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(54) Title: LIGHT SCATTER-BASED IMMUNOASSAY WITHOUT PARTICLE SELF AGGREGATION

(57) Abstract

A homogeneous immunoassay method for the simultaneous determination of one or more antibody, antigen or hapten analytes in a fluid sample, that comprises the quantification of the effect of said analytes on the statistical changes in a dimension of a light scatter pulse height distribution histogram of relatively large diameter monodisperse binding molecule-coated polymeric microspheres induced by the binding to said microspheres of polydisperse binding molecule-coated colloid metal particles of relatively small diameter. For simultaneous assays of multiple analytes, different diameter or refractive index microspheres are assigned to each analyte. The assay may be used in forward binding, displacement, inhibition, and competition type systems, with the direction of the change in histogram dimension depending on the system. A convenient dimension to measure is the normalized peak width of a graphical representation of the histogram. For simultaneous assays for multiple analytes, a monodisperse polymeric microsphere of unique diameter or refractive index is dedicated to each analyte, so as to generate multiple histograms, one for each analyte. Polydisperse binding molecule-coated colloid metal particles, when used in effective concentrations, will also serve as a scavenger means to reduce the interfering effects of non-specific substances in the analyte-containing fluid samples, particularly when said samples are of biological origin. An improved sheath flow cell for an FPA is also described.

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LIGHT SCATTER-BASED IMMUNOASSAY WITHOUT PARTICLE SELF AGGREGATION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates broadly to homogeneous immunoassay methods for measuring an antigen, antibody or hapten analyte in a fluid sample by measuring light scatter signals from monodisperse particles in a flow particle analyzer. More particularly, the invention relates to measuring changes in light scatter signals from binding molecule-coated monodisperse polymeric microspheres as the result of the analyte-mediated binding to such microspheres of binding molecule-coated polydisperse colloidal metal particles.

Description of the Prior Art

Antibodies, antigens and many haptens exhibit high affinities, not only for their complementary proteins, but also for certain solid surfaces such as those found in the wells of plastic microtiter plates, walls of plastic test tubes, polymeric microspheres, and colloidal metal particles. Exploitation of these properties has led to a revolution in the field of diagnostic assay methods for the aforementioned analytes in fluid samples such as serum.

The ability to carry out antigen-antibody interactions on solid supports has greatly simplified

the separation of analyte-containing immunocomplexes from unused reactants and interfering substances, such as those often present in biological fluids. Such systems are generally referred to as "solid phase immunoassays" or "immunosorbent assays", and fall within the genus of "heterogeneous immunoassays." While the phase separation steps in heterogeneous immunoassays are valuable in reducing interferences by nonspecific binding substances that generally have an adverse effect on the sensitivity of the assay method, such assays are cumbersome and expensive, and are a focal point for reliability problems in automated systems.

Additionally, such heterogeneous systems have the additional disadvantage of requiring that one or another member of the immunocomplex be labeled with a molecule that can be easily quantified. Such molecules are generally referred to as "reporter molecules" and include radioisotopes (radioimmunoassays, RIA), enzymes (enzyme-linked immunoassays generally coupled with a chromophore, ELISA), fluorescent molecules (fluorescence immunoassays, FIA), chemiluminescent molecules(CIA), gold particles, photosensitive molecules, and the like. For a review, see Kemeny, D.M., et al., Immunology Today, 7: 67 (1986). Further, because of the limited number of chromophores and fluorophores available as receptor molecules, and the extensive overlap of emission spectra of such molecules, simultaneous assay of multiple analytes are not suitable using these reporters. For example, Cambridge Biotech's simultaneous EIA assays for C. difficile Toxin A and Toxin B, HTLV-I and HTLV-II, and HIV-1 and HIV-2, are not separable. The present invention greatly simplifies separability of signals

from multiple analytes in a single reaction mixture.

"Homogeneous immunoassay" is the term applied to immunoassays in which no phase separation occurs. Such systems, which include binding protein-coated particle agglutination assays, are useful because they have fewer steps to automate, and automation is mechanically, fluidically and electrically simple. Examples of immunoassays requiring no phase separation steps include: latex microsphere agglutination, hemagglutination, and fluorescence depolarization assays. Examples of latex bead agglutination assays for single analytes are found in U.S. patent nos. 4521521, 4184849, 4279617, 4191739 and 4851329, and for multiple analytes in a single fluid sample in Hansen, copending U.S. patent application no. 883574. Nephelometric or turbidimetric automated systems for agglutination or fluorescence depolarization assays are simple, inexpensive to construct, and, unlike heterogeneous assays, do not require frequent maintenance of the complicated phase separation apparatus.

The presence of interfering substances in body fluids has, however, inhibited otherwise promising homogeneous immunoassay approaches from meeting the high sensitivity requirements of many medically important tests, such as are met by ELISA and RIA. For reviews of this problem see, for example, Masson et al., Methods in Enzymology, 74:115(1981) and Collett-Casssart et al., Clin Chem., 27:64 (1981). One important aspect of the present invention is that it is a homogeneous immunoassay that is free from non-specific interferences, at least to a sensitivity level of about 5×10^{-13} M. This level of sensitivity is two to three orders of magnitude greater than prior art homogeneous latex bead agglutination assays. See,

e.g., alpha-fetoprotein (3 x 10^{-10} M, Collett-Cassart et al. above), urinary HCG (6 x 10^{-11} M, Lentrichia et al., J. Immunol. Meth., 89:657(1986) (but exhibiting only an 87% correlation with RIA at analyte levels 20 times the claimed sensitivity limit), and serum digoxin by a fluorescence depolarization method (3 x 10^{-10} M, S. Wong in D. Chan, ed., Immunoassay Automation, Academic Press, 1992, p. 329).

Prior art approaches to eliminating or decreasing the undesirable effects of non-specific interfering substance on homogeneous immunoassays have been generally unsatisfactory. These include: high dilution of body fluid sample (Fritz et al., J. Immunol., 108:110(1972), but this proportionally decreases sensitivity; using antibody fragments (Masson, Id.), but this approach is expensive and unpredictable; and, use of special conditions of pH, ionic strength, and buffer type, and/or addition of chelators or other scavengers (Masson, Id.), but these introduce multiple dependent factors that must be optimized for each analyte, and can become prohibitively expensive and cumbersome (Lim et al., J. Clin. Chem. Clin. Biochem., 20:141(1982).

Other approaches to solving the non-specific interference problem have included using IgG-coated latex ultramicrospheres to inhibit non-specific reactions in a latex sphere agglutination assay that uses antibody fragments. The sensitivity of one such agglutination method using a Coulter principle electronic resistance flow particle analyzer with a 30 μm orifice was reported to be about 5 x 10^{-13} to 4 x 10^{-12} M. Sakai et al., Chem. Pharm. Bull., 37:3010 (1989). The disadvantages of this approach is that the additional reagent (non-specific, IgG coated

ultramicrospheres) has an incremental manufacturing, quality control and storage cost associated with it. The present invention removes this important disadvantage by combining the action of specific immunoreactivity with the action of improving specificity, all in one reagent. In addition, although the Coulter principle particle counter used by Sakai et al. yields quantitative results, the need for the small (30 μm) orifice in order to sense agglutination has the well known problem of clogging during agglutination reactions (Masson, Id.). The present invention uses a sheath flow particle analyzer with a 250-300 μm orifice, which eliminates clogging. However, in the Sakai et al. approach, the distribution of specifically agglutinated particles (dimers, trimers, etc) presents a problem in Coulter volume overlap if simultaneous assays of more than one analyte are attempted, a problem not encountered in the present invention as multimers are not formed and multiple simultaneous assays can be performed without algorithms to remove the problem of overlap.

Another problem encountered in prior art agglutination immunoassays is the need for agitation by mechanical mixers during the entire reaction period of reaction mixtures containing particles of one micron or greater (Masson, Id.), thus requiring stringent washing between samples to prevent carryover of samples. A further advantage of the present invention, insofar as automation is concerned, is that agitation of samples is not needed to complete the agglutination reaction during useful time frames.

Schutt et al., EP 0254430 and United States Patent No. 5017009, show a scattered total internal reflectance (STIR) assay method for an analyte in which

colloidal gold particles are used as a label for proteins that bind to a coated macroscopic plastic plate of optical quality. This immunoassay relies upon the detection of back scattered light from an evanescent wave disturbed by the presence of a colloidal gold label brought to the interface by an immunological reaction. This evanescent wave is said to be the result of a totally internally reflected incident light wave. A disadvantage of this system is that the expensive optical-quality plastic plate is not reusable and must be discarded after a single use.

