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Comprehensive Review

Latex Immunoagglutination Assays*

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Latex immunoagglutination assays continue to be widely used in biology and medicine for the detection of small quantities of an antibody or antigen of interest in fluid test samples. Main characteristics of preparation and use of these assays are examined here. Physical adsorption of proteins onto latex particles surface, with special relevance to immunoglobulins, is analyzed with major attention to those factors that influence adsorption: medium conditions such as pH and ionic strength, surface characteristics as type and amount of charge, or hydrophobicity. Different functionalized latexes for covalent linking are also presented, as well as the corresponding chemical reactions. Techniques for the detection and quantification of the immunoreaction are briefly summarized, including visual observation, light scattering, turbidimetry, nephelometry, and angular anisotropy. Finally, some problems of colloidal stability of these latex assays are analyzed, as well as the different solutions applied by scientists to solve them.

I. Introduction

Recent years have heralded an increase in the use of clinical diagnostic methods involving immunological procedures because they are specific and have high sensitivity. Of the many heterogeneous and homogeneous immunological assay methods available, those based on the agglutination of latex particles continue to be widely used in biology and medicine for the detection of small quantities of an antibody or antigen in a fluid test sample. Some advantages of these assays are that the procedures are simple, widely applicable, and nonhazardous, and test results are obtained in a very short time. The agglutination reaction involves in vitro aggregation of microscopic carrier particles (usually of polymeric nature, referred to as latex). This aggregation is mediated by the specific

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reaction between antibodies and antigens, one of which is immobilized on the surface of the latex particles to enhance the sensitivity and extend the point of equivalence. In one format, a fluid containing the ligand of interest is introduced into a suspension of the sensitized carrier particles, and the presence of agglutination is noted as indicative of the ligand. The degree of agglutination plotted as a function of the agglutinant concentration follows a bell-shaped curve similar to that for precipitin. The agglutination reaction may be used in several different modes to detect an antigen or antibody (the ligand of interest), and each has its own limitations and applications:

1. *A direct latex agglutination test for the detection of the presence of an antigen or hapten in a biological sample.* The biological sample is mixed with a suspension containing antibodies against that antigen bound to latex particles (Fig. 1). If antigen is present in the sample it will react with the antibodies to form an aggregate. If no antigen is present in the sample the mixture will keep its appearance as a smooth suspension. This method is applicable to the detection of polyvalent antigens, e.g., proteins and micro-organisms.
2. *An indirect latex agglutination test for the detection of an antibody in a biological sample.* This works based on similar principles whereby antigens of the antibody corresponding are bound to latex particles (Fig. 1). This approach is applicable to mono- and polyvalent antigens, e.g., drugs, steroid hormones, and proteins.
3. *An agglutination inhibition mode using antigen immobilized particles.* A fixed quantity of antibody is mixed with a dilution of the test sample containing the ligand of interest. This reaction mixture is then combined with the antigen immobilized carrier particles. The degree to which the ligand of interest (antigen) in the test sample inhibits the aggregation of the carrier particles that would otherwise have occurred, indicates the concentration of ligand present in the sample.

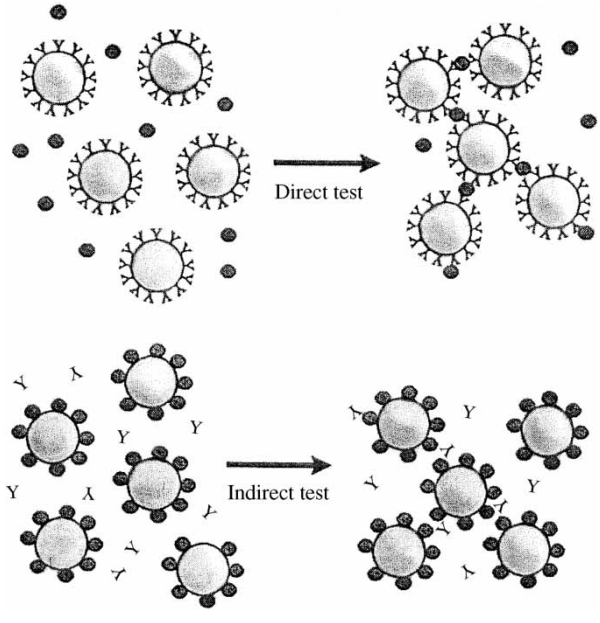


Figure 1. Latex immunoagglutination assay: antibody-coated particles agglutinated by antigen molecules (direct test); antigen-coated particles agglutinated by antibody molecules (indirect test).

4. *An agglutination inhibition mode with antibody immobilized particles.* A fixed quantity of antigen is mixed with a dilution of the test sample containing the ligand of interest (a specific antibody) which inactivates a portion of the antigen. This reaction mixture is then combined with the antibody-immobilized carrier particles. The degree to which the ligand (antibody) present in the test sample inhibits the aggregation of carrier particles, in comparison to that which would otherwise have occurred, indicates the concentration of antibody present.

Latex immunoagglutination assay was first described in 1956 by Singer and Plotz (1) and applied to rheumatoid factor. One can realize the importance of this kind of assays when perusing the specialized literature. In the last decade alone more than 400 publications in medicine and veterinary journals reported the use of latex immunoagglutination assays as analysis or research tools. The popularity of this diagnostic technology is illustrated by the fact that in 1992 there were over 200 commercial reagents available employing this approach to detect infectious diseases from "strep throat" to AIDS (2) These include bacterial, fungal, parasitic, rickettsial, and viral diseases. The tests are also useful for cancer detection and for identification of many other substances (hormones, drugs, serum proteins, etc). The most familiar application of latex immunoassays is the pregnancy determination. In this procedure, a suspension of latex particles covered by human chorionic gonadotropin (HCG) is mixed with a drop of urine. New latex applications and technologies are still being devised and applied to new analytes.

Immunoglobulins are bifunctional molecules that not only bind to antigens but also initiate a number of other biological phenomena such as complement activation and histamine release by mast cells (activities in which the antibody acts as a directing agent). These two kinds of functional activities are localized to different portions of the molecule: the antigen binding activity to the Fab and the biological activities to the Fc portion of the molecule. Structurally they have a tetrameric arrangement of pairs of identical light and heavy polypeptide chains held together by non-covalent forces and usually by interchain disulfide bridges. Each chain consists of a number of loops or domains of more or less constant size. The N-terminal domain of each chain has greater variation in amino acid sequence than the other regions, and it is this factor that imparts the specificity to the molecule. There are five types of heavy chains, which distinguish the class of immunoglobulins IgM, IgG, IgD, IgA, and IgE, and two types of light chains. Of these, immunoglobulin G (IgG) is the most abundant and its structural characteristics are better understood.

In the main immunoagglutination assays, the particles employed to adsorb the antibody or the antigen are latex particles rather than another kind of solid support (e.g., sheep or human erythrocytes, metal sols, etc.). This is due to the following factors: (1) Uniform size particles can be synthesized with diameter in the range 50–10,000 nm. The monodispersity is an important property for detection of immunoagglutination by light scattering techniques. (2) A wide selection of functional groups can be incorporated onto the latex surface to bind proteins covalently or to achieve colloidal stability. (3) Biological molecules adsorb strongly to the hydrophobic surface of latex particles.

In the recent past, numerous researchers have done extensive work in an attempt to optimize the multiple variables affecting the reproducibility, detection limit, analytical range, sensitivity, and reliability of latex immunoassays. Some of the parameters that must be taken into account are the size of the particles used to immobilized the biological molecule, the surface charge density and hydrophilic-hydrophobic nature of the particle surface, the means of attachment of the biological molecule to the latex, the experimental

conditions for immobilization and agglutination, and the optical method for detecting the extent of immunoaggregate formation (3).

The attachment of molecules to latex particles can be achieved through physical adsorption or covalent coupling. Polymer engineering has facilitated the synthesis of latex particles with surface reactive groups that enable covalent coupling of protein molecules to the particle. In addition, spacer groups may be introduced between the particle surface and the immunoproteins. The spacer groups are thought to permit a degree of freedom to the reagent moiety separating it from the particle surface, thereby lending enhanced specificity.

Apart from some visual methods for detecting qualitatively the agglutination of sensitized particles, there exist optical techniques to quantify the agglutination. The most important ones are turbidimetry, nephelometry, angular anisotropy, and photon correlation spectroscopy (light scattering measurements). The require particle size is different for qualitative (bigger particles) than for quantitative methods (smaller particles). In general, for visual slide agglutination the particle diameter range is 0.2–0.9 μm, whereas for light scattering immunoassays the diameter range is 0.01–0.3 μm.

The major problem of particle-based assays, which require careful attention, is the nonspecific agglutination. The presence of this nonspecific agglutination has been one of the main reasons why, for a long time, latex immunoagglutination tests were considered to be semiquantitative at best (4). Nonspecific agglutination can be caused by a variety of factors:

(1) Many body fluids, such as serum, often contain other undefined substances in addition to the particular analyte of interest. Such substances can cause or inhibit agglutination. The mechanisms by which they interfere are poorly understood, and no particular causative agent or set of conditions is responsible for these effects. Moreover, interferences of these types cannot be corrected by comparison of the assay results with a similar assay using a sample not containing the analyte in question as a blank sample because the blank may not be truly representative of the serum under test. As a result, much time and effort has been expended in the search of eliminating nonspecific interferences. Some methods of reducing nonspecific interferences in latex immunoagglutination assays are as follows: massive dilution of the test sample; addition of detergents: covering of the bare surface of the sensitized particles with inactive proteins; rigorous pretreatment of the test sample including heat treatment for 30 minutes at 56°C; and enzymatic treatment with proteases reaction. These procedures are time consuming and can carry with them the undesirable effect of drastically reducing the potential sensitivity and accuracy of the immunoassay as a result of the required manipulations.

(2) Sometimes nonspecific agglutination occurs by a bridging mechanism. This mechanism assumes that the biomolecule attached to the particle has chains or loops extending to the dispersion medium sufficiently far to encounter another particle, provoking the unspecific linking of the two particles. The agglutination by bridging phenomena is important at low degrees of coverage. This process can be eliminated using an inactive protein to cover the free surface of antibody-coated particle.

(3) After the protein coating procedure, the latex particles show low colloidal stability and the aggregation occurs at pH and ionic strength values reproducing the physiological conditions. This self-aggregation process is undesirable. The difficulty in keeping the protein-coated particle system colloidally stable is the main reason that half of all latex immunoagglutination testing is unsuccessful.

II. Physical Adsorption

Under most conditions globular proteins, such as enzymes and immunoglobulins, show a strong tendency to adsorb at interfaces. This surface-active behavior of proteins is utilized in various biomedical and biotechnological applications. In many of those applications the sorbent material is supplied as a dispersion to reach a large surface area to volume ratio and, hence, to accommodate sufficiently large amounts of adsorbed protein in a given volume. Examples are the immobilization of enzymes on solid matrices in biocatalysis (5) and of immunoglobulins in clinical diagnostics (6). Possible advantages of immobilization are, among others, reusability and apparent stabilization of the protein, as well as visual amplification of the antigen–antibody reaction in the case of immunodiagnostic tests.

Effective control of any of these processes requires an understanding of the driving force(s) for adsorption, which is a complex process. Investigations on simple "model" systems, consisting of a well-characterized protein, a well-characterized sorbent, and an aqueous solvent containing only nonbuffering ions, have provided the most reliable and meaningful data on the process of protein adsorption (7–10). For instance, protein and sorbent hydrophobicity, charge distribution, protein structural stability, solution pH, and ionic strength are known to influence the affinity of a protein for a given interface. These findings form the basis of a qualitative theory, originally proposed by Norde and Lyklema (11–16), which indicates that four effects—namely, structural rearrangements in the protein molecule, dehydration of the sorbent surface, redistribution of charged groups in the interfacial layer, and protein–surface polarity—usually make the primary contributions to the overall adsorption behavior. However, many important questions remain unanswered, and a unified, predictive theory is not in sight. Thus, protein adsorption research is in need of novel theoretical and experimental approaches that complement and expand our knowledge of the adsorption mechanism.

The mechanism of adsorption can be studied by systematically changing the physical properties of the protein, the sorbent surface, and the solution. In this way information is obtained about the nature of the interactions responsible for the adsorption process. A detailed understanding of the influence of the various interactions on the adsorbed state of the proteins is required to optimize the functioning of the immobilized proteins in their applications.

A. Adsorption Isotherms

When protein adsorption occurs it takes a certain time before the adsorbed amount and the protein concentration in solution reach their equilibrium values. When the equilibrium adsorption values are determined as a function of the equilibrium protein concentration in solution, an adsorption isotherm is obtained. Measurement of an adsorption isotherm is the starting point of most protein adsorption studies. By measuring adsorption isotherms under several experimental conditions one tries to determine which of the above-mentioned interactions plays an important role in the adsorption process. It depends on the experimental conditions, such as pH, ionic strength, charge of the adsorbent surface, and nature of the protein, and gives information on the affinity of the protein for the surface. In an adsorption isotherm two parts can be distinguished. The rising initial part at low concentrations indicates the affinity of the protein for the surface. When the isotherm rises very steeply it is called a "high-affinity" isotherm. This means that almost all protein present in the system is at the surface and the amount remaining in

solution is minimal. The slope of the isotherm at low surface coverage is a measure of the first three of the four interactions discussed previously. In general, the adsorption increases up to an amount where the total surface is saturated with protein. This amount is called the "plateau value" of the isotherm, and in general it depends on the experimental conditions, the conformation of the adsorbed molecules, the affinity of the proteins for the surface, and the surface coverage–dependent lateral interaction.

