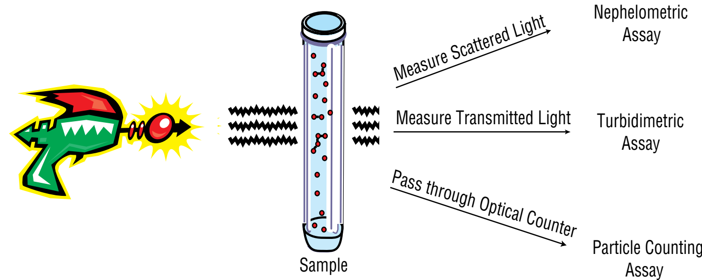
TechNote 304

Light-Scattering Assays

9025 Technology Dr. • Fishers, IN 46038-2886
800.387.0672 • 317.570.7020 • Fax 317.570.7034
info@bangslabs.com • www.bangslabs.com

Bangs Laboratories, Inc.

BEADS ● ABOVE THE REST™



CONTENTS

I. Background
- A. Principles of Light-Scattering Immunoassays
  1. Turbidimetry
  2. Nephelometry
  3. Particle Counting
- B. Kinetics of Immunoprecipitation

II. Particle-Enhanced Immunoassay
- A. Assay Formats
- B. Assay Instrumentation
  1. Spectrophotometer/Fast Centrifugal Analyzer
  2. Nephelometer
  3. Optical Cell Counter
- C. Assay Parameters
  1. Particle Size
  2. Optimum Wavelength
  3. Kinetic vs. Endpoint Monitoring
  4. Antibody/Antigen Considerations
  5. Antigen/Antibody Loading
  6. Buffers and Ionic Species/Optimal pH
  7. Influence of Polymers
  8. Effect of Temperature
- D. Roles of Different Types of Microspheres
  1. Size/Size Distribution
  2. Density
  3. Refractive Index
  4. Colloidal Stability
  5. Streptavidin-Coated Microspheres
  6. Polystyrene/Magnetic Polystyrene
- E. Options for Ligand Attachment
  1. Adsorption
  2. Covalent Attachment
  3. Streptavidin/Biotin Coupling

III. New Developments

IV. References

I. BACKGROUND

A. Principles of Light Scattering Immunoassays

Immunoassays based on the measurement of scattered or absorbed light are an extension of the basic principles underlying latex agglutination tests. The change in the light scattered or blocked by Ab (or Ag) solutions is used to measure the amount of Ag (or Ab) which causes the immunological Ab-Ag precipitation reaction or agglutination reaction (if latex is used).

In a basic light-scattering immunoassay, polyvalent antigens react with divalent antibodies to form large complexes, the antibody effectively forming a bridge between antigen molecules. A protein antigen, which can be considered multivalent, with possibly multiple copies of the same epitope as well as different epitopes, can produce a large immune complex made up of several molecules.

1. Turbidimetry

Turbidimetry is the measurement of light-scattering species in solution by means of a decrease in intensity of the incident beam after it has passed through solution.[1] For turbidimetric assays, the change in the amount of light absorbed (inverse of amount transmitted) can be related to the amount of agglutination which occurs. Hence, the amount of analyte (the species causing agglutination) in the sample can be easily determined.

2. Nephelometry

Nephelometry is the technique for measuring the light-scattering species in solution by means of the light intensity at an angle away from the incident light passing through the sample.[1] Nephelometric assays present an indirect method of measurement of the amount of analyte in a sample by measuring the amount of light scattered or reflected at a given angle (typically 90°) from the origin. In the presence of the protein antigen, the antibody reacts with the antigen, and a precipitation reaction begins. The measurement is taken early in this precipitation reaction time sequence. A quantitative value is obtained by comparison with a standard curve, which has been established previously. In order to increase the sensitivity of the detection, you can adsorb or covalently attach the protein to polymeric microspheres. In this way, a greater signal is produced with less reagent.

(The main practical difference between these two approaches is the

concentration of the solutions used in the assay. Nephelometry is best performed with dilute solutions, as at higher concentrations, destructive light scattering might cause a loss of sensitivity. Conversely, turbidimetry requires a higher density of particles to achieve a measurable and precise signal.)

3. Particle Counting

Particle counting is unique in that it requires a particle-enhanced format. It is an immunoassay technique that recognizes microspheres that are not agglutinated (i.e., it determines the difference between the amount of signal generated when antigen is present or absent). The reagent antigen or antibody is coupled to microspheres and then forms immunocomplexes with the sample antibody or antigen, respectively; the number of unagglutinated microspheres is thus inversely proportional to the analyte concentration.

Table 1 shows a comparison of the detection limits for the measurement of proteins in biological samples1 by turbidimetry, nephelometry, or particle counting detection methods.