There remains an important need for an immunoassay method for antigens, antibodies, and haptens in fluid samples that combines the mechanical simplicity and low cost of particle agglutination homogeneous assays with the reduction in deleterious effects of interfering substances enjoyed by solid support based heterogeneous assays, and that does so with increased efficiency and scope when compared to prior art agglutination assays. This need is now fulfilled by the invention described in detail below.

SUMMARY OF THE INVENTION

The invention relates to a novel immunoassay method for simultaneously detecting and quantifying the concentration in a single fluid sample of one or more antigen, hapten or antibody analytes. The invention is based upon the serendipitous and unexpected observation that the binding of relatively small polydisperse binding molecule-coated colloidal metal particles to the surface of relatively large monodisperse binding molecule-coated polymeric microspheres produces changes in the dimensions of light scatter pulse height distribution histograms of these polymeric

microspheres, and that these changes in histogram dimension can be correlated with the concentration of the analyte that induces the dimension changes. Preferred light scatter is substantially low angle forward light scatter or substantially right angle light scatter. A convenient histogram dimension to determine for this purpose is the normalized peak width of a graphical representation of the histogram, that is, the peak width at one-half peak height.

In a forward binding reaction (sandwich) embodiment, a monodisperse immunocomplex is formed between large monodisperse polymeric microspheres coated with a first binding molecule, small polydisperse colloid metal particles coated with a second binding molecule, and an analyte that is complementary to both binding molecules. The dimensions of the light scatter pulse height distribution histogram of the monodisperse particulate immunocomplex are compared with control histogram dimensions (obtained with monodisperse coated polymeric microspheres in the absence of metal particles and linking analyte tested before, after, or during the analytical run), and the statistical increases in histogram dimension due to the presence of the analyte are correlated with the concentration of the analyte in the fluid sample. In a displacement embodiment of the invention, an immunocomplex reagent is first formed between monodisperse polymeric microspheres coated with the analyte and polydisperse colloidal metal particles coated with an anti-analyte antibody, the light scatter histogram dimensions of the immunocomplex is determined before and after its exposure to the analyte, and the statistical changes of the histogram dimension after exposure to the analyte correlated with analyte

concentration in the fluid sample. In a competition embodiment of the invention, an analyte competes against anti-analyte antibody-coated polydisperse colloidal metal particles for binding to monodisperse polymeric microspheres coated with a first binding molecule complementary to the anti-analyte antibody. A reduction by analyte in the extent of immunocomplex formation between the microspheres and metal particle, reduces a dimension of the light scatter pulse height distribution histograms in proportion to the concentration of analyte in the fluid sample. In an inhibition reaction embodiment, the immunoassay relies upon the ability of analyte to inhibit the binding of anti-analyte-coated polydisperse metal particles to binding molecule-coated monodisperse polymeric microspheres.

In one aspect of this invention, there are disclosed details and scope of forward binding, displacement, competition and inhibition embodiments of the aforementioned inventive method.

In another aspect there are disclosed polymeric microspheres and colloid metal particles suitable for carrying out the method of the invention.

In still another aspect there are disclosed examples of the application of the inventive method to the estimation of specific analytes in biological fluid.

It is yet another aspect of the invention to disclose application of the inventive method to the simultaneous assay of multiple analytes in the same fluid sample in a single analytical run.

It is yet another object to disclose the concurrent use of coated metal particles, not only for analytical purposes, but also as scavengers that

advantageously remove interfering non-specific substances present in biological samples from immunoassay reaction mixtures.

These and other objects will become apparent by reference to the following detailed description of the preferred embodiments and the appended claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows polymeric microspheres and metal particles (A), and light scatter pulse height distributions before (B) and after (C) binding of metal particles to monodisperse polymeric particles. Figure 1(D) shows the simultaneous analysis of multiple analytes in a single sample by a combination of the present embodiments (histograms D1, D2, D3, D4).

Figure 2 shows (A) the normalized peak width parameters and (B) calibration (i.e., standard) curve for a forward binding (i.e., sandwich) assay.

Figure 3 shows a human serum TSH assay standard curve (A) and comparison with other methods (B).

Figure 4 shows a T4 standard curve (A) and comparison with other methods (B), in human serum.

Figure 5 (A) shows a latex-latex agglutination assay for IgE and (B) the correlation curve for the method of the invention.

Figure 6 shows the kinetics of binding of colloidal gold particles to latex microspheres brought about by analyte.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to novel immunoassay methods for antigen, antibody and hapten analytes in a fluid sample based on flow particle analysis. The methods take advantage of the serendipitous and unexpected

discovery that a statistical change occurs in measured values of certain physical properties of a preparation of relatively large monodisperse polymeric microspheres when relatively small polydisperse metal particles bind to the former microspheres in an immunochemical reaction induced, proportionally to concentration, by an analyte. Examples of particle properties that can be monodispersed, as measured by flow particle analyzers, are: the so-called Coulter-volume of insulating particles in a given orifice diameter in an electrical impedance flow particle analyzer; the fluorescent emission by single particles when illuminated at a given wavelength, also in an optical flow particle analyzer; and, the light scatter pulse height distribution histogram of monodisperse microspheres substantially in a given direction when illuminated in an optical flow particle analyzer. The last-named property is used in the present novel method for concurrently measuring one or more antigen, antibody, or hapten analyte in a single fluid sample, a method that is also capable of avoiding or reducing the deleterious interfering effects of non-specific substances in biological fluid samples, in prior art agglutination immunoassays.

The optical flow particle analyzer (FPA) used in the invention uses a sheath flow cell through which passes a guided narrow sample stream, and light scatter, preferably substantially low angle forward light scatter or substantially right angle light scatter, of an incident light beam, preferably a laser beam, to sense and measure the degree of immunochemically-induced complex formation between relatively small polydisperse colloidal metal particles and a population of relatively large monodisperse

polymeric microspheres. The method is based upon the unexpected discovery that the aforementioned polydisperse metal particles, which themselves do not scatter light under the conditions of the invention in the region of the polymeric particles (even after the former are agglutinated by virtue of reaction with nonspecific binding components found often in sera), cause profound changes in the light scatter pulse height distribution histogram dimensions of the monodisperse polymeric microspheres when the metal particles bind to the surface of the polymeric microspheres to form an immunocomplex. Where the polymeric microspheres are coated with a first complementary binding molecule and the metal particles with another, a protein (i.e., antigen or antibody) or hapten analyte that is complementary to both first and second binding molecules will crosslink the metal particles to the polymeric particles, and will bring about a change in the dimensions of the light scatter pulse height distribution histogram of the monodisperse polymeric microspheres compared to the control histogram dimensions obtained in the absence of metal particles and analyte. The aforementioned change in the histogram dimensions has been found to correlate directly with the amount of the analyte present in the fluid sample.

It is an important discovery that the aforementioned change in the histogram dimension occurs in the absence of any significant shift in the position of the monodisperse polymeric microsphere light scatter pulse height distribution histogram, which indicates that self-aggregation of polymeric microspheres is not involved.

The preferred dimension for monitoring changes in

the histogram is the peak width at one half peak height of the graphical representation of the light scatter pulse height distribution histogram. This dimension will be referred to in this specification as "normalized peak width" or "NPW" (see Figure 2A for example). In the forward binding (sandwich) immunocomplex formation method described above, the change in histogram dimension is a widening of the NPW in the graphical representation and, consequently, an increase in the coefficient of variation (CV) around the histogram mean.

It has also been discovered that measurement of statistical changes in the dimension of a light scatter pulse height distribution histogram can be the basis for a quantitative determination of an analyte in a fluid sample in displacement-, competition- and inhibition-type immunoassay methods.

In a displacement embodiment, an immunocomplex reagent is prior-prepared by immunobinding polydisperse colloid metal particles coated with an anti-analyte antibody to monodisperse polymeric microspheres coated with analyte. A base line determination of the light scatter pulse height distribution histogram of this reagent will show a dimension that resembles that which results when the two types of particles are immunocomplexed, that is, a wide NPW if this is the dimension examined. When this reagent is mixed with a solution containing an analyte that is complementary to the binding molecule with which the metal particles are coated, the metal particles will be displaced from the immunocomplex reagent in direct proportion to the concentration of analyte present, and the histogram dimension will return to that resembling control monodisperse polymeric microspheres, that is, the

dimensions of the histogram will be reduced, that is to say, the NPW (and CV) will be reduced. The statistical extent of dimension reduction, as reflected in the NPW, can be correlated by standard means to the concentration of analyte in the fluid sample.