The shape of a typical protein adsorption isotherm is very similar to the well-known Langmuir gas adsorption isotherm, and in some publications the protein adsorption is interpreted accordingly. However, the typical irreversibility of protein adsorption against dilution (17–19) shows that one of the essential conditions for a Langmuir isotherm is not fulfilled in the case of adsorption of most proteins. Many studies demonstrate that protein–surface interaction includes several stages, two of which are always specified: (1) adsorption of protein onto the surface and (2) conformational changes or reorientations of adsorbed protein. For the other proposed models of three-stage and multistage adsorption (20, 21), quantitative descriptions have not been developed.

The protein–surface interaction has often been shown to be quite heterogeneous. Beginning by solid surfaces, some polymers consist of crystal and amorphous phases, and the existence of hydrophilic and hydrophobic domains on the surface of multiblock copolymers (22) can lead to this heterogeneity of protein–surface interactions. Electrostatic interactions between protein and strong charge sites of the surface can lead to dispersion of the entropy of the adsorption when the distance between the sites on the surface is as large as a protein molecular size (23). Regarding proteins, they are known to have anisotropic properties and, thus, the activation energy, as well as other characteristics of adsorption, will depend on which sites of the adsorbing molecule (hydrophobic or hydrophilic, charged or uncharged) directly interacts with the surface. That is why one should consider the heterogeneity of the protein–surface interaction rather than the heterogeneity of the protein and surface separately.

It should be noted also that protein–protein interactions might lead to additional heterogeneity in the adsorption rate constant. Thus, multilayer adsorption can be characterized by a set of adsorption rate constants, which correspond to different mechanisms of formation of each protein layer. Several proteins with different adsorption or desorption rate constants, lateral dynamics, heat adsorption, and so on have been described (24, 25). These observations have suggested that there are weakly and strongly bound proteins on different surfaces. However, the processes underlying such evident heterogeneity are not clear. Many of theses models take into consideration the heterogeneity of the protein–surface interaction, which can affect the kinetics of protein adsorption even more than the electrostatic repulsion (26).

B. Immunoglobulin Adsorption

The literature concerning the study of immunoglobulin G adsorption at solid–liquid interfaces has a long and confusing history (20, 27, 28). We note specifically that (1) experimental adsorption isotherms performed in different laboratories on quite similar systems often conflict; and (2) minor changes in experimental conditions (pH, ionic strength, temperature) may result in major differences in the measured adsorption. These studies are difficult due to the complex interactions involved, and they suggest that immunoglobulin adsorption on solid surfaces takes place with a rather low experimental reproducibility.

Perhaps one of the most striking features that crystallographic studies have revealed is that of molecular flexibility. This kind of flexibility is expected to facilitate the formation of antibody–antigen complexes. The Fab and Fc fragments are relatively compact; however, the whole IgG molecule is not compact (its scattering curves are anomalous and the radii of gyration of the whole molecule are larger than expected for overall close packing of regions). This segmental flexibility could explain why the dimensions of immunoglobulin G vary, and why the distance between binding sites of an antibody on an elongated molecule is 12 nm (crystalline state) but molecules can expand to reach 25 nm (end-to-end solution distance) (29–33). This segmental flexibility might explain the poor agreement between the IgG adsorption data obtained by different authors. The IgG_1, IgG_2, IgG_3, and IgG_4 subclasses of human IgG contain two, four, five, and two disulfide bridges, respectively, between heavy chains, whereas mouse IgG_1, IgG_{2a}, and IgG_{2b} contains three bridges each and guinea pig IgG_2 also contains three (33). Hence, the flexibility of these IgG molecules (Y- or T-shaped molecules) would be different as would their dimensions. Also, the area per molecule depends on the configuration of the IgG at the solid–liquid interface: the projected area in an end-on configuration is 20 nm^2, whereas side-on is 103 nm^2 (34). A monolayer of side-on IgG is reported to correspond to an adsorbed amount of about 3 mg m^{-2}, while a monolayer of end-on IgG corresponds to approximately 15 mg m^{-2} (35).

IgG adsorption is usually an irreversible process; there is practically no desorption of antibodies by dilution of IgG-coated polymer particles when they are diluted at pH 7, as can be seen in Fig. 2 (36). Thus, although adsorption isotherms from solution appear to be of the Langmuir type, it is not possible to determine equilibrium thermodynamics binding constants from this kind of experiment. Adsorption isotherms of IgG on polymer supports

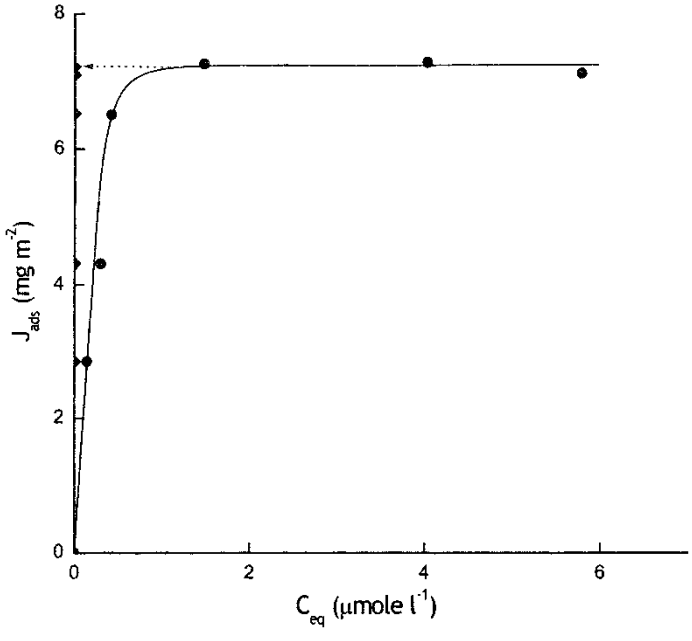


Figure 2. Desorption of IgG from cationic polystyrene (PS) latex by dilution at pH 7.2, 2 mM ionic strength and (20 ± 1)°C. Adsorption values (●) and final values (◆).

usually developed well-defined plateaus that were in the range of those calculated for a close-packed monolayer of IgG molecules (28, 36–43). The results obtained with the adsorption of a monoclonal antibody (MAb) (IgG_1 isotope directed against hepatitis B antigen, HBsAg) on cationic and anionic polystyrene latex particles are shown in Fig. 3. Even when the protein has the same charge sign as its adsorbent, adsorption occurs spontaneously. These results constitute an example of well-defined plateaus (37). It should be noted, however, that step-like adsorption isotherms (27) and others without a clear plateau value (44) have also been reported. These discrepancies stress the necessity for proper characterization of both IgG and polymer supports used in IgG adsorption studies.

The conformational stability of a protein is mainly determined by intramolecular factors and solvent interactions (hydration of interfacial groups). Nevertheless, solubility is determined primarily by intermolecular effects (protein–protein interactions), but protein molecules are solvated, so that hydration effects are also involved in changes in solubility. The energy of the hydration interaction will depend on the groups placed in the interfacial zone of the protein, and then solubility and conformational stability are closely related. Solubility is a good index of denaturation and undergoes a minimum in the neighborhood of the isoelectric pH. From the adsorption point of view the solubility of a protein is of major importance, as the method for determining adsorbed protein amount is based on the difference between the initial and the supernatant concentration. If protein molecules denature in the process, they form aggregates and precipitate in the centrifugation step. This amount of protein should be quantified as adsorbed, and it could be a cause of error (37).

Several investigators (28, 36–38, 40, 42, 43) have observed a maximum in the amount of IgG adsorbed with pH, and indicate that it is due to the decrease in

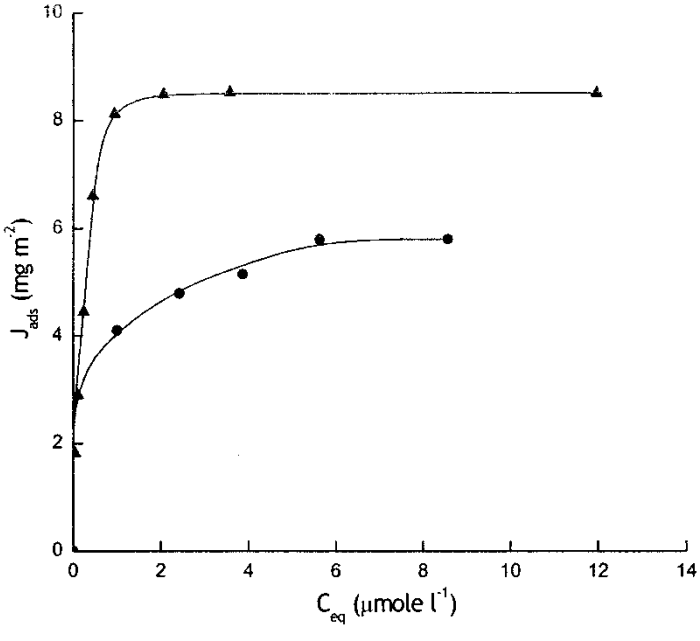


Figure 3. Adsorption isotherms of IgG on cationic (▲) and anionic (●) PS latex at pH 5.5, ionic strength 2 mM and (20 ± 1)°C.

conformational stability of the IgGs with increasing net charge on the molecule. This results in a greater tendency for structural rearrangements of the adsorbing molecules that create a larger surface area per molecule and cause a small amount of IgG to be adsorbed. Furthermore, at pH values away from the isoelectric point of the IgG, there is an increased electrostatic repulsion between adsorbed molecules that leads to a smaller amount of adsorbed IgG. Maximal protein adsorption around the isoelectric point (IEP) has been reported for IgG (28, 37, 38). Figure 4 shows the adsorption plateau values of the above MAb on cationic and anionic polystyrene latex beads as a function of pH, and we can see that maximal values occur in the neighborhood of the IEP of the protein (36). Nevertheless, some authors have shown that the maximum appears in the IEP of the immunoglobulin–carrier complex (38).

C. Factors that Influence Adsorption

The major types of interactions that are relevant in immunoglobulin adsorption from aqueous solution are (1) hydrophobic interaction, (2) Coulomb interaction, and (3) hydrogen bonding. The effects of electrostatic charge and potential (which can be controlled by varying the pH and ionic strength in the system), the hydrophobicities of the protein and the sorbent surface, and the chemical compositions of the sorbent and the medium on the rate of adsorption and on the adsorbed amount at equilibrium provide insight into the relative importance of the above-mentioned interactions. Other factors that may also influence immunoglobulin adsorption onto surfaces include intermolecular forces between adsorbed molecules, solvent–solvent interactions, strength of functional group bonds, chemistry of solid surface, topology, and morphology. Adsorption

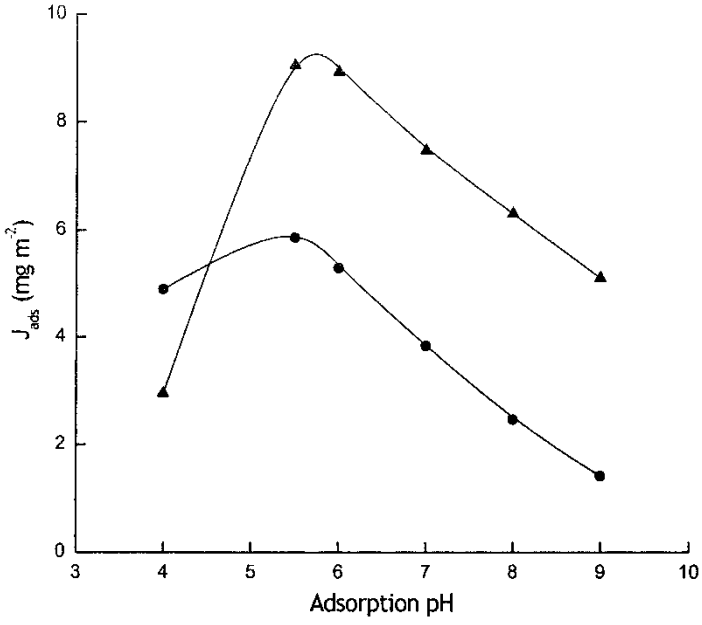


Figure 4. Maximum adsorption of monoclonal IgG on cationic (▲) and anionic (●) PS latex as a function of pH at 2 mM ionic strength and (20 ± 1)°C.

of IgG on hydrophobic surfaces is usually an irreversible process and occurs rapidly. It should be noted that the characteristics of $F(ab')_2$ and IgG adsorption are very similar (45, 46).

