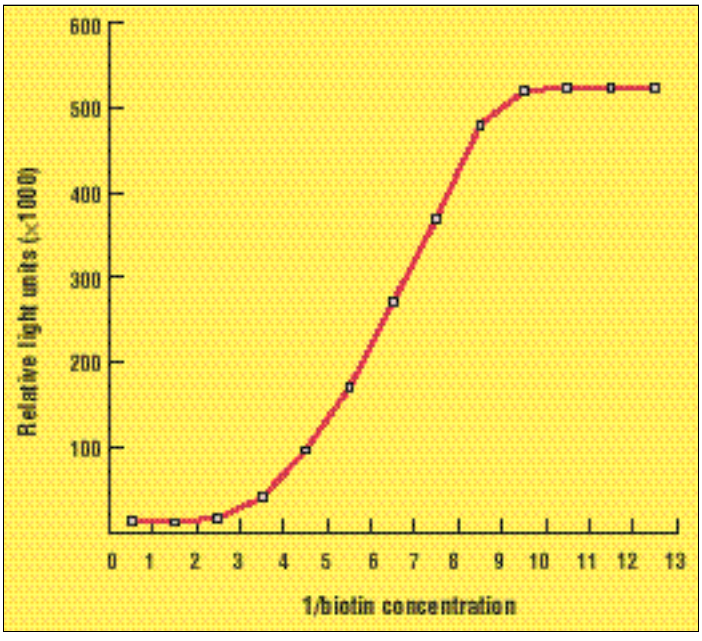

Figure 4. Increasing chemiluminescent signal, as read on a luminometer, results when a lower concentration of free biotin is added in the presence of a constant amount of biotinylated acridinium.

We then serially diluted the free biotin, and incubated this with the same constant concentration of B-ACR used previously, and streptavidin-coated magnetic microspheres. As the luminescent signal increased from the washed particles, this was an indication of the number of free sites left open for binding of the biotinylated acridinium. The dilutions were carried out to complete a sigmoidal binding curve, with the dilutions representing the steepest part of the curve being narrowed for increased precision (see Figure 4). Finally, as it was difficult to identify a direct point on the curve to assess maximum binding, we reasoned that the point on the curve with the steepest slope could be considered to be the equivalence point (with half of the streptavidin binding sites occupied by biotin, and half by B-ACR), and that multiplying this value by two would give a precise binding capacity value for B-ACR.

Factors Influencing Assay Variability

Although the total binding capacity of streptavidin-coated magnetic microspheres is an important factor for most applications, with the highest total binding normally being the best, other variables play important roles. Because binding capacity measurements are given in terms of a weight of bound marker per unit weight of microspheres, the percent of solids in the suspension in which these are supplied is important for obtaining their true binding capacity. Before designing any assay, we recommend measuring this percentage gravimetrically to ensure that the measured percent of solids coincides with the reported percent of solids. Figure 5 illustrates differences that we found in our lab for material supplied by several leading vendors.

Similarly, the total binding can be classified in terms of both specific and nonspecific binding. The former refers to biotinylated ligands that are attached via the streptavidin-biotin bond, whereas the latter refers to ligands that are

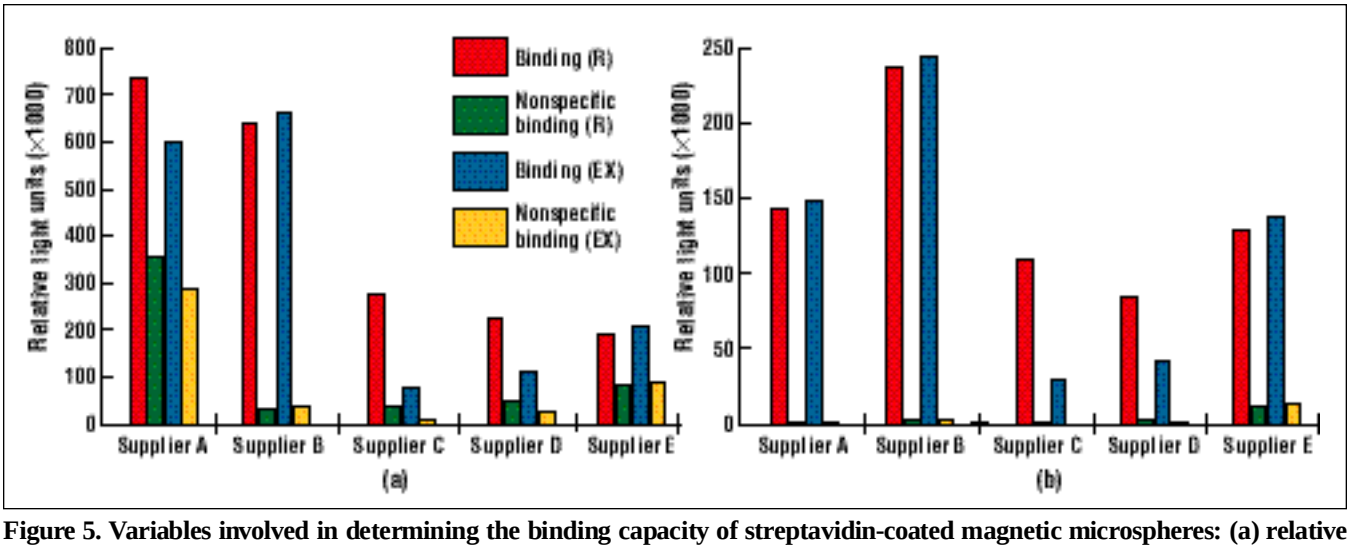

Figure 5. Variables involved in determining the binding capacity of streptavidin-coated magnetic microspheres: (a) relative total binding and nonspecific binding capacity of microspheres from several suppliers based on supplier-reported percent of solids (R) and on experimentally determined (gravimetric) percent of solids (EX); (b) effect of adding and optimizing a blocker to reduce nonspecific binding of the acridinium label.

attached via some other mechanism—normally hydrophobic adsorption to the particle surface (see Figure 5). By designing the binding-capacity assay so that both types of binding can be measured, optimization using various types of blocking molecules can be performed to minimize nonspecific binding.

Conclusion

In the fields of immunology and molecular biology, streptavidin-coated magnetic microspheres offer several advantages over more-conventional solid supports. But to use this type of solid support to the fullest, it is important to first ensure that the binding capacity is fully characterized for the type (size and molecular weight) of ligand that the final application will ultimately use.

Expectedly, as the technology involved in making this type of solid support has advanced, so has the sensitivity and simplicity of the assays used to characterize them. As immunoassays have evolved from radioactive to enzymatic to chemiluminescent detection, binding-capacity assays have followed.

The point that we have tried to stress in this article is that once the choice has been made to base an immunoassay on streptavidin-coated magnetic microspheres, it is important to select the microspheres based on their performance in the assay system under development. Variables that influence performance are not just total binding, but binding of molecules with steric characteristics similar to those of the molecules that will be used in the actual assay. If this information is not available from the manufacturer, one of the assays described here can be used for appropriate characterization. Further, among the streptavidin-coated magnetic microspheres currently on the market, the characteristics of the base particles vary greatly. In developing these binding-capacity assays, we looked at particles from a number of suppliers, and made comparisons based not only on total binding, but on characteristics such as nonspecific binding, ease of handling (how rapidly they could be pulled to a standard magnet), measured versus reported percent of solids, and so on. We feel that these considerations are vital in ensuring that the reagent selected is truly the best choice for a particular application.

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