In a competition embodiment, binding molecule-coated monodisperse polymeric particles and analyte compete for binding to binding molecule-coated polydisperse metal particles. The NPW for the polymeric particles is inversely related to the concentration of fluid sample being analyzed, that is to say, a high analyte concentration will "capture" a relatively larger fraction of the metal particles, leaving a relatively smaller fraction of metal particles to bind to the polymeric microspheres. This will produce a relatively narrow histogram, that is, a small NPW. As in the previous two embodiments, the analyte may be an antigen, hapten or antibody, and it is well within the skill of the assayist, following the guidelines provided by the invention, to select appropriate binding molecules with which to coat the microspheres and metal particles.

In an inhibition embodiment, the analyte-containing sample is incubated with anti-analyte antibody-coated polydisperse metal particles and incubated for a finite time which does not necessarily include the time necessary to reach equilibrium, which may be on the order of hours. Analyte-coated monodisperse polymeric microspheres are then added and incubation is continued for another finite time, which is of the order of minutes, during which period the analyte inhibits the binding of metal particles to the polymeric microspheres. Histogram dimensions are determined as above. High concentrations of analyte

will produce narrow histogram peak widths as the result of its sequestering of the metal particles; low concentrations of analyte will produce the reverse.

As noted above, it is an important feature of the embodiments of the invention that incubation mixtures used need not be stirred during reaction periods. When adding components of reaction mixtures, only brief mixing (1-2 seconds) to produce homogeneity of the mixture is required.

A variety of commercially available polymeric particles may be used in this invention, although highly preferred are uniform latex microspheres. Bangs, L.B., Uniform Latex Particles, Seragen, Indianapolis, 1984. Although the term "latex", strictly speaking, refers to the polyisoprene of which milk sap is composed, the definition of this term has been expanded, and will be used herein to include synthetic polymers such as polybutadiene, polystyrene and the like. Uniform latex microspheres of average diameter ranging between 0.05 and 10 μm, preferably between about 0.5 and about 5.0 μm, and having stable hydrophobic surface groups to which proteins bind strongly so as to produce stable hydrophilic colloidal suspensions, are preferable diameters for use in the invention, although diameters ranging up to about 100-120 μm are available commercially. The 0.5 to 5.0 μm diameter latex microspheres with highly monodispersed diameters are available from Polysciences, Inc., Warrington, PA 18976 and from Interfacial Dynamics Corp., Portland, OR 97220. The standard deviation of diameters expressed as per-cent of the mean (i.e., coefficient of variation, or CV) are approximately 1% to 2% for these commercial preparations, and it is highly preferred that CV's of 2% not be exceeded.

The CV of the light scatter pulse height distribution histogram obtained from these spherical particles is a strong function of the relative diameter of the sample stream through the flow cell of the FPA and the focal dimensions of the incident (e.g., laser) light. If the sample stream is relatively large, then particles can flow through different intensities of the incident light beam and yield an undesirably large CV of pulse heights, even though the CV of the particles themselves is small. Therefore, it is essential that a narrow sample stream of constant dimensions be maintained in order to practice this invention optimally. Means for accomplishing this will be described below.

Although central bore diameters for the optical capillary flow cell ranging between 100 μm and 500 μm are suitable for purposes of the present invention, a central bore diameter of about 250 μm is preferred. It is preferred to center the fluid sample stream and to confine it to a diameter range of about 3 μm to 10 μm. In a highly preferred system, this diameter amounts to about 1% to 3% of the width of the laser light beam. Under these conditions, CV values for light scatter pulse height distribution histograms of less than 2% for monodisperse polymeric microspheres can be obtained, and are most preferred. As used in the present context "monodisperse" is taken to mean a population of polymeric microspheres that produce a low angle forward scattered light pulse height distribution histogram with a CV of no greater than about 2%. When the sample stream diameter is not controlled so as to give a histogram CV of about 2% or less for non-immunochemically sensitized control monodispersed spherical particles, the sensitivity of the method is

adversely affected, and the symmetrical broadening or narrowing of the histogram are not readily observed.

A variety of fluid drive means can be used to achieve the aforementioned narrow fluid stream. These include stepped motor controlled syringe pumps and vacuum devices. Persons skilled in this art will recognize that the best mode would be one in which the stream diameter could be either directly or indirectly regulated during a measurement and controlled to the proper dimension range. It is also desireable to provide the flow cell with a stream centering means so as to confine the narrowed fluid sample stream as precisely as possible in the center of the sheath flow. To monitor both the narrowing and centering features, it is possible to monitor the sample stream before, after and during the times during which the immunoreacted particles are measured. This can be accomplished in either case with a control population of monodisperse polymeric microspheres of the same or different diameters as that of the analytical microspheres and that do not participate in any immunobinding reactions. Such control microspheres can serve as a component of a sentinel system that includes an electronic sensing means that senses departures from the desired CV value, and that feedback regulates both or either the stream narrowing means and stream centering means so as to achieve the desired CV value.

As noted above, to carry out the inventive method in the forward binding (sandwich) reaction embodiment, both the relatively large monodisperse microspheres and the relatively small polydispersed metal particles are coated with different complementary binding molecules. Where the analyte is an antigen or hapten, monodisperse

polymeric microspheres are coated with a first complementary anti-antigen or hapten analyte antibody, which may be a monoclonal antibody directed to a first epitope on the antigen. The polydisperse metal particles are coated with a second anti-antigen analyte antibody, which may be a monoclonal antibody directed to a second epitope of the antigen analyte. In the presence of antigen, a typical "sandwich" reaction takes place, with the antigen crosslinking the smaller metal particles randomly on the surface of the larger polymeric microspheres. When this occurs, and the resulting particulate immunocomplexes are passed through the optical flow cell described above, we have found unexpectedly that the CV and standard deviation of the monodispersed light scatter pulse height distribution histogram, as reflected in a graphical representation of the histogram, produces peaks that broaden approximately symmetrically about the peak mean. We have also discovered that this broadening is directly and quantitatively related to the concentration of the analyte in the fluid sample.

The principle of this embodiment of the invention is shown in Figure 1. In Figure 1A are shown the relative diameters of monodisperse latex microspheres (about 1.0 μm diameter) and the polydisperse colloidal metal particles (about 20 to 120 nm in diameter). In the preferred particle diameters described above, it can be seen that the ratio of polymer microsphere diameter to metal particle size ranges between 15-30:1. This characteristic provides the potential for a large number of colloidal metal particles to be bound randomly on each polymeric microsphere. The light scatter pulse height distribution histogram for the latex microspheres is shown in Figure 1B. The pulse

height signal from the latex microspheres is high, and the width of the peak representing the histogram is very narrow. As is evident, the small amount of light scatter from self agglutinated metal particles does not interfere, i.e., does not overlap, with the histogram produced by the microspheres. After analyte-mediated binding of the metal particles to the latex microspheres, a broadening of the latex histogram peak occurs (Figure 1C) in a forward binding reaction. Even when multiple analytes in a single sample are analyzed simultaneously by a combination of the present embodiments (histograms D1, D2, D3 and D4 in Figure 1D), there is no overlap with the signal generated by unbound gold particles.

Methods for producing stably adsorbed proteins on latex microsphere surfaces are described generally in Seaman, G.V.F., ed., Latex Based Technology in Diagnostics, Health & Science Communications, Washington, D.C. 20005, 1990, which is incorporated by reference.

We have discovered that this invention may also be carried out in a displacement embodiment. To carry out the invention in this mode, the two types of particles are first immunochemically sensitized by incubation separately with complementary binding molecules to form a two component immunocomplex reagent. In one embodiment, the large polymeric microspheres are coated stably with analyte. The metal particles are coated with a binding molecule that is complementary to the analyte. The two suspensions are mixed to form a reagent that may be stored prior to use. This reagent is then mixed with the analyte-containing fluid sample. The analyte displaces a portion of the bound metal particles from the polymeric microspheres by virtue of

immunocomplex formation. This displacement results in a decrease in the dimension of the light scatter pulse height distribution histogram of the aforementioned immunocomplex reagent. This decrease is proportional to the concentration of analyte present in the fluid sample.