The major driving force for protein adsorption onto polymer surfaces is the dehydration of hydrophobic side groups (11, 47), which is almost completely due to the entropy increase in water that is released from contact with hydrophobic components, and the surface dehydration also favors the protein adsorption. It seems reasonable to assume that antibody adsorption on hydrophobic surfaces is driven entropically as well. However, electrostatic forces at low ionic strength can play a certain role in IgG adsorption even on hydrophobic surfaces. This role has been shown by the adsorption of monoclonal antibodies onto surfaces with different signs of surface charge (36, 38, 43, 48). The initial slopes in the adsorption isotherms give information about the affinity between IgG and the adsorbent surface. With this aim, several authors have studied adsorption isotherms on systems that vary the possible electrostatic interactions between the components. Figure 5 shows the adsorption of a monoclonal antibody (IEP 5.5) at neutral adsorption pH on positively and negatively charged surfaces. We can see the differences in affinity between the IgG molecules and the polymer surfaces when the electrostatic forces influence the adsorption process. Effectively, the initial adsorption values for the anionic surface do not coincide with the total adsorption line, showing that electrostatic repulsion between negative charges makes the approach of IgG molecules to the surface difficult.

The electrostatic forces can give rise to fractionation in the adsorption of polyclonal antibodies on charged surfaces. Since polyclonal antibodies are in fact mixtures of IgG molecules with different physical properties, preferential adsorption of any fraction can

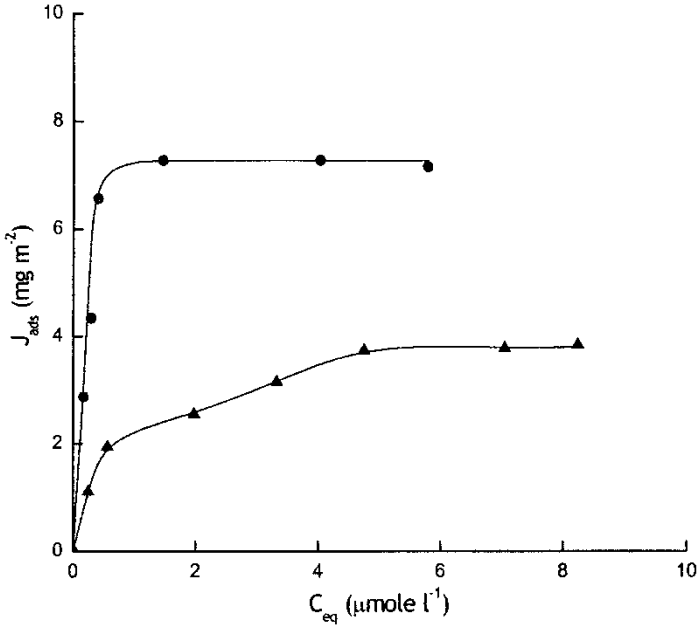


Figure 5. Adsorption isotherms of monoclonal IgG on cationic (●) and anionic (▲) PS latex at pH 7, 2 mM ionic strength and (20 ± 1)°C.

take place at low ionic strength. To check this possibility, some authors (43) have analyzed by isoelectrofocusing (IEF) the supernatants after IgG adsorption onto charged surfaces. These authors have demonstrated that, at pH 7 and 9, preferential adsorption is partly determined by electrostatic factors; the IgG molecules with the highest IEP are preferentially adsorbed on negatively charged surfaces, whereas at pH 5 no preferential adsorption is observed. Most single-component IgG adsorption from buffer studies simulates physiological conditions, implying that the ionic strength is relatively high. Under those experimental conditions the electrostatic forces between protein and adsorbent are negligible. An exception is the serum competition, where IgG adsorption from a multicomponent protein solution is a phenomenon completely distinct from single-component IgG adsorption from buffer.

Antibody adsorption to, and desorption from, adsorbent surfaces is a function of the nature of both antibody and the surface, and can be dependent on time, temperature, ionic strength, pH, protein concentration, and surface tension (39). IgG molecules adsorbed onto a surface are in a dynamic state. Although adsorbed IgG molecules generally do not desorb as a result of simple dilution, they can be displaced by an increase in ionic strength. Certainly, ionic strength exerts a pronounced effect on the adsorption of IgG molecules on charged surfaces. As ionic strength increases, the electrostatic forces between the IgG molecules and the adsorbent decreases. Under these experimental conditions, hydrophobic interactions are predominant in the adsorption mechanism of IgG molecules on a surface. Figure 6 shows the plateau values of adsorption as a function of pH at increasing ionic strength. In this case, the adsorbed IgG maximum is less dependent on pH at high ionic strength. Nevertheless, the effect of ionic strength on the adsorbed amount is different at adsorption pH 4, 7, and 10, as can be seen in Fig. 7.

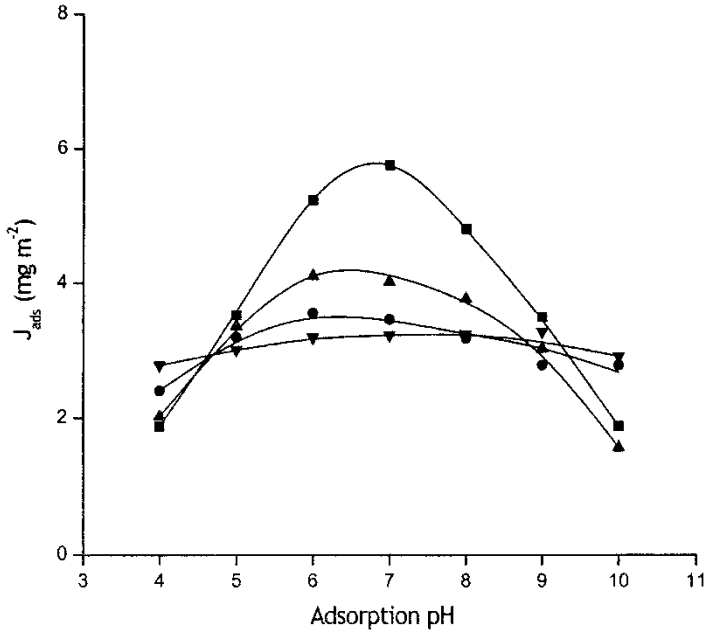


Figure 6. Maximum adsorption as a function of pH at different NaCl ionic strengths for rabbit IgG: 2 mM (■), 20 mM (▲), 50 mM (●), 100 mM (▼).

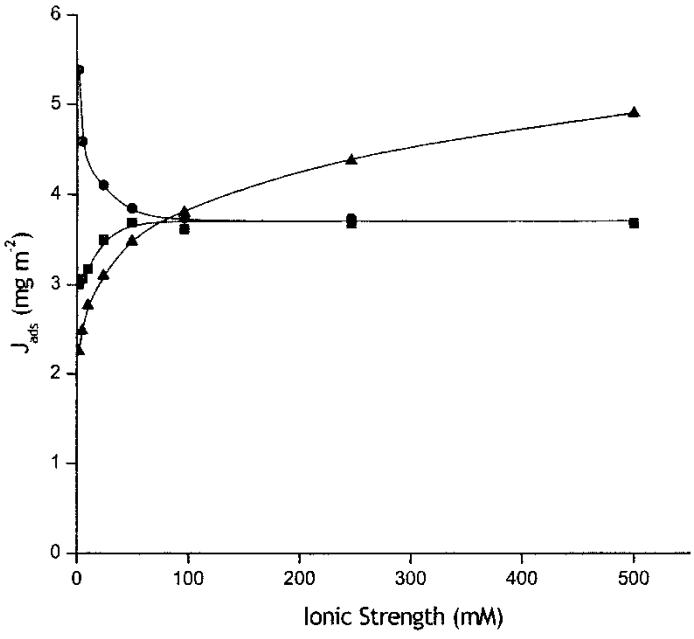


Figure 7. Maximum adsorption as a function of NaCl ionic strength at different adsorption pH for rabbit IgG: pH 4 (▲), pH 7 (●), pH 9 (■).

At neutral pH an increase in the ionic strength implies a decrease in the plateau value, whereas at pH 4 and 10 this value increases. This trend seems to indicate that the structural stability of polyclonal IgG molecules decreases as ionic strength increases at neutral pH, whereas when the polypeptide chains are highly charged (pH 4 and 10) an increase in electrolyte concentration provokes a larger screening of the net charge on the IgG molecules and, thus, an increasing conformational stability of the IgG.

D. Electrokinetics

To determine the role of electrostatic interactions in IgG adsorption, several authors (36–38, 40, 42, 43) have studied the electrokinetic behavior of IgG-coated surfaces. Furthermore, the electrophoretic mobility of IgG-coated surfaces can be suitable to predict the colloidal stability of these systems. Figure 8 shows the electrophoretic mobility of IgG-coated polystyrene beads (IgG-PS) as a function of the amount of adsorbed IgG (Γ). With increasing Γ the absolute value of mobility decreases to reach a plateau value. This decrease is dependent on the pH, i.e., on the charge of the IgG molecules. In Fig. 9 mobilities at complete coverage of the IgG-PS complexes are given as a function of resuspension pH for both types of charged surfaces. There is a significant difference between IgG adsorbed on negatively and positively charged surfaces. This difference can be related to electrostatic interactions between IgG and the charged surface. The IEPs of the polyclonal IgG-PS complexes are 6 and 8 for the anionic and cationic PS beads, respectively. This difference indicates that the surface charge must compensate, at least partly, the charge of the IgG molecules. This effect has also been seen with monoclonal antibodies on positively and negatively charged

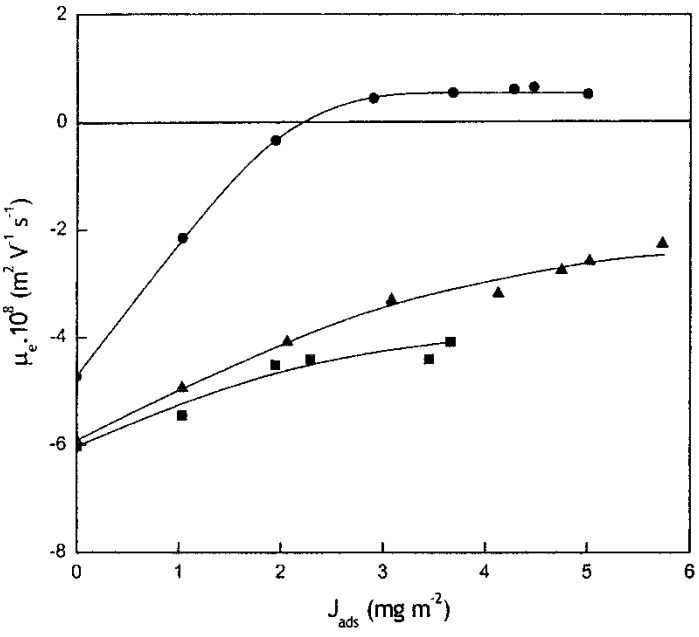


Figure 8. Electrophoretic mobility vs. adsorbed amount of rabbit IgG at 2 mM NaCl and three different adsorption-resuspension pH values: pH 5 (●), pH 7 (▲), pH 9 (■).

surfaces (36, 38). Also, it should be noted that, in both cases, the mobility of the IgG-PS complexes is decreased in comparison with the mobility of the bare PS beads, which could explain the extremely low colloidal stability of the polyclonal IgG-coated surfaces. The structure of the electrical double layer (EDL) of polyclonal IgG-coated polystyrene beads has been studied by Galisteo et al. (49). The main conclusions

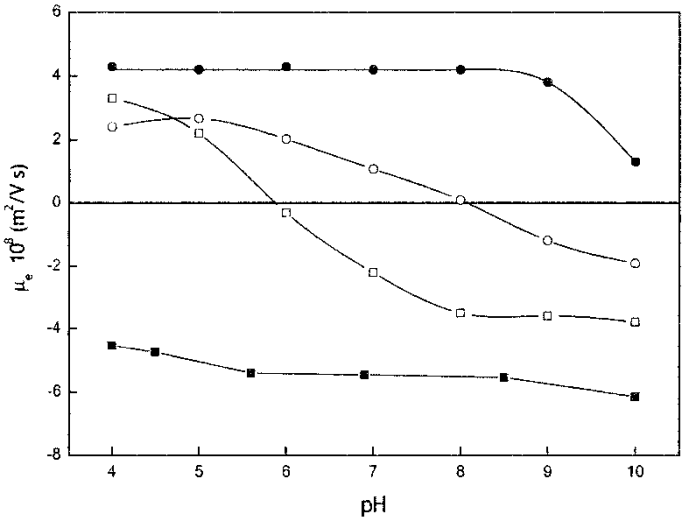


Figure 9. Electrophoretic mobility vs. resuspension pH: bare cationic PS beads (●) saturated cationic PS beads (○), bare anionic PS beads (■), saturated anionic PS beads (□).

drawn from these studies are (1) that ions in the electrical double layer surrounding the IgG–polymer surface (especially those under the hydrodynamic slipping plane) have a greater ionic mobility when the electric charge in the protein molecule has the same sign as the electric groups in the particle surface; and (2) that the anomalous surface conduction mechanism is more pronounced in this case in the surface charge region.

A different approach to the electrophoretic mobility of antibody-carrying latex particles has been used by Nakamura et al. (50, 51). According to these authors, the ζ potential loses its meaning for the IgG-latex particles, since the electrophoretic mobility is insensitive to the precise position of the slipping plane. Upon analysis, they conclude that the depth of the bound IgG layer varied from 3.5 to 8.5 nm, and it decreased with increasing ionic strength, suggesting that conformation of the bound IgG becomes more compact following addition of electrolyte. It is considered that the addition of excessive electrolyte ions reduces the intramolecular and intermolecular electrostatic interaction of the IgG molecules bound to the surface of latex particles. This new approach to the interpretation of the electrophoretic mobility even provides conformational information about the IgG that is fixed on polymer surfaces, which is its major advantage against the classical electrical double-layer theory.