Table 1: Comparison of Detection Limits

Monitoring System	Analyte	Sample	[Conc.]	Molar
Turbidimetry: Nonenhanced	Human placental lactogen	Serum	1.6mg/L	$5.5x10^{-8}$
Turbidimetry: Latex particle-enhanced	Retinol-binding protein	Urine	25µg/L	$12x10^{-9}$
Turbidimetry: Gold sol particle-enhanced	Chloriogonadotropin	Serum	50µg/L	$1.6x10^{-10}$
Nephelometry: Nonenhanced	Immunoglobulin M	Cerebral Spinal Fluid	6.1mg/L	$6.3x10^{-9}$
Nephelometry: Latex particle-enhanced	Myoglobin	Serum	6.1µg/L	$3.4x10^{-10}$
Rate Nephelometry	Immunoglobulin M	Cerebral Spinal Fluid	11.1mg/L	$1.1x10^{-9}$
Particle Counting:	C-reactive protein	Serum	1.0µg/L	$0.9x10^{-11}$

B. Kinetics of Immunoprecipitation

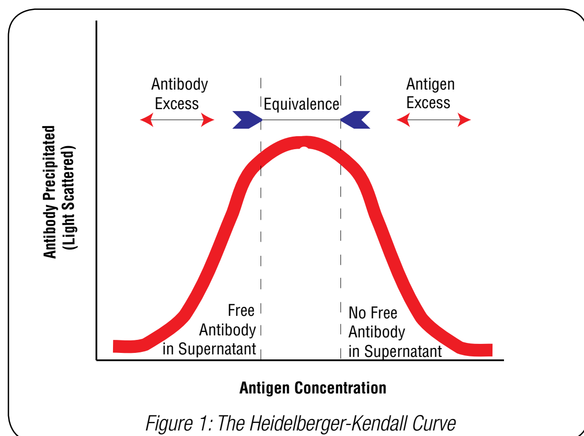


Figure 1: The Heidelberger-Kendall Curve

Figure 1 shows the general relationship between antigen concentration and antibody precipitated.2 There are three distinct elements to the curve in Figure 1. The first region, in which the antigen concentration increases, is one of antibody excess when the immune complexes are small, with some bridging. The second region, 'equivalence,' represents an optimum ratio of antibody bridging in relation to antibody concentration, i.e., enough polyvalent antigen molecules bind both 'arms' of the divalent antibodies in solution; this is the point of maximum lattice, and thus precipitate, formation. In the third region, antigen excess, there is, in effect, a reduced supply of 'bridging' antibody molecules in relation to the amount of antigen now in solution.

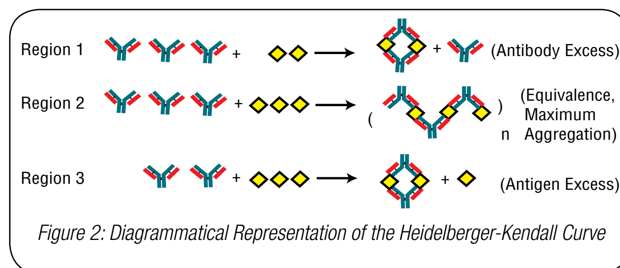


Figure 2: Diagrammatical Representation of the Heidelberger-Kendall Curve

Figures 1 and 2 illustrate two important considerations when developing these types of assays. First, that there are two potential antigen concentrations that will generate the same apparent light-scattering signal; one when there is antibody excess and one when there is antigen excess. For this reason, the time at which measurements are made is an important factor. Second, assuming that the antigen used is divalent, the largest complex formed outside of the region of equivalence will be a triplet (Antibody-Antigen-Antibody). Depending on the sophistication of the instrumentation and the size of the antibodies and antigens used, this is normally not large enough to be measured. As is shown in Figure 1, the region of equivalence represents a relatively high concentration of sample (antigen). For this reason, the direct turbidimetric immunoassay lacks sensitivity for analytes for which small concentrations may hold clinical significance. The solution to these limitations is the particle-enhanced immunoassay, for the following reasons:

1. Sensitivity is increased by increasing the relative light-scattering signal;
2. It provides the opportunity to use a different assay format. Specifically, the ability to test for haptens, whose single epitopic sites make them unsuitable for a direct turbidimetric assay; and
3. The problems that can result from antigen excess, as discussed previously, can be avoided with assay optimization.

II. PARTICLE-ENHANCED IMMUNOASSAY

A. Assay Formats

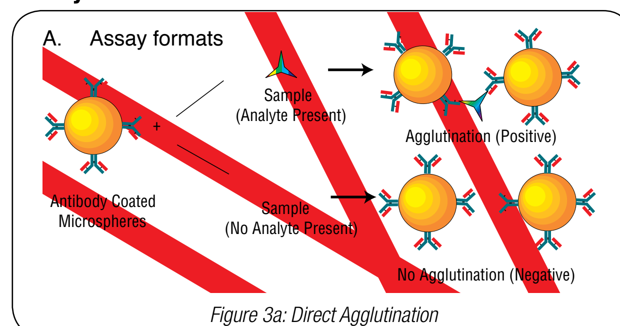


Figure 3a: Direct Agglutination

Figure 3a illustrates a format known as direct agglutination. This format can be used for turbidimetric or nephelometric monitoring, and is only useful for polyvalent antigens, such as proteins and microorganisms. Here, the amount of antibody conjugated to the microspheres can be varied considerably, provided that it is present in excess of the amount of antigen in the sample (preferably at or near the equivalence point shown in Figure 1).