In a competition-type embodiment, an analyte and an analyte-coated polymeric microsphere compete for binding to anti-analyte-coated metal particles. After an appropriate incubation period, the suspension is subjected to FPA and histograms determined. A control consists of the coated polymeric particles alone.

As noted above, in an inhibition embodiment, analyte is incubated with anti-analyte binding molecule-coated polydisperse metal particles for a finite period of time, e.g., 5-30 minutes, during which period the analyte binds to and "sequesters" a portion of the metal particles. Unbound metal particles are free to bind to polymeric microspheres coated with a binding molecule (which may be a carrier binding molecule that contains analyte) that are then added and incubated. FPA of control and experimental monodisperse polymeric microspheres after a brief period, e.g., 5-10 minutes, of incubation will reveal the amount of unbound metal particles which, in turn, is inversely related to the concentration of analyte.

While it has been convenient to describe the forward binding (sandwich) reaction, inhibition, displacement and competition embodiments of the invention in terms of an antigenic or hapten analyte and antibody-coated particles, it should be understood that the scope of this invention includes assays in which the analyte is an antibody present in, for example, human serum and the like. In such assays

carried out in the forward binding mode, both types of particles may be coated with the antigen that is complementary to the analyte or, optionally, one type of particle may be coated with the antigen and the other with a second antibody directed against the antibody analyte. In this mode, in the presence of the antibody analyte, the small polydisperse metal particles will complex with the monodisperse polymeric particles, and the abovedescribed statistical changes in the light scatter pulse height distribution histogram measured.

The foregoing has described assaying for a single analyte in a fluid sample. It is within the scope of this invention to analyze concurrently multiple analytes in the same fluid sample without the need to split the sample for multiple assays as must be done in prior art methods. This may be accomplished by assigning to each analyte to be determined a polymeric microsphere of a unique diameter and/or refractive index and a type of binding reaction. The coatings on the different size microspheres will, of course, be determined by the type of assay system to be assigned to the analyte, e.g., forward binding, inhibition, displacement or competition. The assay systems for the multiple analytes may be the same or different. This embodiment of the invention relies upon the electronic components of the FPA to monitor the light scatter signals produced by each differently sized polymeric microsphere. Electronic systems for monitoring each size of microsphere are disclosed in copending United States patent application serial no. 883,574, which is incorporated by reference. Briefly, signals from light detectors that receive the light scatter signals from polymeric microspheres may be analyzed by either of two

analytical systems. In a preferred analytical system which is software based, pulses from the light scatter detector are fed to an analog-to-digital converter which samples the peak height of each pulse and passes these peak height values to a computer which sorts the peak height values by size and then arranges them in a histogram, which may be a smoothed histogram, one for each analyte.

Binding molecule-coated polymeric microspheres may be purchased commercially (Polysciences, Inc.) or prepared as described in Seaman, 1990, above. In a typical coating procedure, suspensions of microspheres are incubated with a buffered (pH 7 to 8) solution of a first binding molecule at a concentration and for a period of time (typically, 0.5 to 16 hrs) sufficient to reach equilibrium binding of the molecule to the microspheres. Coated microspheres are recovered by brief centrifugation, and nonspecific binding sites blocked by a brief (e.g. 15 min) exposure to a solution of an inert protein (e.g., nonfat dry milk solids or serum albumin). Coated microspheres are then washed at least three times with an at least 4-fold volume of cold storage buffer. Any storage buffer that provides stability to the microsphere suspensions on storage may be used. Typical storage buffers are 0.5% BSA-0.1% NaN_3 in 0.154 M NaCl, pH 7.4, or 0.1% BSA-0.01% NaN_3 in 10 mM HEPES buffer, pH 7.5.

The polydisperse metal colloids may be of metals and metal compounds, such as metal oxides, metal hydroxides and metal salts. Examples include the metals gold, platinum, silver and copper; gold is highly preferred. Methods of production of colloidal gold of the desired range of particle diameters, and methods for coating metal particles with proteins, are

described generally in Roth, J., "The Colloidal Gold Marker System for Light and Electron Microscopy Cytochemistry", in Bullock, G.R. et al., Techniques in Immunochemistry, 2: 217 (19), in Horisberger, M., SEM 11: 9 (1981), in Weiser, H.B., Inorganic Colloid Chemistry, J. Wiley, N.Y. 1931, p. 1, in Leuvering, J.H.W., United States patent no. 4,313,734, and in Frens, G., Nature, Physical Science, 241:20 (1973), all of which are incorporated by reference.

Polydisperse colloidal gold particle suspensions may be obtained from E-Y Laboratories, Inc., San Matteo, CA 94401 or prepared as described in the above-cited references. In a preferred method, polydisperse colloidal gold particles of particle size 10 nm to 120 nm diameter are produced by the method of Frens (1973) above. Although this method produces a relatively broad range of gold particle sizes, the dimensions of the range is not a critical factor in the present invention. As noted above, gold particles of such sizes do not produce measurable and interfering light scatter under the conditions of the invention. Within the present context, therefore, by "polydisperse metal particles" is meant a population of colloidal metal particles whose diameters range between 20 nm and 120 nm.

In one method of coating gold particles with antibody, the above-described solution of gold particle is titrated to pH 7.5 using K_2CO_3. The coating molecule, e.g., antibody is dissolved in 10 mM HEPES buffer, pH 7.5, containing 0.02% BSA as a stabilizer, and one-tenth volume is added to the gold particle solution. After 60 mins of mixing, a one-tenth volume of 0.1% non-fat dried milk solids is added in order to block nonspecific binding sites. The particles are

washed three times by centrifugation in the storage buffer (10 mM HEPES, pH 7.5, 0.1% BSA, 0.01% NaN_3, 1% mannitol) and used immediately or stored at 4°C. Other methods for coating gold particles are described in Leuvering above, which is incorporated by reference.

Using the present invention, highly sensitive assays for a multi-epitopic, high molecular weight antigen such as thyroid stimulating hormone (TSH) may be carried out by either passively or covalently binding appropriately complementary polyclonal or monoclonal antibodies to the colloidal metal (e.g. gold) particles and polymeric (e.g. latex) microspheres. The exact method of protein binding is not critical provided that the immunological integrity of the protein is not compromised, and that the binding is stable under the conditions of the assay. Particularly preferred for this assay are 1.62 μm diameter latex microspheres with a CV of about 2% (Interfacial Dynamics Corp.). Colloidal gold particles with polydispersed sizes in the range of 50 to 80 nm, as determined by electron microscopy, made as described above, are particularly preferred.

As noted above, I have found that colloidal metal particles, when used at high densities relative to polymeric microspheres such as from 2 to 100,000 to 1, act as "scavengers" for nonspecific interfering substances commonly found in fluid samples of biological origin, such as human serum. That is to say, the metal particles not only provide the basis for the quantitative immunoassay of the invention, but also reduce the deleterious effects of interfering substances. Although the useful particle ratio in this regard is very broad, metal particle to polymeric microsphere ratios of the order of 1000 to 10000:1 are

preferred.

The following examples are provided merely to illustrate several embodiments of the invention, and are not intended to delimit the scope of the invention which is encompassed by the specification and included claims.

EXAMPLE 1

FORWARD (SANDWICH) BINDING REACTION ASSAY FOR SERUM TSH

Polydisperse colloidal gold particles (50-80nm) were coated with a first anti-TSH monoclonal antibody (BioDesign International, Inc., Kennebunkport, ME 04046) as follows. A suspension of colloidal gold particles was brought to pH 7.5 using 0.2 M potassium carbonate. To this suspension was added a 0.1 volume of the coating antibody diluted in 10 mM HEPES-0.02% BSA, pH 7.5. After mixing for 1 hr, a 0.1 volume of a solution of non-fat dried milk solids were added with mixing. The particles were washed three times by centrifugation in 10 mM HEPES buffer (pH 7.5) containing 1% BSA, 0.01% NaN_3 and 1% mannitol.

Polystyrene (latex) beads of 1.62 μm diameter (approximate diameter CV of 2.0%) were coated with a second anti-TSH monoclonal antibody as described in Hansen, United States patent application serial no. 883,574, which is incorporated herein by reference.