III. Covalent Coupling

A. Why Attach Covalently?

One of the several advantages for the selection of latex particles as solid support for immunoagglutination tests is that latex may be "custom synthesized" to fit the needs of a given application. A wide variety of monomer combinations may be chosen to produce latex with desired surface characteristics. In this way, reactive groups can be incorporated into microspheres by an emulsion copolymerization process in which, for example, one monomer is styrene and the other is methyl methacrylate, methacrylic acid, chloromethylstyrene, acrylamide, etc. A small amount ($<5\%$) of the functionalized monomer is generally used in the copolymerization reaction. Other functional groups can be obtained by using different initiators. These functional groups can be employed as reaction sites to covalently bind different ligands as proteins. On the other hand, the nature of the particle surface may be modified by introducing a given degree of hydrophobicity or hydrophilicity by monomer selection. In fact, it is considered advantageous for immunomicrospheres to be hydrophilic in order to avoid nonspecific interactions. The selection of latex particles as solid support for medical diagnostic tests offers a great deal of flexibility in the design of the reactive.

The ideal polymer support for latex immunoagglutination should allow the attachment of proteins in a controlled manner, resulting in a colloidally stable system with the required surface concentration of the immobilized proteins, and retaining a maximum of their biological activity. This immobilization could be achieved not only by physical adsorption, the most conventional approach, but also by covalent coupling. The latter might have, in principle, some advantages from the point of view of its application in the development of new immunodiagnostic tests:

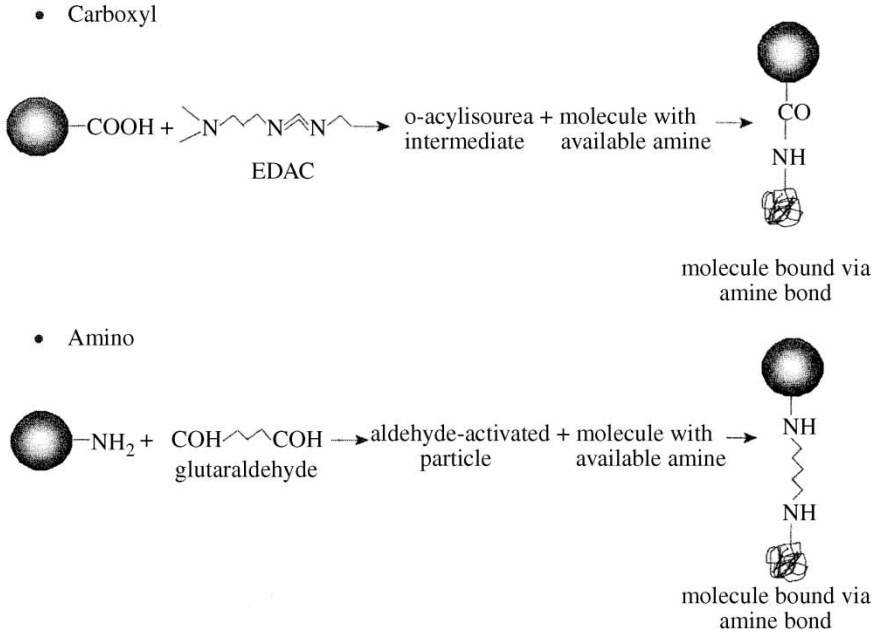
1. The functioning of latex immunoassays depends on the capability of the immobilized antibodies to bind antigens. It is important that antibodies adsorb retaining a

maximum of their biological activity. However, the antibody molecules immobilized onto polystyrene by physical adsorption retained only a small fraction ($\leq 10\%$) or even lost completely their binding activity due to the protein denaturation on the surface (52–54). This adsorption-induced reduction in the effective immunological activity can be caused by changes in the protein structure. Furthermore, surface activity could be enhanced if the antibodies are covalently coupled, by reducing the rearrangement of the protein molecules during and after adsorption (17, 55).

2. Covalent attachment is permanent, leaving no unbound material after clean-up. It may prevent elution of bound protein during storage, thus increasing shelf life.
3. The hydrophobic surface of polystyrene beads may contribute to nonspecific binding of other ligands, producing false positives in the immunoagglutination tests. Chemical binding of the antibodies would aid in the elimination of false results.
4. Covalent coupling is a uniform coating procedure. As a consequence of having reactive groups over the entire surface of the latex, it is possible to completely cover the surface with protein. On the other hand, the protein coverage is more easily controlled by covalent coupling, especially when the desired quantity of adsorbed protein is low.
5. Achieving the correct spatial orientation for the bound protein can be difficult via physical adsorption. Covalent attachment, on the other hand, can orient the molecule properly, if the correct coupling chemistry is chosen, improving the activity of the bound proteins and resulting in lower reagent consumption.
6. In a standard technique of immunoassays, washing buffers containing surfactants are used to remove loosely bound proteins from the device surface and to avoid the nonspecific adsorption of proteins such as the first and the second antibodies on the surface. Since there is a possibility that adsorbed proteins on the latex may be desorbed by surfactants, it is advisable to use covalent coupling of the proteins.
7. Smaller molecules, such as antigens or $F(ab')_2$ fragments, do not adsorb strongly to the hydrophobic surface of polystyrene beads. These smaller ligands are candidates for covalent coupling to the particle surface. Protein A or protein G covalently attached can be used to capture a variety of immunoglobulins.
8. In order to reduce nonspecific reactions and self-aggregation of the particles, a large variety of blockers (bovine serum albumin, casein, nonionic surfactants, polyethylene glycol, etc.) are added to the storage buffer to block the exposed hydrophobic surface of the polymeric microspheres. This is especially useful with antibody-coated particles because the IgG molecule has a low charge density and presents low colloidal stability. Also, a separate incubation in a higher concentration of blocker is recommended before storage in order to saturate the latex surface. The additives could displace the antibodies physically adsorbed (56, 57). For a lot of antigen–antibody systems, blocking agents of proteinic nature cannot be used because it is possible to find cross-reaction and alteration of the antigen–antibody reactivity that falsify the result of the immunodiagnostic test (58).
9. Some evidence indicates that one can attach 10–40% more protein via covalency than for physical adsorption (59, 60).
10. There are latex applications that require thermocycling. In these cases, reactives with protein covalently bonded are desired because the covalent bond is more thermally stable.

B. Functionalized Particles

Many kinds of functionalized latexes with reactive surface groups suitable for covalent protein immobilization have been described. Some of the more common choices include the following:



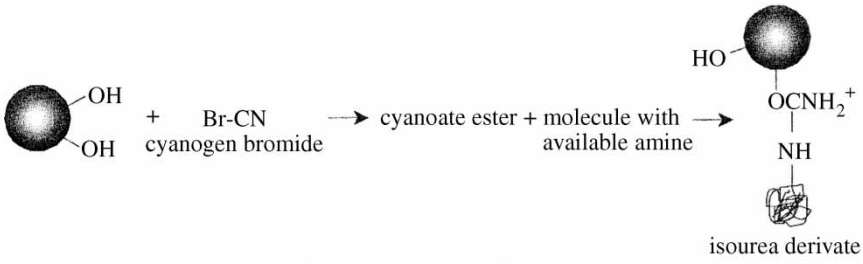
The most commonly used surfaces for attachment of ligands are carboxyl and amino groups, for the following reasons:

1. These groups have proven to be very stable over time.
2. The chemistries involved in attaching ligands to either of these groups have been widely explored, and several options exist for each.
3. The existence of terminal amino and carboxyl groups on proteins is universal, ensuring their availability for complementary attachment to one or the other functional group on the surface of the microspheres.

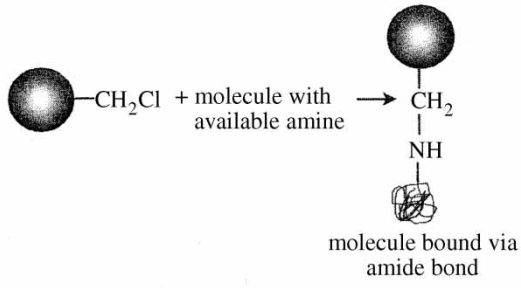
A number of special linkers can be used to convert one surface functional group on a microsphere to another. For example, amino microspheres can be converted to carboxylic particles by reacting with succinic anhydride (61). Conversely, carboxylic particles can be converted to amino microspheres through water-soluble carbodiimide-mediated attachment of a diamine (62). Also, sulfhydryl particles can be made by reacting amino particles with iminothiolane (63).

The functionalized groups may be used as sites for the attachment of spacer arm molecules. These spacer arms have functional groups at the distal end for the flexible bonding of proteins. In this way, antibody molecules extend away from the latex surface into the aqueous medium. This approach may minimize protein denaturation and the antigen–antibody recognition could be easier because the coupled antibodies are set off from the surface (64, 65). For example, it is possible to attach amine spacer arm molecules to activated chlorine groups at the latex surface.

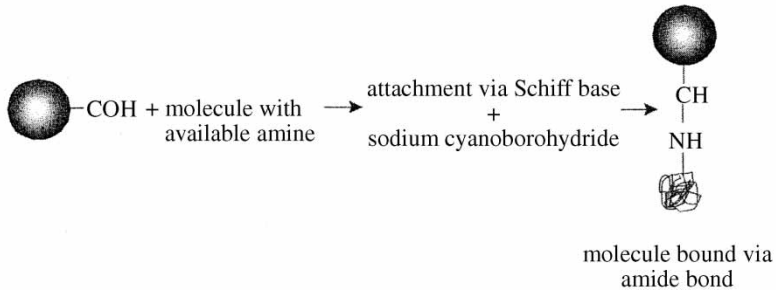
- Hydroxyl



- Chloromethyl



- Aldehyde



Some functional groups borne by the latex are unreactive as such and need to be activated prior to protein immobilization: cyanogen bromide is used to bind hydroxyl groups from the latex surface to amine groups in the protein at alkaline pH, and glutaraldehyde is used to link amino groups present on the latex to amino groups on the antibody molecule. Most of the work on the covalent binding of proteins has been conducted on carboxylated latexes (66, 67). The methodology required for coupling proteins to unactivated latexes is tedious (involving several steps before the activated groups react with free groups of the protein), expensive, and more time consuming than one-step coupling. The carboxyl groups have to be activated by the 1-ethyl-3-(3-dimethylarninopro-py1)carbodiimide (EDAC). The intermediate obtained on reaction of a carboxylic acid group with a carbodiimide is fairly unstable, especially in water, and has to be quickly mixed with the protein to be immobilized. This is the major drawback of any immobilization protocol based on the use of highly reactive and unstable intermediates. The balance between covalent coupling and unproductive side reactions depends to some extent on activation reaction conditions. The ionic

composition of the reaction, the pH, and the buffer type can greatly enhance or inhibit the total binding of protein to particles. A number of variations exist for EDAC-mediated coupling. The degree of coupling is dependent on the density of the reactive groups, i.e., carboxyl groups on the polymer. Sufficient amounts of functional groups should be present to provide adequate coupling of antibody. The covalent attachment of the IgG molecule to carboxylated particles improves the immunoreactivity of antibodies when compared with physical adsorption, maintaining its immunoreactivity after long periods of storage (68).

Some authors have described the preparation of diazotized polystyrene for use in the separation and purification of antibodies (69, 70) and for latex agglutination testing (71). This functionalized latex with active diazo groups can be coupled to phenol and imidazole groups of antibodies via a covalent bond linkage. This immunopolystyrene diazonium latex reagent showed a positive agglutination reaction of 78–91% when mixed with serum from patients with leptospirosis.

Different authors have indicated that the use of aldehyde groups could simplify the covalent bond of the protein due to the direct reaction between the aldehyde groups of the latex and the primary amino groups in the protein molecules (72, 73) by forming an imine derivate with concomitant water elimination. Rembaum et al. (74) described polymerization of acrolein to prepare microspheres that could be used as substrates for immobilization of proteins, but the microspheres were porous. Bale et al. (75) proposed a method for providing beads nonporous since reaction between an immobilized ligand and other reactant is expected to be faster on nonporous surfaces due to diffusional considerations. Alternatively, preformed polymeric latex could be modified to contain aldehyde groups (76).

In general, hydrophilic surfaces may have a lower level of nonspecific interactions than hydrophobic surfaces (77, 78). In this sense, Koning et al. (79) proposed the synthesis of core-shell particles containing a hydrophilic polymeric shell with aldehyde groups. These particles have been used to detect human chorionic gonadotropin (HCG) in urine and serum. The results were compared with hydrophobic latex particles with the same antibodies physically adsorbed. The results showed that the functionalized latex presents a less nonspecific interaction and a higher detection limit. Hydrophilic particles with functional aldehyde groups can be produced by polymerization of glutaraldehyde and of acrolein at high pH (80). New approaches can be accomplished by producing particles with a uniform distribution of functional group areas separated by hydrophilic areas (81, 82). The former can be used for attaching proteins, the latter for inhibiting nonspecific effects. Ideally, particles should be available with different percentages of the two types of areas in order to optimize assay concentration ranges.