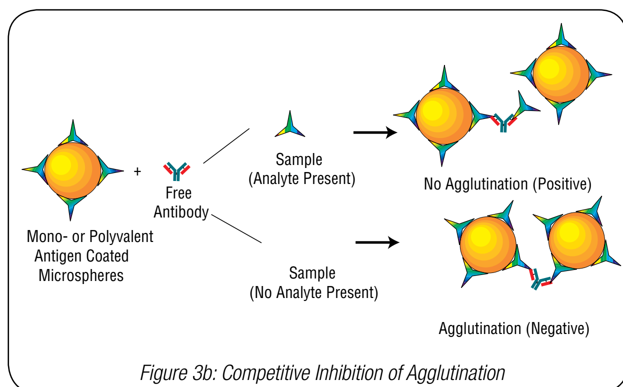


Figure 3b: Competitive Inhibition of Agglutination

Figure 3b illustrates a format known as competitive inhibition of agglutination. This format is used most often to measure monovalent antigens, such as haptens. The antigen can be directly attached to the microsphere surface, or can be coupled to the carrier, such as a protein. Bovine serum albumin is frequently used for this purpose. Here, assay sensitivity is inversely related to the amount of antigen conjugated to the surface of the microspheres; the lower the loading, the greater the sensitivity.1 However, the concentration of surface-conjugated antigen must be sufficiently high to allow aggregate formation at zero analyte concentration, in order to measure a suitable baseline for the assay.

A variation of the format shown in Figure 3b is the dual particle assay. This is also a competitive inhibition assay, but offers the potential for increased sensitivity, as both antibody and antigen are conjugated to microspheres. For this reason, a lower concentration of sample antigen is needed for detection.

B. Assay Instrumentation

1. Spectrophotometer / Fast Centrifugal Analyzer

Turbidimetric measurements can be made with a spectrophotometer, and the signal will be a function of several factors, including monochromator wavelength, spectral bandwidth, stray light, cuvette path length and geometry, light source, and detector stability.[1] As the sophistication of spectrophotometers has increased over the years, so has the popularity of their use as turbidimeters in this type of assay. Centrifugal analyzers and other discrete analyzers, in which either the reaction cuvettes or the optics are rotated while the other remains stationary, creating a regular scanning mode with respect to time, have also proved to be very precise turbidimeters.

2. Nephelometer

This instrument, used for reaction monitoring, is strongly influenced by the angle from the incident beam at which detection occurs. The forward angle offers the greatest potential sensitivity for larger scattering species (such as those found in particle-enhanced immunoassays).[1] However, forward angle measurement can be difficult to achieve from an engineering standpoint, as its use will require that the forward scattered light can be differentiated from the incident beam. Secondly, it is often desirable for many clinical applications that both scattered and transmitted light can be measured with the same analyzer. For these reasons, 90˚ light scatter is typically used for nephelometric monitoring. One advantage of nephelometric monitoring is that the sensitivity can be adjusted to specific assay requirements by setting the detector sensitivity appropriately. In this case, one must also take into account the increased noise that will result. For these instruments, the intensity of the light source also plays a large role in the sensitivity of the assay, with laser illumination offering the greatest sensitivity.

3. Optical Cell Counter

Particle counting assays rely on this type of equipment, as these are designed to recognize a narrow range of particle sizes, thereby ensuring that agglutinated particles are not detected. These can be used with either the direct or competitive inhibition formats described previously, and particle detection is either via a change in electrical resistance as they pass through a counting chamber with a controlled aperture, or by light scattering.[3] In other words, if using 0.2µm particles, light scatter or change in electrical resistance from any particles less than 0.2µm or greater than 0.4µm can be ignored electronically. One feature of using this type of instrumentation for reaction monitoring is that dimers can be detected easily, and therefore there is an increased sensitivity over monitoring with a spectrophotometer or nephelometer, which require larger immune complexes in order to be accurately quantified. This type of detection is the basis for the Copalis® Multiplex Technology developed by DiaSorin.

C. Assay Parameters

1. Particle Size

The two primary considerations when choosing the correct microspheres for light-scattering immunoassay formats are their size and size distribution.