Coated gold particles were mixed with coated latex particles in a ratio of 10,000 to 1 to form Reagent A. Reagent A was added to TSH-containing serum in a volume such that the final latex microsphere density was about 2×10^6/mL, and the serum was 40%. After 60 minutes of incubation at room temperature, the mixture was measured in a sheath FPA as described above using low angle forward light scatter. Normalized peak width

units ("NPW" in Figure 2) as a function of analyte TSH concentration in the serum over the concentration range of 0 to 1.2 μIU/mL is shown in Figure 3. The sensitivity of this assay was about 5 x 10^{-13} M in serum.

EXAMPLE 2

INHIBITION ASSAY FOR THYROXINE (T4)

Monodisperse latex microspheres (1.62 μm diameter, Interfacial Dynamics Corp.) were coated with human thyroglobulin (Calbiochem Inc.) as follows to form Reagent A. The microspheres were incubated overnight with antigen thyroglobulin in 10 mM HEPES buffer, pH 7.5. Microspheres, recovered by centrifugation, were washed with HEPES buffer containing BSA and NaN_3 as described above, and stored in the same buffer containg mannitol.

Polydisperse colloidal gold particles (50 - 80 nm diameter) were coated with an antibody to T4 as follows to form Reagent B. One-tenth volume of 10 mM HEPES buffer (pH 7.5) containing antibody and BSA was added to the gold particles. For the T4 assay, an IgG-purified polyclonal antibody specific to T4 (OEM Concepts) was used for coating. Coated particles were then post-coated with a solution of non-fat dry milk powder to block nonspecific binding sites. Particles were recovered by centrifugation and washed with 10 mM HEPES buffer (pH 7.5) containing BSA and NaN_3. Washed particles were stored to the same buffer, to which mannitol was added as a stabilizer. Coated gold particles were diluted into an assay buffer containing 50 mM glycine (pH 9), 10 mM EDTA, 0.01% aminonaphthosulfonic acid (Sigma Chem. Co., St. Louis, MO), 0.1% BSA, 0.3 M KI and 0.01% NaN_3.

Serum samples containing T4 were incubated with Reagent B for 30 minutes, and then Reagent A was added

to deliver a microsphere density of 2 x 10^7/mL. After an additional 30-minute incubation, samples were analyzed in the FPA for histogram peak width determination using low angle forward light scatter.

Serum T4 inhibits the binding of gold particles with latex thyroglobulin. High concentrations of serum T4 produced narrow histogram peak widths, and low concentrations of serum T4 produced the converse (Figure 4A). The curve in Figure 4A was generated using calibrator sera (Biomerica, Inc., San Luis Obispo, CA) and served as the standard curve for further comparisons with standard EIA methods, i.e., the EMIT method (Syva Corp., Palo Alto, CA). The correlation data are shown in Figure 4B. The correlation was > 95% over the analyte concentration range shown.

EXAMPLE 3

SIMULTANEOUS ASSAY OF TSH AND T4 IN THE SAME FLUID SAMPLE

The TSH and T4 immunoassays described in Examples 1 and 2 were combined in order to demonstrate that multiple analytes may be analyzed in the same fluid sample. The T4 and TSH assays were carried out with monodispersed latex microsphere populations having mean diameters of 0.95 μm and 1.62 μm, respectively. The final serum concentration was 15%, the gold particle concentration was 1 x 10^{10}/mL, and the latex microsphere concentrations were 1 x 10^8/mL for T4 and 5 x 10^6/mL for TSH.

Reactions were carried out in the following sequence. The TSH-assay reaction was performed as described in Example 1. After 30 minutes of this reaction, Reagent B of Example 2 was added. Twenty-

five minutes thereafter, Reagent A of Example 2 was added and the mixture incubated for five minutes. FPA analysis was then performed.

When midrange concentrations of the analytes were assayed with the FPA system and methods of the present invention, the results were 4 μg/mL for T4 and 2 μIU/ml for TSH. When a sample with no T4 and a TSH midrange level of 2 μIU/mL was assayed by the present method, the measured T4 value was zero, and the measured value for TSH was 2 μIU/mL ± 10%. With a sample with a known low value for TSH (0.3 μIU/mL) and a known midrange value of 4 μg/mL for T4, the present method correlated within 10% with the known values.

This experiment demonstrates the way in which the present invention permits simultaneous assays can be performed with separable results, and with no detectable cross-interference between assays in a single run.

EXAMPLE 4

COMPARISON OF THE PRESENT METHOD WITH A STANDARD LATEX-LATEX AGGLUTINATION METHOD FOR IgE DETERMINATION

In order to demonstrate the efficacy of the present invention in suppressing the adverse effects of non-specific binding, an assay for human IgE was conducted in two formats. In the first format, ordinary latex microsphere-latex microsphere agglutination was carried out with anti-IgE polyclonal antibodies passively coated on the latex particles, according to the method of Masson et al., above, in which the disappearance of monomeric particles is monitored. The latex particle diameter was 1.62 μm. The second format consisted of the forward binding reaction embodiment of the present invention.

To coat gold particles, a suspension of gold particles was titrated to pH 7.5 with 0.2 M K_2CO_3. To this suspension was added a one-tenth volume of mouse monoclonal anti-human IgE (Biodesign International, Inc., Kennebunkport, ME, 04046) in 10 mM HEPES buffer, pH 7.5, containing 0.02% BSA. After 60 minutes of mixing, a one-tenth volume of 0.1% non-fat dried milk solids solution was added. The particles were isolated by centrifugation washed three times with the same buffer, then stored in a storage buffer consisting of 10 mM HEPES, 0.01% BSA, 0.01% NaN_3, 1% mannitol.

To coat latex microspheres, 1.62 μm latex microspheres (Interfacial Dynamics Intnl., Portland, OR 97220) were diluted to a density of 0.5% in 10 mM HEPES, pH 7.5, the suspension was incubated overnight at 4°C with a 100 μg/mL solution of affinity-purified goat anti-human IgE, microspheres were isolated by centrifugation and washed three time with HEPES buffer, and finally stored in 10 mM HEPES, 0.1% BSA, 0.01% NaN_3, 1% mannitol.

For assay, latex and gold particles were diluted to 1 x 10^8/mL and 2 x 10^{10}/mL, respectively, in assay buffer. A 50 μl aliquot of analyte serum sample was added to 450 μl of the particle mixture, and mixed by vortexing; the final serum dilution was 10%. After 15 minutes of incubation at 23°C, the reaction mixture was subjected to FPA analysis. The assay buffer was 0.05 M glycine 0.1% BSA, 0.3 M KI, 0.01% NaN_3, pH 9.5.

A commercial ELISA IgE assay method for serum IgE(Ventrex Laboratories, Portland, ME) served as the reference standard for both formats. The correlation curve for the latex-latex agglutination format is shown in Figure 5A and that for the method of the invention in Figure 5B. It is clear from the data that the

correlation is poor and that the sensitivity is limited for the latex agglutination method, likely due to the influence of interfering substances in the analyte serum sample. In sharp contrast, the latex microsphere-gold particle method of the present invention shows an excellent correlation with the reference method (Figure 5B).

EXAMPLE 5

KINETICS OF HISTOGRAM PEAK WIDTH BROADENING

The assay of Example 1 was carried out in a manner that permitted low angle forward light scatter FPA analyses of the reaction mixture (containing 6 μIU/mL TSH) at intervals.

In the absence of analyte (-o-, Figure 6), coated gold particles did not bind to coated latex microspheres, and there was no change in histogram dimension. However, in the presence of analyte (-□-, in Figure 6) binding was detected from the first time point (2 min.), as reflected by a broadening of the histogram derived from the monodisperse latex microspheres. This broadening continued at a linear rate for the first 20 minutes of reaction, and reached a plateau at about 100 minutes.

I CLAIM:

1. A polymeric particle light scatter-based immunoassay method for simultaneously measuring one or more antibody, antigen or hapten analytes in a single fluid sample essentially without self-aggregation of said polymeric particles, comprising the steps of:

a) combining with said fluid sample for each said analyte first binding molecule-coated monodisperse polymeric microspheres of unique diameter or refractive index, such that the light scatter pulse height distribution histogram obtained in an optical sheath flow particle analyzer for each said unique diameter or refractive index microsphere is resolvable from all other unique diameter microspheres, and second binding molecule-coated colloidal metal particles; wherein said first and second binding molecules are selected such that the extent of binding of the colloidal metal particles to the polymeric microspheres is dependent upon the amount of analyte in the fluid sample;

b) measuring, in an optical sheath flow particle analyzer equipped with a regulatable means for producing a narrow fluid reaction mixture stream, a regulatable means for centering said narrow stream flowing through an optical capillary sheath flow cell, a source of incident light, and a means for measuring the scatter of said incident light induced by said microspheres, the effect of each said analyte on the statistical change in the dimension of light scatter pulse height distribution histograms of first binding molecule-coated monodisperse polymeric microspheres that are induced by the binding to said microspheres of second binding molecule-coated polydisperse colloidal

metal particles; and

c) correlating said analyte effect with the amount of each said analyte in said single fluid sample.