The aldehyde groups tend to decompose with time, losing the capacity to bind the proteins. As suggested by Kapmeyer et al. (83, 84), a possibility is to produce latex particles with acetal groups on the surface. These groups can be transformed to aldehyde groups at the moment to produce the covalent coupling of the proteins, by moving the medium to acid pH. Peula et al. (85–87) prepared acetal latexes, which permitted the covalent coupling of IgG anti-C-reactive protein (anti-CRP) in a simple way by changing the pH of the suspension to pH 2. The latex–protein complexes showed a good immunological response that was not disturbed by the presence of a nonionic surfactant in the reaction medium and was stable with time.

Preactivated microparticles have been developed with surface groups, which are sufficiently reactive to directly couple with proteins. There is no requirement for a separate

preliminary activating step. This convenience allows fewer handling and transfer steps. An example of preactivated groups is vinylbenzyl chloride (88–90), where the reaction occurs by nucleophilic displacement of the chloride atom of chloromethylstyrene groups. Such latexes have limited shelf life due to the inevitable hydrolysis or oxidation of the reactive groups in aqueous media. The stability of chloromethyl function is strongly dependent on temperature. The hydrolysis rate increases with increasing temperature. At the storage temperature of 4°C some hydrolysis occurs, and after a long period of time (1 year) approximately 20% of the surface chloromethyl groups disappear (91). Nevertheless a significant proportion of the reactive groups are retained. A chloromethylstyrene monomer can be polymerized onto a polystyrene core in any proportion to other nonactivated containing monomer, to produce a shell having from 5% to 100% chloromethylstyrene monomers (92).

Sarobe et al. (93) have studied the covalent immobilization of lysozyme on chloromethyl latexes. As can be seen in Fig. 10, the initial steps of the adsorption isotherm indicate that all the adsorbed protein becomes covalently bound. This result is general if a certain surface density of functional groups exists on the surface ($>21\ \mu mol/g$ polymer). As physical contact occurs prior to chemical linking, a sufficiently high number of chloromethyl groups are needed to ensure that covalent binding can take place. With more adsorbed protein at the surface, the covalent extent decreases to a more or less constant value between 60% and 70% independent of the number of chloromethyl groups and pH.

A method to show the existence of covalency between protein and functionalized latex is treatment of protein–latex system with surfactants under appropriate conditions able to recover all the physically adsorbed protein from the particle surface. In the case of chloromethyl latexes, the determination of free chloride ions after protein

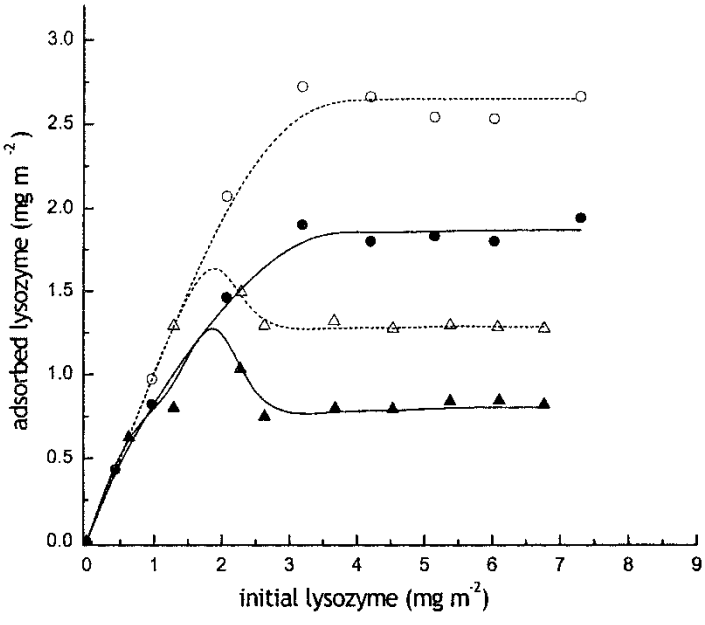


Figure 10. Adsorption isotherm of lysozyme on chloro-activated latex at two pH: (▲) pH 7; (●) pH 11. Total adsorption, open symbols; covalent binding, closed symbols.

adsorption and comparison with a blank could show the covalent attachment (Fig. 11). The kinetic of the aminochloromethyl reaction at the interface is slow. Although physical adsorption achieves saturation after some minutes of sensitization (94), covalent binding is quite slower, needing several hours to be completed (5–6 h). This means that the first contact between the protein and surface is always physical, while chemical linking develops later. Nustad et al. (95) demonstrated that adsorptive binding to core-shell particles occurred rapidly followed by slow covalent coupling. It has been claimed that adsorption is a necessary prerequisite to covalent coupling and that an excessively high charge density on the latex surface will actually decrease the yield of covalent coupling (96).

In practice, with preactivated microparticles a blocking step in the process of preparing antibody-coated latex has to be included to eliminate the unreacted functional groups (in the case of chloromethyl groups with inert amines). Figure 12 shows a functionalization scheme of chloro-activated latexes to obtain functional groups other than chloromethyl groups through a linked spacer arm and without a linked spacer arm (97).

The immunoreactivity of IgG or $F(ab')_2$ antibody molecules covalently bound to the surface of chloro-activated latex has been compared to passive physical adsorption to a conventional polystyrene latex (98, 99) (Fig. 13). For both antibodies an improvement in the immunoresponse is observed for the covalent union to latex particles. The desorption of physically attached protein from the surface with time reduces the period for which latex agglutination tests may be stored. Molina et al. (99) indicated that the storage period for IgG and $F(ab')_2$ antibodies covalently attached to chloro-activated surface is higher than for physical adsorption.

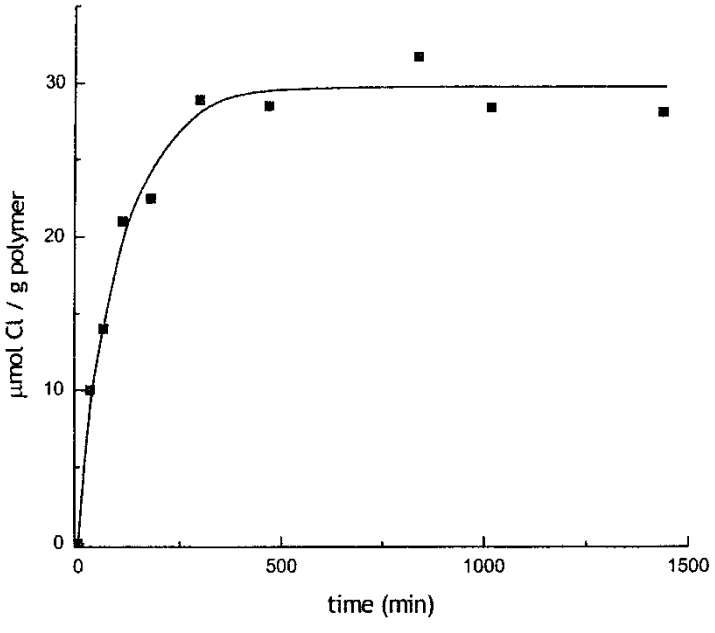


Figure 11. Evolution of chloride ion release as a function of sensitization time for protein-saturated conditions.

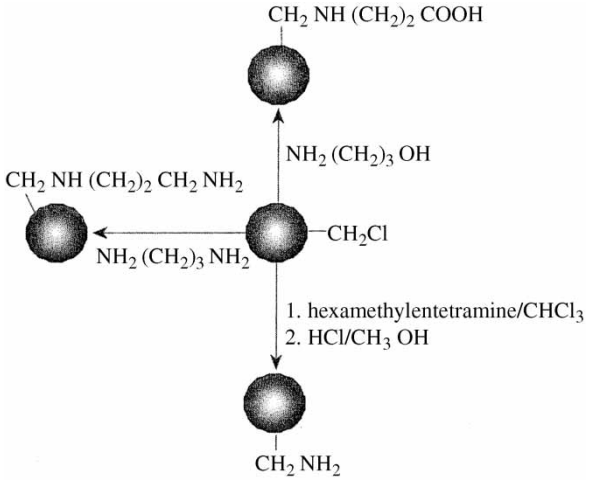


Figure 12. Functionalization of chloromethyl-activated particles.

In covalent coupling of proteins to functionalized latexes a variety of conditions need to be tested for each case. In many of the coupling procedures extensive multipoint binding takes place during covalent attachment of the proteins and latex particle because there is a very large number of functional groups on the particle surface. This multiple binding may render either the antigen or the antibody inactive. The binding procedures have to be adapted to minimize the protein denaturation.

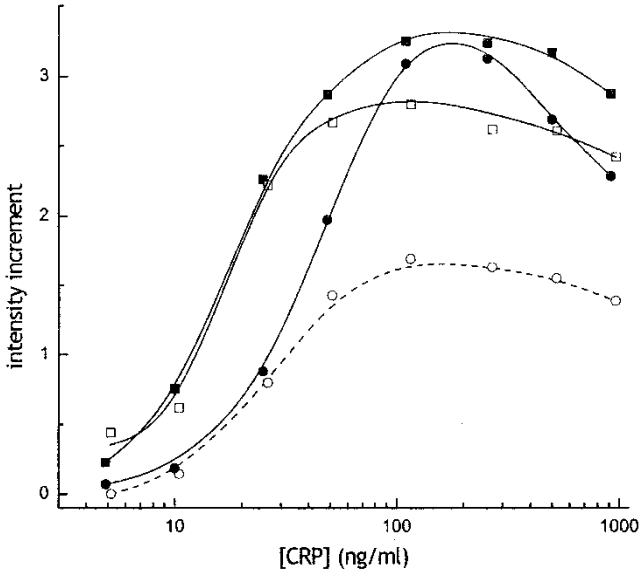


Figure 13. Immunoreactivity of IgG anti-CRP (■) and F(ab′)$_2$ anti-CRP (●) molecules physically (open symbol) and covalently (closed symbol) adsorbed on latex particles. The immunoreactivity is measured by nephelometric monitoring of the immunoaggregation reaction with human CRP for 10 min. Both IgG-latex conjugates have the same protein coverage. Also, both F(ab′)$_2$-latex conjugates have the same protein coverage.

IV. Detecting Immunoagglutination

A. Visual Observation

The light scattered from a monodisperse suspension of particles makes the latex look milky in appearance. If the suspension of particles is aggregated by some process then the microspheres clump together to produce a coarse granular suspension that resembles curdled milk in appearance. Many of the latex agglutination tests developed are performed manually and the agglutination is detected by visual observation. In these tests larger particles of several hundred nanometers have to be used, the most common size being 0.8 μm. It has been established that about 100 clumps must be seen to determine agglutination and that these clumps must be about 50 μm to be seen by eye (100). For a particle size of 0.8 μm, about 10^5 latex particles is required to make one visible aggregate, and about 10^7 particles is needed to determine agglutination in a given test. Based on these calculations and assuming that about 10 bonds are required per particle to hold them together, Bangs has evaluated the sensitivity of such a manual test to be in the order of picograms (100). Although quite useful in the laboratory and cheap due to the absence of equipment needs, the major limitation of these manual assays is that the only quantitation possible is to use serial dilutions of the test fluid and to look for the disappearance of immunoagglutination.

B. Light Scattering Techniques

Latex looks like milky white because the submicrometer particles scatter visible light. Developments in instrumentation have permitted the automatically full quantification of the immunological agglutination by light scattering, thus avoiding the subjectiveness of manual detection. These instrumental methods are far more sensitive than visual detection of the aggregates. Light scattering can be measured with rather inexpensive and simple instruments that are commercially available. The sensitivity, reproducibility, and detection limits of latex immunoagglutination tests depend on the technique used to detect the aggregated product.

Monitoring of the antigen–antibody reaction by measurement of light scattering has been known for the past 50 years, although the broader concept of agglutination had been explored in the 1920s for microbiological assays (101). Light scattering methods can be divided into two major categories: methods that measure time-average scattering (static light scattering) and methods that observe the scattering fluctuation as a function of time (dynamic light scattering). Both methods can give information on the agglutination state of the protein-coated particles. During the immunoagglutination process particles aggregate with increasing diameter, resulting in the appearance of very large particles. The scattering particle size can vary from nanometers to millimeters. This change in the particle size of containing suspension provokes a dramatically increase in the scattered light. Light scattering studies applied to particle immunoassays have been published and reviewed (102–104). Sensitivity, reproducibility, detection limit, reaction time, amount of particles needed, and availability of the required detection device are some characteristics that depend on the chosen technique (105). There are different variables that must be optimized to obtain the best results. Some of them are specific for each technique, whereas others can be applied to all of them (106). All of these techniques allow an increase in sensitivity and improve standardization, and the procedures involved may be automated. The difficulty for users, if they have not already purchased

an instrument, is in how to choose the most appropriate system for their needs. In most cases this has probably been determined by the ease of use of the instrument itself.