Microspheres which scatter light best have diameters approximately equal to the wavelength of the light being scattered. Therefore, for visible light (λ = 380-770nm), the best scattering microspheres have diameters of 380-770nm (0.38-0.77µm). Microspheres outside of this range will not scatter as well. Theoretically, detection of doublets would give the most sensitive assay, and this would be maximized for a particle size that is half the wavelength of the light used for illumination. In practice, there is a great deal of experience with small particles (<100nm, measured using 340nm light) used for light scattering. Particle size selection also depends greatly on the optical system (not just wavelength). Microspheres less than 0.1µm are poor scatterers and, as they agglutinate, quickly grow to a size where they scatter light much better. This change of scattered light vs. analyte concentration can be the basis for very sensitive end-point or rate method immunoassays. UV light requires smaller microspheres (<< 100nm) and infrared light can use ~0.5µm microspheres.

Conversely, one can also start with microspheres which scatter light well (perhaps 0.5µm microspheres) and observe them clumping to sizes where they fall out of solution and do not scatter as well. Most assay systems seem to use the principle of small, poorly-scattering microspheres clumping to form big, good light-scattering clumps.

It is best to use microspheres with a narrow size distribution, because the maximum change in light scattering occurs when single particles combine with others to form dimers. If the size distribution is broad, the instrument used for detection could mistake a dimer for a larger singlet within the microsphere population.

2. Optimum Wavelength

Regarding the optics involved in this type of assay, there are three important points to consider:

a. The optimum wavelength for turbidimetric monitoring (and

nephelometric monitoring to a lesser extent) increases with the size of the immune complex. Thus, for monitoring of protein-antibody complex formation, a wavelength of 340nm (or less) is preferred, partly because it will enable detection of the early stages of complex formation more quickly. Based on a survey of the literature that has been published regarding particle-enhanced light scattering immunoassays, the most common approach is to use 40-60nm particles, with a light source at 340-360nm.

b. Nephelometry appears to be more sensitive to smaller particles than turbidimetry, apparent from the more rapid kinetics in a reaction mixture monitored by both nephelometry and turbidimetry.[1] Destructive light scattering may occur as larger complexes form, and an apparent plateau is reached in the signal, produced more quickly than in the case of turbidimetry.

c. A plateau in the signal (and thus the equivalence point), particularly using nephelometry, is influenced by optical, as well as reagent, considerations and is only therefore applicable to that set of reagents and sample conditions, and the optical characteristics of the monitoring system used.

3. Kinetic vs. Endpoint Monitoring

Given that an instrument is capable of gathering light-intensity data at precise time intervals after the initiation of the reaction, it is widely accepted that kinetic monitoring techniques offer advantages over end-point procedures. The major benefit of kinetic monitoring, assuming that it is possible to take a reading immediately after initiation of the reaction (less than 5 seconds), is effectively the ability to take both a reagent and a sample blank reading. If there is a delay in taking this reading, sensitivity will be reduced. It is thus important when optimizing your reaction conditions to choose a reaction rate that enables this early blank reading.

Although a kinetic mode for reaction monitoring may involve only two data points, the choice of read points can influence the apparent accuracy, when differences in reaction kinetics between sample and calibrator exist. This may lead to significant changes in the calibration curve for different data collection periods. In general, the use of the second data collection point (near the apparent end point) will minimize the influence of sample-to-sample variations in reaction kinetics.

An alternative approach to reaction monitoring is to use continuous monitoring of the reaction, to show that the peak rate of change of light scatter was related to antigen concentration. Assays could be optimized in such a way that the peak rate was reached in less than 40 seconds, the relationship of peak rate to antigen concentration being similar to that of the Heidelberger-Kendall curve (Figure 1).

The important criteria in choosing the right equipment for reaction monitoring include the precision of the optical measurements, the linear response, and the ability to take an early reading. Some of the variables to be considered when optimizing assay reaction monitoring include:

a. The ability to take an early reading of the light scattering will enable the measurement of a sample and reagent blank reading in a single cuvette, along with the monitoring of the reaction.
b. Nephelometry detects smaller particles and consequently appears to give a faster rate of reaction.
c. It is difficult to obtain a sample and reagent blank in the reaction cuvette in the case of nephelometry, because of the fast reaction kinetics.
d. Turbidimetry, because of slower apparent kinetics, will enable monitoring of sample and reagent blanks, as well as the immunochemical reaction, in a single cuvette. As a consequence, turbidimetry generally gives better precision than nephelometry.

Note: Sections 4-8 are the real tools that an assay developer uses to optimize assay performance. (Sections 1-3 are also options, but may be pre-determined.)

4. Antibody / Antigen Considerations

In the case of an antibody particle reagent for a direct aggregation assay, experience has shown that there is an optimum level of protein loading to achieve the best results (i.e., maximum signal change). As the protein is loaded, the overall size increases and multiple protein layers may be introduced. The colloidal stability of the particle can decrease with increased protein loading; the practical implications of this being twofold:

a. Nonspecific aggregation (or aggregation in the absence of the ligand intended to cause aggregation [Figure 3]) may occur when sample is added; and
b. The particle becomes highly susceptible to self-aggregation when polyethylene glycol (PEG) is added.