2. A method of claim 1, wherein said incident light scatter is substantially low angle forward light scatter.

3. A method of claim 1, wherein said incident light scatter is substantially right angle light scatter.

4. A method of claim 1, wherein the binding of said second binding molecule-coated polydisperse colloidal metal particles to said first binding molecule-coated polymeric microspheres is increased by said analyte, and said increase results in an increase in said dimension of said histogram.

5. A method of claim 1, wherein the binding of said second binding molecule-coated polydisperse colloidal metal particles to said first binding molecule-coated polymeric microspheres is decreased by said analyte, and said decrease results in a decrease in said dimension of said histogram.

6. A method of claim 1, wherein said histogram dimension comprises a normalized peak width of a graphical representation of each said histogram.

7. A method of claim 1, further comprising the step of providing a ratio of said metal particles to said polymeric microspheres effective to reduce interference in said immunoassay by nonspecific binding substances.

8. A method of claim 7, wherein said ratio ranges between 2 and 100,000 to 1.

9. A method of claim 7, wherein said ratio ranges between 1,000 and 10,000 to 1.

10. A method of claim 1, wherein said monodisperse polymeric microspheres comprise uniform latex microspheres.

11. A method of claim 1, wherein said microspheres comprise microspheres of average diameter ranging between about 0.02 and about 100 μm.

12. A method of claim 1, wherein said microspheres comprise microspheres of an average diameter ranging between about 0.05 and about 10.0 μm.

13. A method of claim 1, wherein said microspheres comprise microspheres of an average diameter ranging between about 0.5 and about 5.0 μm.

14. A method of claim 1, wherein said colloidal metal particles are selected from the group consisting of gold, platinum, silver and copper particles.

15. A method of claim 1, wherein said colloidal metal particles are gold particles.

16. A method of claim 1, wherein the average diameter of said polydisperse metal particles ranges between about 20 nm and about 120 nm.

17. A method of claim 1, wherein the average diameter of said polydisperse metal particles ranges between about 50 nm and about 80 nm.

18. A method of claim 1, wherein the diameter of said regulatable narrow sample stream ranges between about 3 μm and 10 μm and produces a coefficient of variation around a graphical representation of a histogram mean of no greater than about 2% for control, non-immunochemically sensitized monodisperse polymeric microspheres.

19. A method for detecting an analyte in a fluid sample by analyte-mediated binding reaction comprising the steps of:

a) placing in a reaction vessel said first

binding molecule-coated monodisperse polymeric microspheres, said second binding molecule-coated polydisperse colloidal metal particles, and said fluid sample containing said analyte for a period of time effective to form a reaction product consisting essentially of a monodisperse immunocomplex between the three components;

b) analyzing said monodisperse immunocomplex in said flow particle analyzer;

c) measuring the dimensions of the light scatter pulse height distribution histogram of said monodisperse immunocomplex;

d) comparing said dimensions in c) with the dimensions of a control reaction mixture composed of monodisperse polymeric microspheres analyzed in the absence of analyte or metal particles, to determine the statistical change in said histogram dimension in c); and,

e) relating said statistical change in said histogram dimension to the concentration of said analyte in said fluid sample.

20. A method of claim 19, wherein said analyte is an antigen or hapten, said first binding molecule comprises a first complementary anti-analyte antibody, and said second binding molecule comprises a second complementary anti-analyte antibody.

21. A method of claim 19, wherein said analyte is an antibody, and said first and said second binding molecules are each an antigen or hapten complementary to said analyte antibody, or, optionally, one binding molecule comprises an antigen complementary to said analyte antibody and the other binding molecule comprises a second antibody directed against said analyte antibody.

22. A method of for detecting an analyte in a fluid sample by analyte-mediate displacement reaction comprising the steps of:

a) mixing analyte-coated monodisperse polymeric microspheres and complementary binding molecule-coated polydisperse colloid metal particles or, optionally, analyte-coated metal particles and complementary binding molecule-coated polymeric microspheres, so as to form a monodisperse immunocomplex reagent;

b) mixing said monodisperse immunocomplex reagent with a fluid sample containing said analyte for a period of time sufficient for said analyte to displace a portion of said metal particles from said monodisperse immunocomplex;

c) determining the dimensions of the light scatter pulse height distribution histograms for said monodisperse immunocomplex both before and after displacement with analyte in step b); and,

d) relating the statistical changes in said histogram dimensions to the concentration of said analyte in said fluid sample.

23. A method of claim 22, wherein said analyte is an antigen or hapten.

24. A method of claim 22, wherein said analyte is an antibody, and said complementary binding molecule is the corresponding antigen.

25. A method for detecting an analyge in a fluid sample by a competition reaction comprising the steps of:

a) mixing anti-analyte antibody-coated polydisperse metal particles, analyte, antigen or hapten-coated monodisperse polymeric microspheres, and a fluid sample containing analyte antigen or hapten for

a period of time sufficient for competition immunocomplexes to form;

b) determining the dimensions of the light scatter pulse height distribution histograms for said coated monodisperse polymeric microspheres before and after said competition immunocomplex formation; and

c) relating the statistical changes in said histogram dimensions to the concentration of said analyte in said fluid sample.

26. A method for detecting an analyte in a fluid sample by a competition reaction comprising the steps of:

a) mixing antigen or hapten-coated polydisperse metal particles, fluid sample containing an analyte antibody, and monodisperse polymeric microspheres coated with a binding molecule complementary to said antigen or hapten for a period of time sufficient for competition immunocomplexes to form;

b) determining the dimensions of the light scatter pulse height distribution histograms for said coated monodisperse polymeric microspheres before and after said competition immunocomplex formation; and

c) relating the statistical changes in said histogram dimensions to the concentration of said analyte in said fluid sample.

27. A method for detecting an analyte in a fluid sample consisting of an inhibition reaction, comprising the steps of:

a) coating monodisperse polymeric microspheres with a first binding molecule that is or is conjugated to analyte to form a first reagent;

b) coating polydisperse metal particles with a second binding molecule that is complementary to

analyte to form a second reagent;

c) incubating an analyte-containing fluid sample with said second reagent to form a polydisperse immunocomplex;

d) incubating said polydisperse immunocomplex with said first reagent to form a monodisperse immunocomplex between said microspheres and that fraction of metal particles not bound to analyte;

e) analyzing said monodisperse immunocomplex in said flow particle analyzer;

f) measuring the dimensions of the light scatter pulse height distribution histogram of said monodisperse immunocomplex;

g) comparing said dimensions in f) with a control reaction mixture composed of coated monodisperse polymeric microspheres in the absence of analyte or metal particles to determine the statistical change in said histogram dimension in f); and

h) relating said statistical change in said histogram dimension to the concentration of said analyte in said fluid sample.

28. A method of claim 27, wherein said analyte is a hapten or antigen and said second binding molecule is an anti-hapten or anti-antigen antibody.

29. A method of claim 27, wherein said analyte is an antibody and said second binding molecule is complementary to said antibody.

30. A method for detecting an analyte in a fluid sample wherein more than one analyte is measured in a single fluid sample, comprising the steps of:

a) assigning to each said analyte a forward binding, displacement, inhibition, or competition reaction;

b) selecting for each said analyte a unique first binding molecule-coated monodisperse polymeric microsphere and unique second binding molecule-coated polydisperse colloidal metal particles;

c) carrying out all said reactions in the same reaction vessel;

d) measuring the statistical change in the dimension of each unique light scatter pulse distribution histogram produced by each said reaction; and,

e) relating each said histogram dimension change to the concentration of each said analyte.

31. A method of claims 19-30, inclusive, wherein said histogram dimension is the normalized peak width of a graphical representation of said histogram. .