1. Light Scattering Theory. When the light impinges on a particle, its electrons are subjected to a force in one direction and its nuclei to a force in the opposite direction, causing the electrons about the particle to oscillate in synchrony with the electric field of the incident light. Thus, an oscillating dipole is induced in the particle by the incident light. This oscillating dipole becomes a source of electromagnetic radiation, reradiating light at the same wavelength of the incident light and in all directions. This radiation from the particle is called scattered light. The theories of light scattering can be divided into three different regimes, depending on the relation between the particle size and the wavelength of the incident light (λ). In 1871 Rayleigh developed a theory for light scattering by a very small dielectric sphere (107, 108). When the dimensions of the particle are much smaller than the wavelength of the incident light (diameter $<0.1\lambda$), then the entire particle is subjected to the same electric field strength at the same time. The intensity of the scattered light (I) at an angle θ between the incident and the scattered beam is given by the expression:

$$\frac{I}{I_0} = N\frac{8\pi^2\alpha^2}{r^2\lambda^4}(1+\cos^2\theta) \tag{1}$$

where N is the number of nonabsorbing particles per unit volume, I_0 is the intensity of the incident beam, r is the distance from the particle to the detector face, and α is the polarizability of the spherical particles given by:

$$\alpha = a^3\frac{n^2-1}{n^2+2} \tag{2}$$

where a is the radius of the particle, and $n = n_\mathrm{p}/n_\mathrm{m}$ is the ratio of refractive index of the particle, n_p, to that of the surrounding medium, n_m. As can be seen, the intensity of scattered light is proportional to the square of the particle volume and to $1/\lambda^4$ Hence, the scattering from larger particles may dominate the scattering from smaller particles, and a decrease in the wavelength will substantially increase the scattering intensity. The ratio of light scattered forward to light scattered backward at any pair of supplementary angles centered on 90° is known as the dissymmetry ratio. The scattering pattern for Rayleigh scatterer is symmetrical about the line corresponding to the 90° scattering angle.

When the particles are larger, the particle cannot be considered as a point source and some destructive interference between light originating from different sites within the particle will occur. The Rayleigh theory is no longer valid and must be modified. The physical basis of the modification, known as the Rayleigh-Gans-Debye theory, is that a particle of arbitrary shape is subdivided into volume elements (109, 110). Each element is treated as a Rayleigh scatterer excited by the incident beam, which is assumed to be unperturbed by the presence of the rest of the particle. The Rayleigh-Gans-Debye theory is valid in the region $\lambda/20 < 2a < \lambda$, where the radiation envelope will become asymmetrical with more light being scattered forward ($\theta \leq 90°$) than backward ($90° \leq \theta \leq 180°$). The light scattering intensity at 90° is much less than the intensity at the forward (0°) angle due to destructive interferences. The relatively enhanced forward scattering with increasing particle size can be used as an index to predict the particle size. The evaluation of the dissymmetry ratio during the immunoagglutination process give valuable information, especially at various times after initiating the reaction.

For particles larger than the wavelength of incident light ($2a \geq 10\lambda$) the Mie theory is used (111). For this size particle region the scattered light progressively decreases with increasing θ, and eventually minima and maxima may appear in the radiation diagram (Fig 14). The number and position of minima and maxima which appear depend on the size parameter ($\pi 2a/\lambda$) and the polarizability of the particle. For all previous theoretical considerations the dispersion is assumed dilute; light is scattered by single particles independent of other particles present.

There are a number of techniques based in light scattering phenomena to detect latex particles' immunoagglutination: turbidimetry, nephelometry, angular anisotropy, and photon correlation spectroscopy. Now we will discuss these techniques in regard to their relative merits for latex immunoagglutination assays.

2. *Turbidimety.* Turbidimetry involves measurement of the intensity of the incident beam as it passes through the sample. The light beam may pass through a suspension or be absorbed, reflected, or scattered by the particles. As a consequence, the intensity of light decreases as it is transmitted through the suspension. For nonabsorbing particles the decrease in light intensity due to scattering is expressed as turbidity, τ:

$$\tau = \frac{1}{l} \ln \frac{I_0}{I} \tag{3}$$

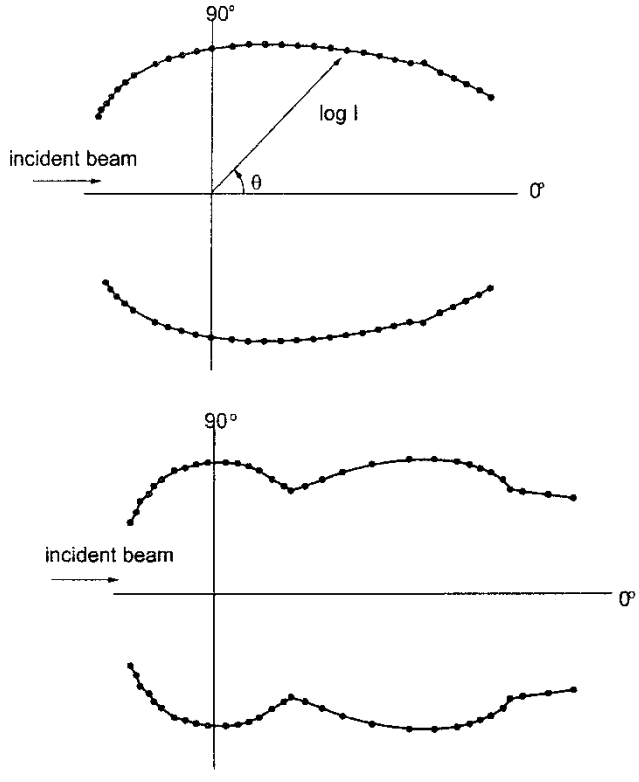


Figure 14. Light scattering intensity distribution for two particle diameters: (top) 186 nm, (bottom) 665 nm.

where I_0, and I are the intensities of the initial and transmitted beams, respectively, and l is the length of the light path, usually the sample thickness. As can be seen, the turbidity is a measure of light attenuation caused by scattering. The spectrophotometer measures increased turbidity (i.e., the reduction in the intensity transmitted light), which is due to the increasing particle size resulting from the immunoagglutination reaction. This increased turbidity is a direct measure of the immunoagglutination caused by the analyte or an indirect measure of the immunoagglutination inhibition caused by the analyte. For dispersions with aggregating particles, turbidity measurements at two wavelengths may also be used to follow the aggregation process.

This technique is rapid and easy to use. With turbidity no special equipment is required other than a spectrophotometer, which is generally available in clinical laboratories. There are fully automatic spectrophotometers that not only measure transmitted light automatically at a desired time but also dilute, pipette, and transfer to the cuvette the convenient volumes of reagents buffers and samples, incubate at a programmed temperature and make the necessary calculations using the selected algorithms and calibration curves (112). The possibility of running latex agglutination tests into these automatic analyzers allows the processing of hundreds of samples in a short time without investment in new instrumentation or personnel.

To optimize the turbitidy change, which occurs during immunoagglutination, it is important to select the appropriate particle size. The number of antigen-antibody bridges between pairs of particles during the immunoagglutination is about 2–10. With larger particles, the shear forces across these bridges may result in disruption of agglutinates when pumped at high speed in automatic machines. Thus, particles of smaller diameter may yield more robust assays. For particles to agglutinate they must first collide so that antigen–antibody bridges can form. For molecules and small particles diffusion is sufficiently rapid to produce the initial collisions necessary for aggregate formation. If the particles are larger diffusion is reduced (i.e., the agglutination kinetics) because the diffusion coefficient is inversely proportional to particle size. Small particles are desirable because of the requirement for increasing the collision frequency between particles or aggregates to enhance the rate of immunoaggregate production.

For the turbidimetric detection of the particle size change it is imperative that the particle size and the incident light wavelength be chosen with care since the turbidity reaches a maximum with time. This maximum occurs when the signal change exceeds the optical limits of the measuring system. It has been observed by photon correlation spectroscopy that changes in aggregates size continue beyond the plateau observed in turbidimetric assays (113). The optimal performance may be a function of the ratio of the particle diameter to the illumination wavelength, and the refractive index of particle. Thus, the selection of particle material, particle size, and wavelength of detection of the immunoagglutination reaction are all important factors in optimizing assay sensitivity. For particles that are small in comparison with the wavelength of light, the scattering increases with the inverse fourth power of the wavelength. Shorter wavelengths, such as 340 nm, give larger signal differences during immunoagglutination than longer wavelengths, such as 450 nm. On the other hand, the higher the refractive index of the particles at the wavelength of choice, the higher the light scattering signal. In general, the refractive index of a material is greater at shorter wave-lengths. Particles with a polyvinylnaphthalene core have been proposed to enhance sensitivity of latex immunoagglutination assays (114). Galvin et al. claimed that for the lowest detection limits particles should be in the size range 40–70 nm, with a high refractive index but low absorbance at the wavelength of light used (115).

Heller and Pangonis gave some information about how to optimize the particle size to wavelength ratio in turbidimetric assays (116). Sharp absorbance changes during the agglutination process can be obtained if the value of the term $2\pi a/\lambda$ is in the range 1–2. This theoretical prediction, obtained from Mie's theory, has often been corroborated experimentally. Different authors provided some evidence that minimal detection limits are achieved if the light wavelength used was in the order of 340 nm for particles in the size range 40–70 nm (117–119). This recommendation has been frequently followed by Price et al. (120–123). These authors even claimed that minimal detection limits are achieved using a wavelength of 340 nm independent of the particle size (101), which is a questionable statement. If latex particles are bigger (i.e., diameters from 100 to 400 nm) the optimal wavelength would be in the 450- to 700-nm range (124–127), as shown in Fig. 15. If larger particles are used instead, the infrared region on the spectrum should be employed (128). Finally, small particles of high refractive index with short wavelength detection are preferred for high sensitivity in the turbidimetric assays. There is a practical limit in the ultraviolet region for measurement of sample in serum because of light absorption by proteins and other components. Thus, convenient wavelengths are those in excess of approximately 320 nm. Turbidimetry measurements require a higher particle number than the other light scattering techniques.

3. Nephelometry. Nephelometry refers to the measurement of the light scattered at an angle θ from the incident beam when the incident beam is passed through the sample. The scattering theories show the importance of choosing a forward scattering angle for the study of particles with size approaching the wavelength of the incident light (Fig. 14). Common nephelometers measure scattered light at right angles to the incident

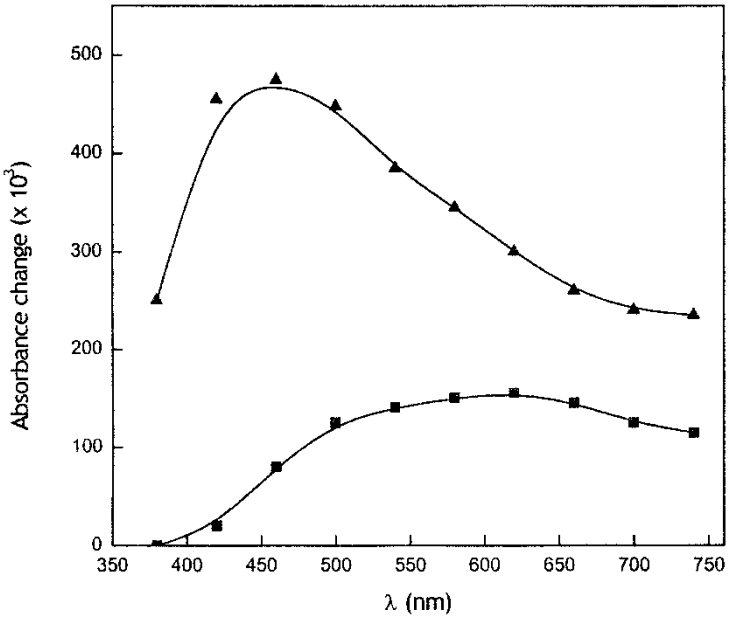


Figure 15. Turbidity change for agglutination of latex-F(ab′)$_2$-aCRP in presence of a constant concentration of CRP as a function of wavelength: (■) particle with 180 nm diameter; (▲) particle with 340 nm diameter.

light. The ideal nephelometric instrument would be free of stray light; neither light scatter nor any other signal would be seen by the detector when no particles are present in solution in front of the detector. However, due to stray light-generating components in the optics path as well as in the sample cuvette or sample itself, a truly dark-field situation is difficult to obtain when making nephelometric measurements. The sensitivity of nephelometric measurements clearly depends on this background signal (129, 130). On the other hand, the sensitivity of this technique also depends on the intensity of the light source with the highest sensitivity being achieved with a laser light source (131). Some nephelometers are designed to measure scattered light at an angle lower than 90° in order to take advantage of the increased forward scatter intensity caused by light scattering from larger particles (immunoaggregates). Unfortunately, forward scattering optical systems are harder to construct but several manufacturers now have forward scattering nephelometers (132). A good example of nephelometers that have been specially designed to operate with latex immunoagglutination is the Behring Nephelometer Analyzer with the following main features: (1) the light source is a red diode with a wavelength of 850 nm; (2) the detector is a photodiode that measure the scattered light in the forward direction at small angles (13–24°); (3) the detection limit is as low as 10 ng/mL for some measuring systems. Dedicated instrumentation is thus required for nephelometry, whereas turbidimetry is more broadly applicable (102).