Clearly, overloading the microsphere surface with antibody may also lead to steric hindrance and less availability of binding sites. Also, it is likely that overloading will result in poor batch-to-batch coupling reproducibility (and thus assay performance from lot to lot).

In the case of an inhibition assay, where the antigen is coupled to the microspheres, sensitivity is related to antigen concentration; the lower the loading, the greater the sensitivity. However, sufficient antigen has to be loaded to ensure a measurable level of aggregate formation. It is then important to block the remaining bare surface of the microspheres with, for example, bovine serum albumin or detergent. This is to prevent nonspecific adsorption.

5. Antigen / Antibody Loading

<u>Direct Agglutination</u>

The two primary considerations here are the type of antisera chosen and the proper loading onto the microspheres. In a direct agglutination assay, the antibody is coupled to the microspheres. As a reagent excess system, the amount of antibody present will determine the rate of reaction (by the law of mass action), and the calibration range. Some general points to consider are[1]:

a. Polyclonal antisera in general are more successful for the development of direct agglutination assays. Experience has shown that many monoclonal antibodies lose functionality when coupled, even when using a cocktail of monoclonals.
b. Final performance will depend on the affinity and avidity of the antisera.
c. The influence of antibody loading on the reaction kinetics for a direct assay format are such that, as the amount of antibody loading increases, the reaction time decreases.
d. Affinity purified antibody preparations generally perform better in quantitative assay systems compared to whole antisera. This allows for both closer control of the density of the reactive species and a higher surface density of antibody molecules, while maintaining surface protein loading within acceptable limits.
e. Use of $F(ab')_2$ fragments will increase the potential binding capacity compared to whole IgG molecules.
f. Use of $F(ab')_2$ fragments will reduce the potential for interference from rheumatoid factor.
g. Covalent attachment or attachment of biotinylated antibodies to streptavidin-coated microspheres (as opposed to passive adsorption to

non-functionalized microspheres) will improve reagent stability, and thus calibration stability.

h. Antibody characteristics may be modified by coupling to particles, and at higher protein concentrations steric hindrance may limit functional capacity of bound antibody (hence the preference for affinity purified antibody over whole antiserum).

Competitive Inhibition of Agglutination

In the case of small molecules using this format, it is important to ensure that the coupling of the hapten is carried out in such a way that the exposure / presentation of the epitope is ensured. Some general considerations here are[1]:

a. Haptens are preferably coupled to the microspheres through a linker molecule, which ensures optimal steric presentation of the molecule, as well as minimal steric hindrance from blocking groups / molecules on the surface of the microspheres.
b. Linker molecules, such as polylysine and polyether polyamines, are designed to separate the hapten from the surface, but not to be of sufficient length to enable the arm to bend over, allowing the hapten to bind to the surface at another point. Typically, these molecules are 4-8 amino acid residues in length.
c. Alternative approaches are to link the hapten to a protein layer on the microsphere's surface (a common choice being bovine serum albumin), or to biotinylate the hapten, with the biotin being attached via a linker, and then attaching the biotinylated hapten to streptavidin-coated microspheres. Linkers of variable lengths and with different active groups are commercially available.
d. Antigen loading must be highly reproducible because the inhibition format is a limited reagent system. Both the microsphere-bound antigen and antibody reagent in solution have an influence on the initial rate of immunoagglutination (i.e., in the absorbance of sample antigen).
e. Every effort must be made to remove any adsorbed hapten after covalent coupling. Subsequent leaching off into the reagent will reduce the sensitivity of the assay, and can be the root cause of apparent assay instability.

6. Buffers and Ionic Species / Optimal pH

It has been shown1 that the primary antibody/antigen reaction light scattering assays are strongly influenced by the nature of the ionic medium in which the reaction is carried out. This can be described by the Hofmeister series (Figure 4), such that ions that promote macromolecular unfolding inhibit immunoprecipitate formation, whereas those that inhibit unfolding promote immunoprecipitate formation.

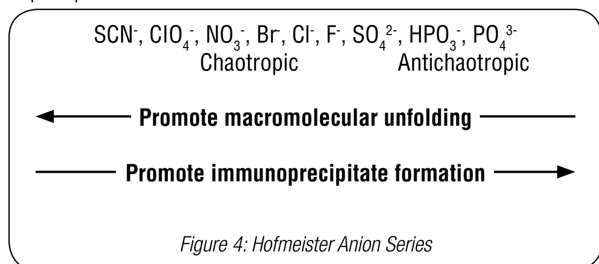


Figure 4: Hofmeister Anion Series

There is also a cationic series described by Hofmeister, as shown in Figure 5.