32. A mercantile test kit for measuring an antibody, antigen or hapten analyte in a single fluid sample which comprises in separate container means:

a) monodisperse latex microspheres of average diameter 0.5 μm to 10.0 μm coated with a binding molecule, said monodisperse latex microspheres having a coefficient of variation around a mean of no greater than about 2%; and,

b) polydisperse colloid gold particles of average diameter of 20 to 120 nm coated with a binding molecule; and, optionally,

c) a reagent comprising a monodisperse immunocomplex between coated monodisperse latex microspheres and coated polydisperse gold particles.

33. In a flow particle analyzer capable of detecting and measuring a light scatter pulse height distribution histogram produced by monodisperse polymeric microspheres contained within a fluid sample stream flowing through an optical sheath flow cell of

said analyzer, the improvement that comprises providing for said optical sheath flow cell: a regulatable fluid drive narrowing means for narrowing said fluid sample stream passing through said cell; a regulatable centering means for centering said narrowed fluid sample stream through said cell; a sensing means for monitoring the histogram dimension of control monodisperse polymeric microspheres; and, a feedback means for controlling said stream narrowing means and said stream centering means so as to maintain said control histogram dimension within desired limits.

34. An analyzer of claim 33, wherein said narrowing of said fluid sample stream is to a diameter ranging between about 3 μm to about 10 μm or between about 1% and 3% of the width of a beam of incident laser light.