In nephelometry the change in the intensity of the scattered light after a time is measured because the scattering species rapidly increase size. The scattered light is proportional to the initial antigen concentrations when measured in the presence of a fixed antibody–latex complex. Calibration curves can therefore be generated by plotting the intensity increment values against antigen concentrations. The concentration of the same antigen in an unknown sample can then be determined by measuring the intensity increment value under identical conditions and extrapolating on the calibration curve. Figure 16a and b show the importance of the angle in nephelometric observations. These figures correspond to the agglutination kinetics of latex particles sensitized with two different $F(ab')_2$ anti-CRP coverages for various low angles of measurement of the light scattering intensity (5°, 10°, and 20°). The intensity of light scattered by small clusters is weak and linear (low antibody coverage or beginning of the process), and is better monitored at higher angles (Rayleigh's scattering). Nevertheless, in the case of high coverage, after some time aggregates grow and light scattering amplification increases, preferably at lower angles (Mie's scattering).

Figure 17a and b show the immunoreactivity (scattered light intensity increment) as a function of CRP concentration at three different angles after 10 min of reaction for two latex-$F(ab')_2$ anti-CRP complexes. As can be seen, the main response features (intensity increments, detection limit, and sensitivity) are dependent on the scattering angle and antibody coverage. For the lowest coverage (Fig. 17a) the intensity increments increase with increasing the light scattering angle, and the shape of these curves coincides with that of the precipitin curve proposed by Heidelberger and Kendall (133). Such response can be explained considering that an antigen molecule acts as a bridge to coagulate two sensitized particles. It is easy to understand why before reaching the maximum the immunological response increases as CRP concentration does. At higher antigen concentrations the system seems to lose reactivity. It may be due to the blocking of the antibody active sites by antigens; thus, the bridging process is unfavored. Nevertheless, in the case of a higher protein coverage (Fig. 17b) the change in the scattered intensity for the 20° angle does not show the typical bell curve of the immunoprecipitin reaction, and gives an apparent plateau when the CRP concentration is above 50 ng/mL. This plateau

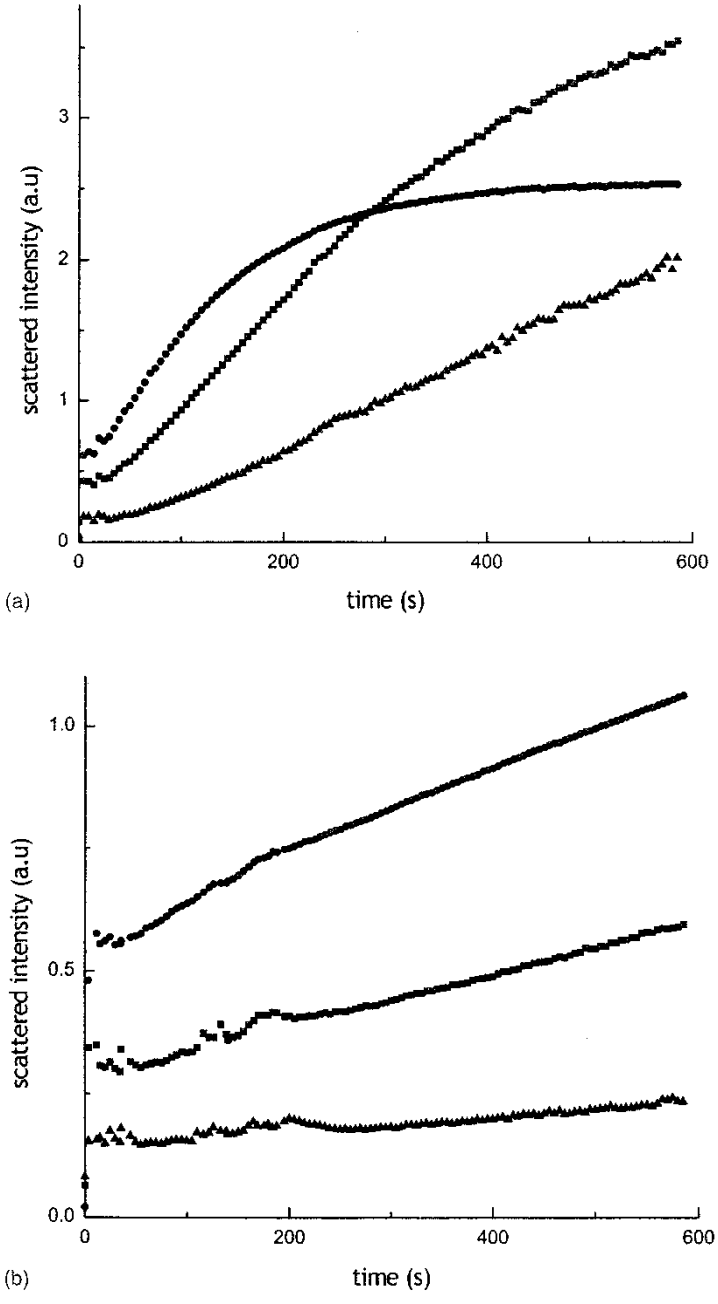


Figure 16. Scattered light intensity at various angles for the immunoagglutination of particles covered with $F(ab')_2$ anti-CRP. (▲) 5°; (■) 10°; (●) 20°: (a) 0.9 mg/m^2; (b) 2.1 mg/m^2.

represents a limitation of the nephelometric technique for high concentrations of antigen at 20°. It is important to emphasize that equilibrium and equivalence points in these assays are illusory. They are produced not only by the classical Heidelberger-Kendall immune aggregation phenomena but also optically (102). When monitoring the light scattered

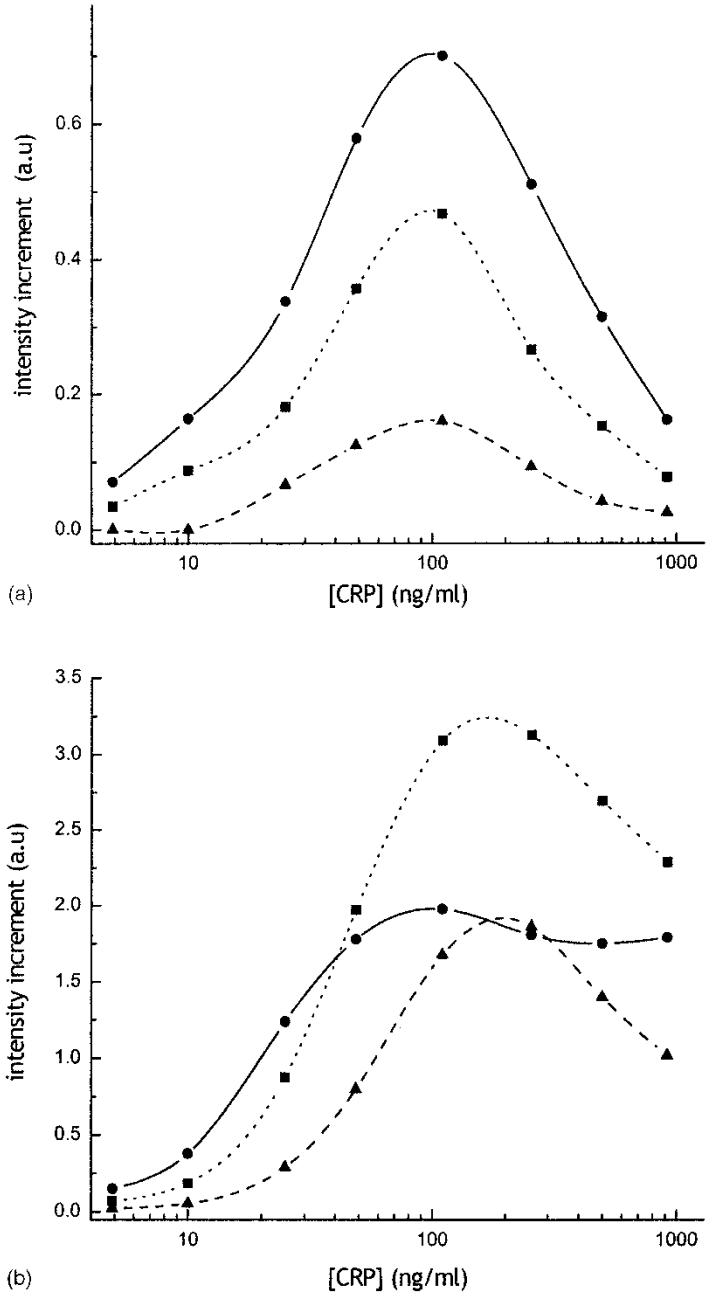


Figure 17. Immunoreactivity as a function of CRP concentration at three different angles (▲) 5°; (■) 10°; (●) 20° for two latex-F(ab′)$_2$ anti-CRP complexes: (a) 0.9 mg/m^2; (b) 2.1 mg/m^2.

by an immunoaggregate, further optical considerations may influence the apparent kinetics.

The preceding figures demonstrate that it is advisable to choose a 20° angle for agglutination processes of short-duration, low-antigen concentration or complexes with

low antibody coverage. In these cases, the measurements made at 20° are more sensitive than at 10° or 5° angles because the size of the aggregates is small enough. Nevertheless, if the size of the aggregates (or the initial single particles) is high, a smaller angle is preferred. Montagne et al. has followed the immunoagglutination of latex particles at different scattering angles (from 9.8° to 40.2°), concluding that the best light scattering amplification is obtained at small angles (9.8° and 12.2°) (134).

Although some authors have claimed that turbidimetric responses are more reproducible and much simpler to reach than those obtained by nephelometry (135, 136), the latter has been successfully employed by many over the last 20 years (128, 137–139). Ortega et al. has demonstrated that both techniques provide similar detection limit, although turbidimetry is slightly more reproducible (105). No longer reaction times are used with turbidimetric and nephelometric detection systems. On the other hand, nephelometry is best performed in dilute dispersion (in which background signal is reduced).

4. Angular Anisotropy. Angular anisotropy is a technique in which the ratio of the intensity of light scattering at two different angles is measured (dissymmetry ratio), usually one above and one below 90°. If the nephelometer measures the angular distribution of the scattered intensity a calibration curve can be obtained where the dissymmetry ratio is shown as a function of antigen concentration. The intensity of the light scattered at small angles is directly proportional to the square volume of the aggregates, whereas for higher angles this dependence on volume is drastically reduced. This feature was studied by Von Schulthess et al. who proposed a new strategy to analyze latex agglutination immunoassays (140). These authors applied angular anisotropy to the detection of human chorionic gonadotropin (HCG), measuring the scattered light at 10° and 90° to maximize sensitivity and to skip source light fluctuations. This method yields high sensitivity, at least theoretically (140), provided that the two angles and the carrier particle size are properly chosen. The above authors demonstrated that the particles acting as antibody carriers should have a radius (a) in the range $\lambda/4 < a < \lambda$. They also showed that the angular anisotropy technique could be very useful if one angle is lower than or equal to 15°, and the other is in the 60–100° range. Ortega et al. has shown that angular anisotropy is a very sensitive technique detecting 1 ng/mL of CRP, after longer reaction time (105).

5. Photon Correlation Spectroscopy. Photon correlation spectroscopy (PCS) is based on the fact that the intensity of light scattered from a latex suspension, when it is illuminated with a coherent light, fluctuates with time, depending on Brownian movement and therefore on the average diffusion coefficient, which could be correlated to the particle size (112). A photon correlation spectroscopy instrument is essentially a multiangle laser nephelometer. The PCS-based immunoassays generally have greater sensitivity than nephelometric and turbidimetric detection systems, although to achieve this longer reaction times are used due to the reduced particle numbers present in the dispersion. The device for PCS experiments is relatively expensive, as it is necessary to use a correlator. Furthermore it is difficult to find clinical laboratories with the sophisticated and extremely delicate equipment for carrying out dynamic light scattering measurements. This technique was first applied to latex immunoagglutination test for the detection of anti-bovine serum albumin by Cohen and Benedek in 1975 (141) and they claimed a sensitivity of 20 ng/ml in a highly reproducible way. Different authors have indicated that photon correlation spectroscopy offers lower detection limits, and use little reagent, but have longer assay times than the classical optical techniques of turbidimetry and nephelometry (105, 142).