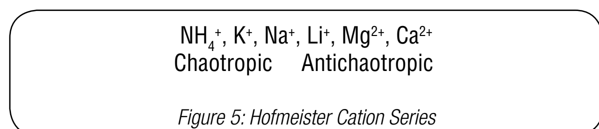


Figure 5: Hofmeister Cation Series

The reaction pH will also influence the rate of aggregate formation, although the rate of reaction is fairly consistent over the pH range of 6-8. Reduction of the reaction pH will lead to some proteins having a net positive charge (those with a pI above the reaction pH), leading to agglutination with negatively charged proteins or particles.

The ionic strength of the reaction environment can also have a profound effect on the rate of the antigen-antibody reaction. As the ionic strength increases, the depth of the electrical double layer that forms around a charged molecule is compressed, reducing the distance over which repulsive forces that keep molecules apart can act. This, in effect, leads to the promotion of aggregation. The reduction in charge will also influence the electrostatic attraction between oppositely charged species, which may then reduce specific binding. This increase in ionic strength can be used to minimize nonspecific interactions; however, further increases in ionic strength may inhibit the antigen-antibody reaction.

7. Influence of Polymers

A variety of polymers have been shown to influence the solubility of proteins, possibly by exclusion of water from the reaction microenvironment. It has been shown that the forces between two charged species are repulsive at large distances, but decrease at short distances and become attractive as van der Waals forces take over. However, there is a repulsive force component caused by water molecules in a hydration layer. It is argued that water molecules are squeezed out as the molecules bind. Thus, any means of assisting the removal of water will enhance the rate of binding (or complex formation in the case of antigen-antibody reaction). Non-ionic polymers, such as polyethylene glycol (PEG), enhance the rate of immunoaggregation, increase the light-scattering signal, and extend the antigen concentration at which equivalence occurs.

The effect of polymers is dependent on both concentration and size, with higher concentrations of smaller polymers having an effect similar to lower concentrations of larger polymers. In practice, however, the smaller polymers are more manageable because of their greater solubility.

PEG is available in a number of molecular weights. The most common size for this type of assay is PEG 6000.[4] When determining how to incorporate the polymer into your assay development scheme, some points to consider are:

a. Increasing the PEG concentration will increase the apparent rate of agglutination. In the case of a turbidimetric assay system, this may also increase the signal change.
b. There is a close link between protein loading on a particle and PEG concentration. As the protein loading increases, the sensitivity to PEG increases. In other words, less PEG is required to achieve an increase in the apparent rate of agglutination. Above a critical concentration of PEG at a given protein loading, the protein loaded particles will agglutinate in the presence of any other protein, increasing problems of nonspecific aggregation. Microsphere auto-agglutination can also increase.

8. Effect of Temperature

Although not conventionally considered to be temperature-dependent, the antigen-antibody reaction rate will obviously vary with reaction temperature. In one study of a range of antisera used in a turbidimetric assay format, a considerable variation in temperature dependency of reaction rates between antisera has been demonstrated.1 Increasing the reaction temperature will decrease the equilibrium constant because the molecules move more quickly. While a light-scattering assay is not an equilibrium reaction, it is correct to say that the rate of association will increase with temperature.

D. Roles of Different Types of Particles

Several of the variables to consider when choosing the appropriate microspheres for a light scattering assay include size (and size distribution), density, refractive index, presence or absence of surface functional groups, and colloidal stability.

1. Size / Size Distribution

Table 2: Relative Size of Microspheres (Before Aggregate Formation) for the Different Types of Detection Methods (d = microsphere diameter, in nm)

Detection Method	Microsphere Diameter (nm)
Naked eye	300 < d < 1000
Turbidimetry	d < 100
Nephelometry (Light Scattering Angle)	d < 100
Nephelometry (Small Scattering Angle)	100 < d < 800

It is best to use polymeric microspheres with a narrow size distribution. Because maximum change in light scattering occurs when single microspheres combine to form dimers, a broad size distribution would make singlet/dimer differentiation difficult. Smaller microspheres have the benefit of providing the largest surface area (relative to volume), and thus the potential for higher antibody loading. In this respect, protein loading will in itself obviously increase the size of the microspheres (a monolayer of IgG increases the diameter by approximately 10nm).[1]

2. Density

Smaller and near-neutral density microspheres offer the benefit of greater movement in the liquid phase, while also minimizing the degree of settling, thereby negating the need for constant mixing of the reagent to maintain homogeneity. The composition of the storage buffer also plays a large role in the microsphere settling time.

3. Refractive Index

The basis for the light-scattering immunoassays described is the ability of the aggregates to scatter light to a greater degree than that which can be achieved by the suspending medium alone. If this suspending medium is water (refractive index = 1.333 at 569nm), polystyrene microspheres, which have a refractive index of 1.591 at 569nm, serve as a reliable solid support, and light scatterer, to yield good sensitivity in the assay.

The work by Price, Newman, et al.,[5] has prompted interest in very small microspheres with higher refractive indices. For polyvinylnaphthalene, n_D = 1.69 at 569nm. These "brighter" microspheres scatter light better, especially when they agglutinate to the optimum scattering size. One can also get a higher refractive index for polystyrene by using a shorter wavelength of light (n_{400nm} = 1.63).

4. Colloidal Stability

The surface charge of the microspheres plays an important role in assay performance. It is determined by the nature of the particles' original surface chemistry, the nature of the protein or other ligand coupled to the particle, the type and amount of detergent or other blocking molecules present, and the nature of the reaction buffer. In general terms, neutral microspheres will tend to self-aggregate, whereas highly charged microspheres will remain dispersed because of repulsion between microspheres. Too great a charge may result in no aggregation in the presence of sample analyte, because the binding energy of the antigen-antibody reaction is too low to overcome the repulsion.

5. Streptavidin-Coated Microspheres

A rate-dependent turbidimetric immunoassay for theophylline has been described,[6] which allows for simplified ligand attachment by utilizing the natural affinity that streptavidin has for biotin. This work used a competitive inhibition format, but could be used for direct agglutination formats as well.

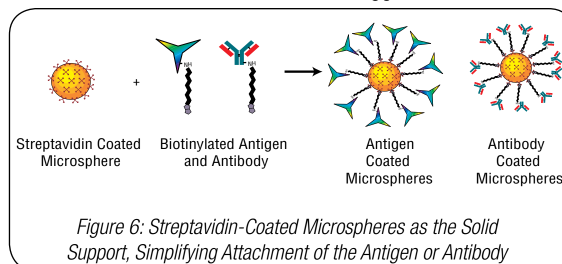


Figure 6: Streptavidin-Coated Microspheres as the Solid Support, Simplifying Attachment of the Antigen or Antibody

The use of streptavidin-coated microspheres for assay development presents several advantages over conventional means of ligand attachment, including:

a. Biotinylation, or attaching biotin to the ligand of interest, is a simple process, and virtually any ligand can be biotinylated using commercially available kits.
b. The biotin-streptavidin attachment is strong (K_d ~ 10^{15}), and therefore makes a more stable and permanent reagent than if the ligand were passively adsorbed, without the optimization required for covalent coupling protocols.
c. The difficulties of hapten attachment (e.g., the need for preliminary attachment to a carrier protein, such as BSA) are eliminated, as the biotinylated hapten can be attached directly to the microspheres.

6. Polystyrene / Magnetic Polystyrene

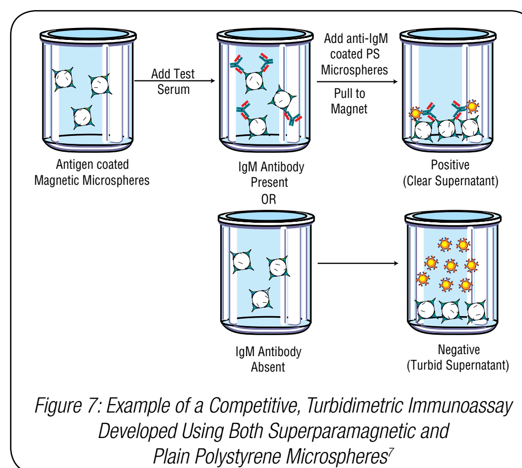


Figure 7: Example of a Competitive, Turbidimetric Immunoassay Developed Using Both Superparamagnetic and Plain Polystyrene Microspheres[7]

There are two stages in this test procedure. Initially, test analyte, if present in the sample, is introduced to antigen-coated superparamagnetic microspheres, forming an antigen/antibody complex at the surface of these microspheres. Next, small, anti-(test analyte) antibody-coated polystyrene microspheres are added to the suspension. If the analyte of interest is present in the sample, agglutination will occur between the magnetic and polystyrene microspheres. By gravimetric or magnetic separation, these complexes will settle out of solution, leaving the supernatant clear. If the test analyte is not present, no agglutination will occur, and the small polystyrene microspheres will remain suspended by Brownian motion. The presence of these microspheres can be detected by measuring the turbidity of the supernatant. If the supernatant remains turbid after the magnetic microspheres have settled, the test is negative. The degree of turbidity in relation to the amount of polystyrene microspheres added can be used to make the assay quantitative.

E. Options for Ligand Attachment

There are three basic approaches to attaching ligands to microspheres.

1. Adsorption

This is advantageous in terms of the simplicity of attachment. However, possible drawbacks are that the hydrophobically adsorbed ligands could possibly become detached under harsh environmental conditions, and that many haptens are not hydrophobic enough to be efficiently adsorbed. If adsorption is chosen as the means of ligand attachment, a general protocol can be found in our TechNote 204.

2. Covalent Coupling

Although the chemistry involved in this type of attachment is more involved than with passive adsorption, the covalent bond allows for a more permanent reagent, thereby increasing the shelf-life of the assay kits.[8] A wide variety of surface functional groups, and hence coupling chemistries, are available. An overview of the choices, along with protocols for attachment to each surface functional group, can be found in our TechNote 205.