V. The Problem of Low Colloidal Stability

A. Colloidal Stability

One of the basic requirements for a protein–latex complex to be applicable to clinical diagnostics is colloidal stability under immunological conditions. However, as indicated in the introduction, the most serious problem in latex immunoagglutination assays is that the system can lose its colloidal stability after antibody adsorption step. This low colloidal stability of latex–antibody complexes in the reaction medium may provoke the nonspecific agglutination of particles. The isoelectric point of most polyclonal IgG molecules used in latex immunoassays tests is in the range 6.5–8.5; in addition, they present a low charge density. Therefore, when the particles are covered by IgG the nonspecific agglutination process takes places under physiological conditions (pH 7.4 and ionic strength 150 mM), since there is almost no electrostatic repulsion between them. Antibody-coated particles must be completely stable in the absence of the antigen. That is, agglutination must only be triggered by the presence of the specific antigen rather than by the experimental conditions of the test. But, what is colloidal stability? When a cube with 1 cm of edge inmersed in a fluid medium is divided into many small colloidal cubes with 10 nm of edge, the surface of the system increases from 6 cm^2 to 600 m^2 (Fig. 18). This increasing area process is accompanied by a change in the free energy given by the expression $dG = \gamma_{SL} dA$, where γ_{SL} is the solid–liquid interfacial surface tension expressed in J/m^2. If the interfacial surface tension is positive, the colloidal dispersion is thermodynamically unstable ($\Delta G > 0$) and the particles tend to assemble to reduce the interfacial area (aggregation phenomena). These colloids are generally called lyophobic. On the other hand, if the interfacial surface tension is negative, the colloid is said to be lyophilic, the free energy of the system is negative, and the particles are thermodynamically stable. An example of lyophobic colloid is latex particles, whereas a typical lyophilic system is microgel. The term colloidal stability is refer to the ability of a suspension to resist aggregation. The colloidal stability may be either thermodynamic or kinetic. Lyophilic colloids are systems thermodynamically stable whereas lyophobic colloids are kinetically stabilized. The kinetic stability is a consequence of an energy barrier opposing collisions between the particles and possible aggregation subsequently. The stability control of suspensions warrants detailed attention because development of different applications of these systems to biophisics, pharmacy, agriculture, medicine, and modem technologies is dependent to a large extent on a better understanding and manipulation of the colloidal stability.

The tendency to aggregation of lyophobic colloids is attributable to the universal attractive van der Waals forces. In some cases, this attractive force between particle and medium is stronger than that between particles, with the result that the colloidal

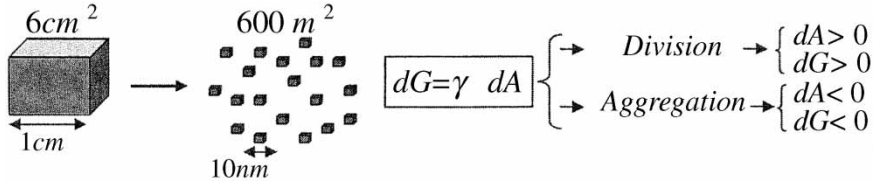


Figure 18. Fragmentation process of a cube.

state is preferred, i.e., the system is lyophilic (143). For lyophobic colloids only when the attractive van der Waals force is counteracted by a repulsive force can some degree of stability be obtained. When the particles have charges on the surface the colloid may be electrostatically stabilized. In some cases, a suitable polymer can be adsorb on the particle surfaces. As the two surfaces are brought closer together the concentration of polymer units increases in the overlap region with a resulting increase in the osmotic pressure. This tends to bring in solvent from the surrounding medium, with a consequent repulsive force to separate the particles. This polymer-induced stability is referred to as steric stability.

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability occupies a central position in colloid science. According to this theory, the stability is predicated on the notion that two independent types of forces govern the interaction between similar colloidal particles immersed in polar (especially aqueous) solutions: attractive van der Waals forces and repulsive electrostatic forces due to the net charge of the particle (144, 145). Electrostatic repulsion decays approximately exponentially with the separation distance H between two particles, whereas the van der Waals forces are proportional to H^{-1}. As a consequence, the interaction–energy distance curve is characterized by the presence of a shallow, secondary minimum at longer separation distances, an interaction barrier closer to the surface, and a deep primary minimum at short separation. The maximal potential represents the energy barrier opposing aggregation. If particles approach each other with sufficient kinetic energies to overcome this energy potential, aggregation occurs and the suspension is destabilized. Adding salt to a dispersion initiates aggregation by suppressing the electrostatic repulsion between particles. The energy barrier decreases with increasing electrolyte concentration and disappears above certain salt concentration called critical coagulation concentration (CCC). The study of this concentration is a practical way to determine colloidal stability.

The DLVO theory has been extensively tested and reviewed (146–149), and it stands today as the only quantitative theory of the colloidal and biocolloidal sciences. However, experimental investigations of the aggregation properties of a wide range of colloidal dispersions suggest that not all systems can be explained using the DLVO theory. When water is the dielectric medium in which colloidal particles are suspended, the theory generally fails to predict the stabilities of very hydrophobic or very hydrophilic particle suspensions. For example, the colloidal stability of silica at its isoelectric point (150), prevention of bubble coalescence at high ionic strength (151), deposition of PS latex on glass surfaces (152), swelling of clays (153), and many hydrophilic colloidal particles, and most biological surfaces and macromolecules, remain separated in aqueous solution even in high salt or in the absence of any net surface charge (154, 155). Reported results with amphoteric charged latex (156) also points to a deviation in the behavior respect to the classical DLVO theory. Many studies based on atomic force microscopy have thrown light on the limitations of the classical DLVO theory. Direct investigations of the interaction potential between silica surfaces (157–159) and mica surfaces (160) in aqueous electrolyte solutions have revealed agreement with DLVO at separations above a few nanometers, but at smaller separations a short-range repulsive force appears, often termed a "hydration" interaction.

B. Improvement of the Colloidal Stability of Antibody-Covered Particles

Different strategies have been proposed in the literature to solve the problem of the false diagnoses resulting from the nonspecific agglutination of the immuno-latex for low

colloidal stability. These strategies have been proposed to increase the stability of the antibody–latex system. The most relevant are the following:

1. Posttreatment to cover nonoccupied parts of the latex surface of the sensitized microspheres with a second inactive protein acting as a stabilizer (161, 162). On many occasions the biomolecule used is bovine serum albumin (BSA), a globular protein that is able to emphasize the colloidal stability of antibody-covered particles. The BSA molecule is a highly-charged protein at physiological pH. It supplies electrostatic stabilization to antibody-covered particles (163, 164). Moreover, BSA is easily obtainable in significant amounts, which is why it is commercially available at reasonably low prices. There are two different methods for preparing latex–antibody complexes with coadsorbed BSA: (1) sequential adsorption where in a first step the antibody adsorption is carried out and after centrifugation the complex is resuspended in a solution of BSA at constant concentration (165–167); (2) competitive coadsorption whereby the adsorption of both proteins occurs in a single step (166, 168). For competitive coadsorption the adsorption phenomena become more complicated because two types of proteins are in the medium. Peula et al. observed that the colloidal stabilization of IgG-covered particles appears when the coverage of coadsorbed BSA is high and at pH 7 and 9, and not stable at pH 5 (isoelectric point of albumin) (169). These authors also indicated that it is necessary to find the adequate equilibrium between the amount of IgG that produces a good immunological response and the amount of BSA responsible for the colloidal stability. These ways of stabilizing antibody–latex particles may have some disadvantages, e.g., only antibody–latex complexes with low antibody coverage can be stabilized or coadsorption with inactive proteins may involve partial or complete displacement of the preadsorbed molecules of antibody.

Other proteins coadsorbed together with the antibody molecules on the particle surface are casein or rabbit serum albumin (170).

2. The use of a detergent as stabilizer molecules can also have powerful effects on nonspecific agglutination. They mainly stabilize antibody-coated particles by means of steric forces, although electrostatic repulsions can also be generated by ionic surfactants. The surfactant concentration requires careful optimization because excess surfactant inhibits the antibody–antigen reaction. The aim is to balance producing a stable antibody-coated particle and not producing inhibition of the immunological reaction. However, this strategy to preserve the colloidal stability of particles presents some disadvantages, since such molecules can desorb the previously adsorbed antibody (171, 172). Only when covalent coupling was used to attach the antibody to particle surface is the use of detergent advisable. But, even in this case, some surfactants are capable of unfolding polypeptide structures, making the antibody lose its own immunoreactivity (172, 173).

3. Coadsorption of lipids and antibody on the polymer surface. The goal of this strategy is to obtain interfacial structures that are similar to biological membranes (174–176), This stabilization strategy has not been greatly investigated, probably because the lipids usually show a high insolubility in aqueous media, which is a considerable drawback (177).

4. Use of monoclonal antibodies with isoelectric point far from the physiological pH. The stability of such systems improves considerably (178), but the use of monoclonal antibodies would increase the cost of test.

5. An IgG molecule consists of three domains; two of them, the so-called Fab parts, contain the antigen binding site which is located on the top, whereas the third domain is the Fc region. The $F(ab')_2$ fragment can be obtained from IgG molecule by pepsin digestion.

It has been demonstrated that the use of the $F(ab')_2$ antibody fragment is useful in the development of latex immunoagglutination tests (179–183) because the colloidal stability of $F(ab')_2$–latex complexes is higher than that of the IgG–latex complexes. As the $F(ab')_2$ fragment shows an isoelectric point range more acidic than the whole IgG molecule (4.6–6.0) at physiological pH, the $F(ab')_2$-covered particles are electrostatically stabilized. On the other hand, as the divalent $F(ab')_2$ fragment has a similar avidity for antigen as the intact IgG molecule it can be used perfectly in the development of latex assays. Moreover, the use of $F(ab')_2$ instead of the whole IgG eliminates false positives in diagnostic tests due to the presence of the rheumatoid factor (184).

6. Covalent coupling of IgG on hydrophilic particles instead of hydrophobic surfaces (185). As previously indicated, the hydrophilic particles present highest colloidal stability but the physical adsorption of proteins on hydrophilic surfaces is energetically unfavored (nonspontaneous).

7. Colloidal particles covered with IgG or $F(ab')_2$ fragment present an "anomalous" colloidal stability (not explained by the DLVO theory) at high electrolyte concentration (186, 187). As can be seen in Fig. 19, the stability diagram presents two regions: a DLVO region and a non-DLVO region. In the DLVO region the colloidal stability proceeds as expected, decreasing with increasing the electrolyte concentration until a minimum is reached at the so-called critical coagulation concentration (CCC) where every interparticle collision is effective. At the CCC the repulsive part of the total interaction potential is completely shielded. Nevertheless, above a certain electrolyte concentration, known as critical stabilization concentration (CSC), a change in the trend is observed and the coagulation diminishes with increasing ionic strength (non-DLVO region). This phenomenon is, of course, quite contrary to the DLVO theory, since addition of electrolyte is generally expected to cause coagulation. This anomalous

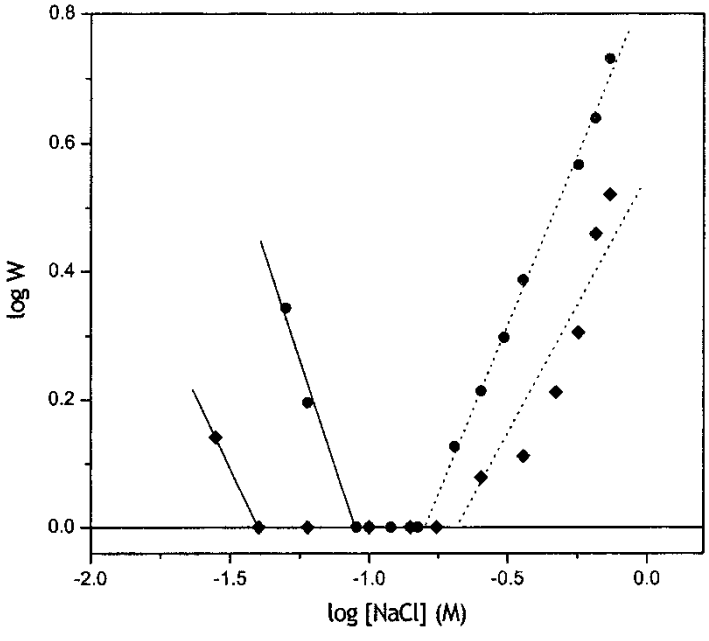


Figure 19. Dependence of the logarithm of stability factor (W) on the logarithm of NaCl concentration for IgG-latex conjugate (●) and $F(ab')_2$-latex conjugate (◆).

stability at high electrolyte concentrations has been explained by the so-called hydration forces (126, 186–188). It is well established that water molecules and hydrated cations strongly bind to the hydrophilic patches of the latex–protein complex. A simplified explanation of the hydration force is that it represents the opposition exerted by water molecules surrounding ions, charges, or polar groups on the complex surface to be removed, thus imposing an energy barrier to a close approximation between particles (189). The repulsive hydration force strongly depends on the type and concentration of electrolytes present in solution, and becomes stronger for increasing hydration energy of the cations involved (190, 191).

It is clear, as shown in Fig. 19, that particles coated by antibody become stable at high salt concentrations. Now the question is, will the presence of the specific antigen trigger the agglutination of such a stable system? The answer is affirmative, as demonstrated by Molina et al. (99, 138). Stable antibody–latex conjugates in 170 mM NaCl plus 300 mM $Mg(NO_3)_2$ aggregate when the antigen is added to the sample. Therefore, this colloidal stabilization strategy based on adding salts to the reaction medium aims to be totally successful in the development of latex inmunoagglutination tests. Moreover, it is cheap and requires minimal handling.

In a latex immunoagglutination test the aim is to selectively allow agglutination to occur with an analyte. The initial latex reagent test system must be sensitive, specific, stable, and reproducible in behavior. In order to accomplish this, a delicate balance between the attractive and repulsive forces must be achieved by manipulation of pH and ionic strength of the reaction medium, as well as by the extent of particle surface coverage by both nonspecific and specific molecules such that the repulsive forces maintain particle stability at short range (less than the bridge length of an antibody molecule) while at the same time keeping the repulsive energy less than the antigen–antibody bonding energy.

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