3. Streptavidin / Biotin

This ligand coupling scheme combines the ease of passive adsorption with a bond strength nearing that of covalent attachment. In addition, biotinylation kits are commercially available that use varying linker lengths, between the ligand and the biotin, thereby simplifying linker length optimization. If the linker is too long, a hydrophobic hapten can double back on itself and interact with the microsphere surface. (Ask us about the availability of a wide range of ProActive® Streptavidin, Protein A, or secondary antibody-coated microspheres.) Protocols and hints for attaching biotinylated ligands to these microspheres can be found in our TechNote 101.

III. NEW DEVELOPMENTS

A recent novel development involving this type of test is the use of an ultrasonic standing wave to enhance the rate of aggregation. Microspheres suspended in an ultrasonic standing wave (not sufficient to induce cavitation or acoustic streaming) rapidly concentrate at positions of potential energy minima in the field. The concentrated microspheres also experience sound-induced particle-particle interactions, the extent of these being dependent on size, density, and compressibility of the particles. A decrease in reaction time between 14- and 50-fold has been found compared to conventional agglutination systems.[11] Another reference,[12] using ultrasound to enhance the rate of agglutination of *Legionella pneumophila*, found that agglutination occurred with an antibody 512 times more dilute than occurred without ultrasound. In addition, detection of agglutination for two identical samples was 100 times faster using ultrasound than otherwise. These findings point to ultrasound as a means of both increasing reaction times and lowering the sensitivity limits of light-scattering immunoasssays.

Work has been done on making light scattering useful for point-of-care (POC) applications. In order for this to be practical, it is necessary to design a self-contained device where all of the components, including diluents, are encapsulated in a complex disposable plastic unit that only requires addition of sample. These devices generally require an instrument for reading a photometric endpoint. An example of a POC, light-scattering immunoassay that is currently on the market is the Biotrack 516 from Roche.[9] A study has been done that shows that results obtained on this device compare well to Abbott's TDx®, for the measurement of theophylline levels in blood. Also, analytes not traditionally measured by light-scattering immunoassays, such as haemoglobin A1c, can be combined with related analytes to create a diagnostic panel with only the need for one sample. An example would be one analyzer that could measure blood glucose, cholesterol and HbA1c, such as the Dimension® analyzer made by Dade-Behring.[10]

IV. REFERENCES

1. **Price, C.P., D.J. Newman.** 1997. *Principles and practice of immunoassay.* New York: Stockton Press, p. 445-472.
2. **Thompson, J.C., et al.** 1997. Kinetics and proposed mechanism to the reaction of immunoinhibition, particle-enhanced immunoassay. *Clin Chem,* 43(11): 2384-2389.
3. **Bernard, A.M., et al.** 1982. Latex immunoassay of retinol-binding protein. *Clin Chem,* 28(5): 1167-1171.
4. **Wojciechowicz, D., et al.** 1988. A turbidimetric assay for measuring proteins in culture media. *J Immunol Methods,* 106(1): 57-61.
5. **Medcalf, E.A., D.J. Newman, E.G. Gorman, C.P. Price.** 1990. Rapid, robust method for measuring low concentration of albumin in urine. *Clin Chem,* 36(3): 446-449.
6. **Oh, C., J. Kim, et al.** 1993. A rate turbidimetric immunoassay for theophylline using biotin-avidin system. *Clin Chem Acta,* 218(1): 59-71.
7. **Lim, P.L., K.H. Ko, W.F. Choy.** 1989. A two-particle turbidimetric latex immunoassay for the detection of specific IgM antibodies. *J Immunol Meth,* 117(2): 267-273.
8. **Thakkar, H., et al.** 1991. Stabilization of turbidimetric immunoassay by covalent coupling of antibody to latex particles. *Clin Chem,* 37(7): 1248-1251.
9. **Klotz, U.** 1993. Comparison of theophylline blood levels measured by the standard Tdx assay and a new patient-side immunoassay cartridge system. *Ther Drug Monit*, 15(5): 462-464.
10. **Holowina, P., E. Bishop, D.J. Newman, et al.** 1997. Adaptation and validation of a particle enhanced assay for percent glycated hemoglobin to a DuPont Dimension® analyzer. *Clin Chem,* 43: 76-84.
11. **Thomas, N., W. Coakley.** 1996. Measurement of antigen concentration by an ultrasound-enhanced latex immunoagglutination assay. *Ultrasound in Medicine and Biology*, 22: 1277-1284.
12. **Grundy, M.A., R.A. Barnes, W.T. Coakley, et al.** 1993. Rapid agglutination testing in an ultrasonic standing wave. *J Immunol Meth*, 165: 47-57.

JD - 12/98

Copyright 1999, Bangs Laboratories, Inc.
All Rights Reserved