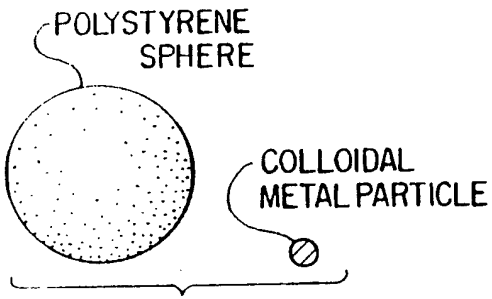


FIG.1A

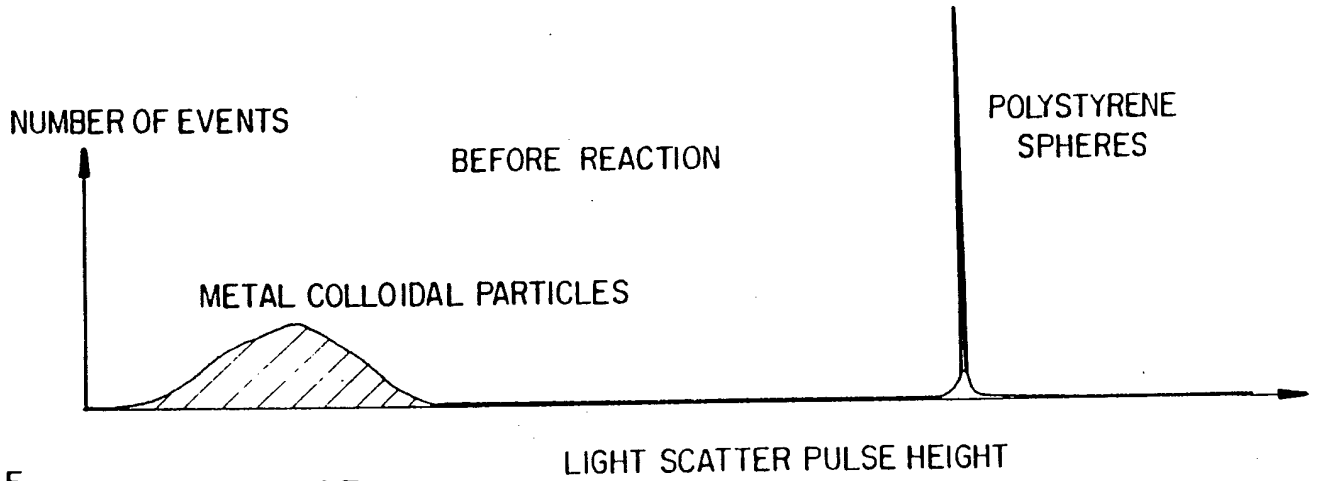


FIG.1B

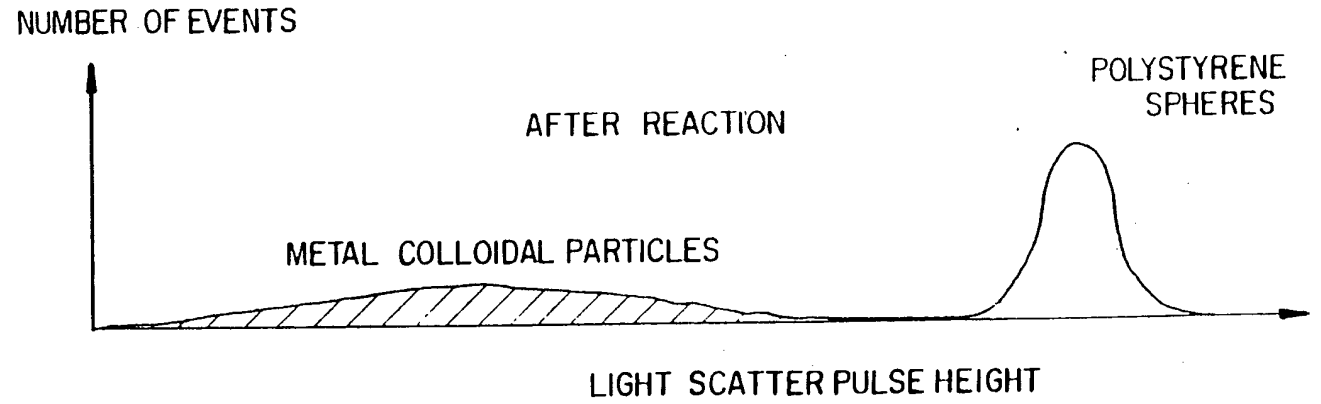


FIG.1C

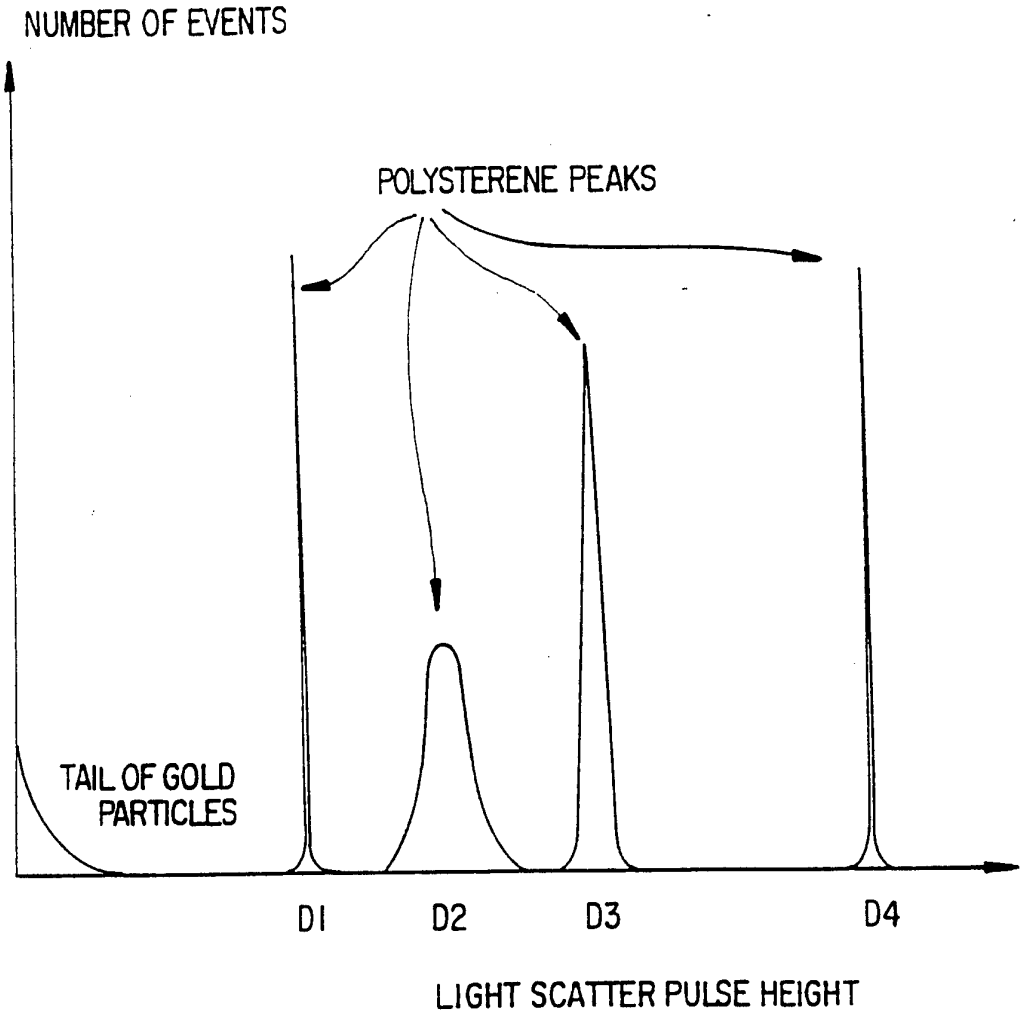

NUMBER OF EVENTS
POLYSTERENE PEAKS
TAIL OF GOLD PARTICLES
D1
D2
D3
D4
LIGHT SCATTER PULSE HEIGHT

FIG. 1D

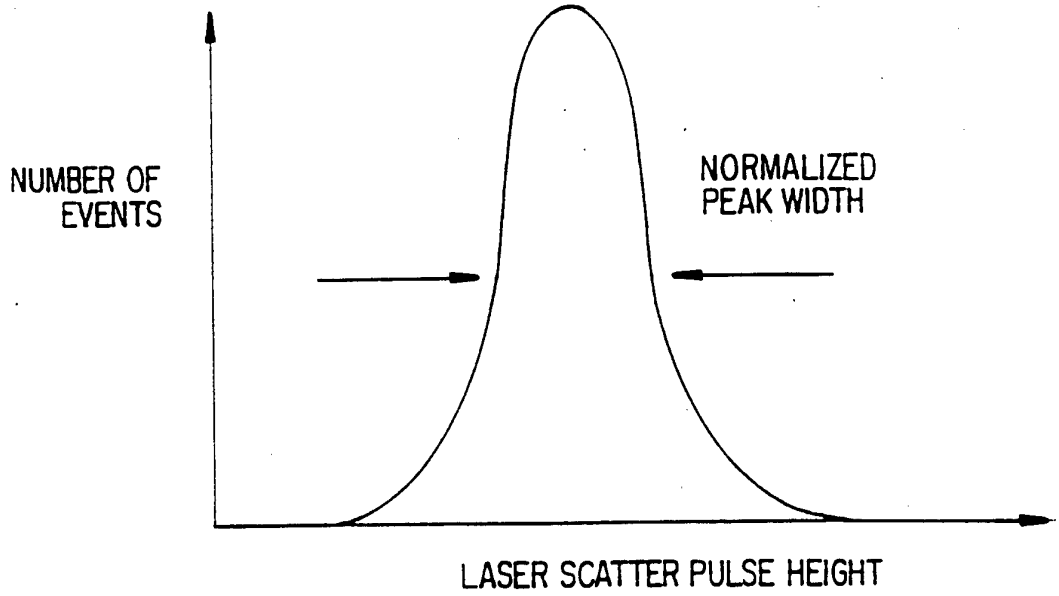

NUMBER OF EVENTS
NORMALIZED PEAK WIDTH
LASER SCATTER PULSE HEIGHT

FIG. 2A

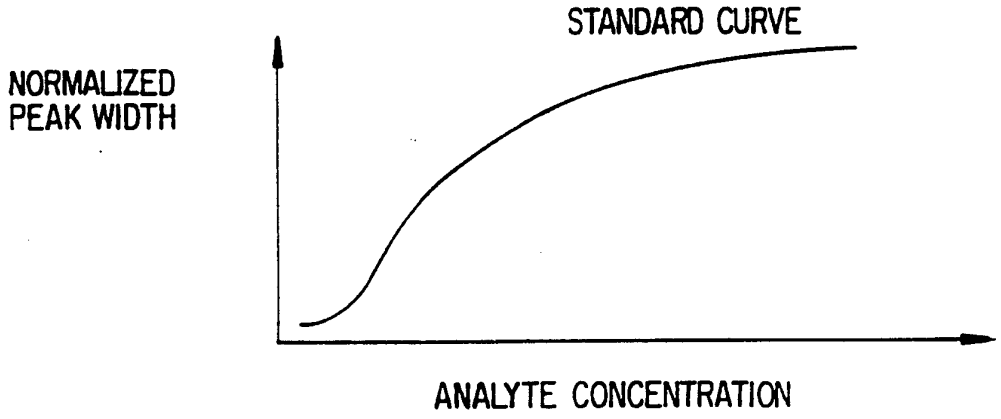

STANDARD CURVE
NORMALIZED PEAK WIDTH
ANALYTE CONCENTRATION

FIG. 2B

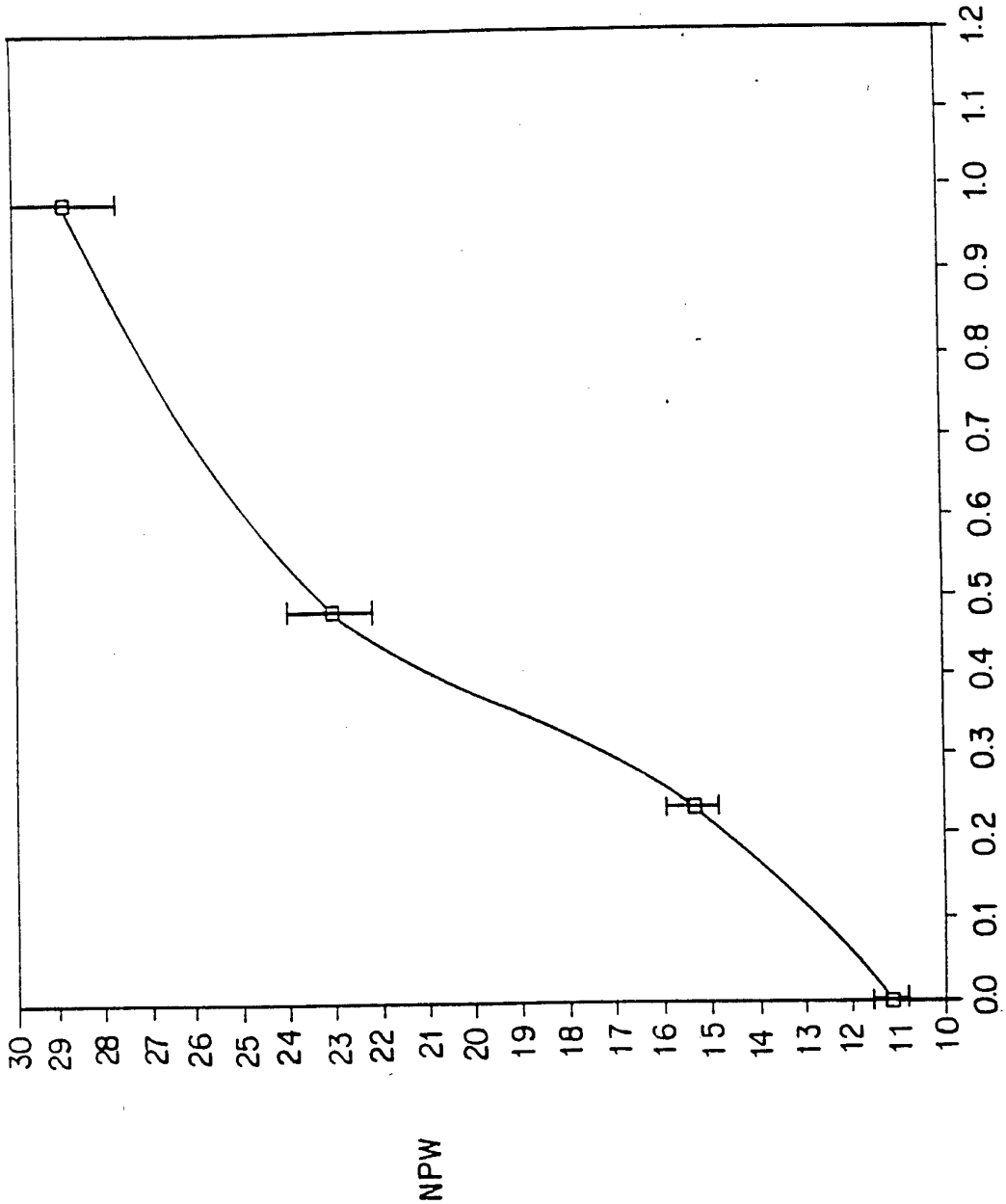
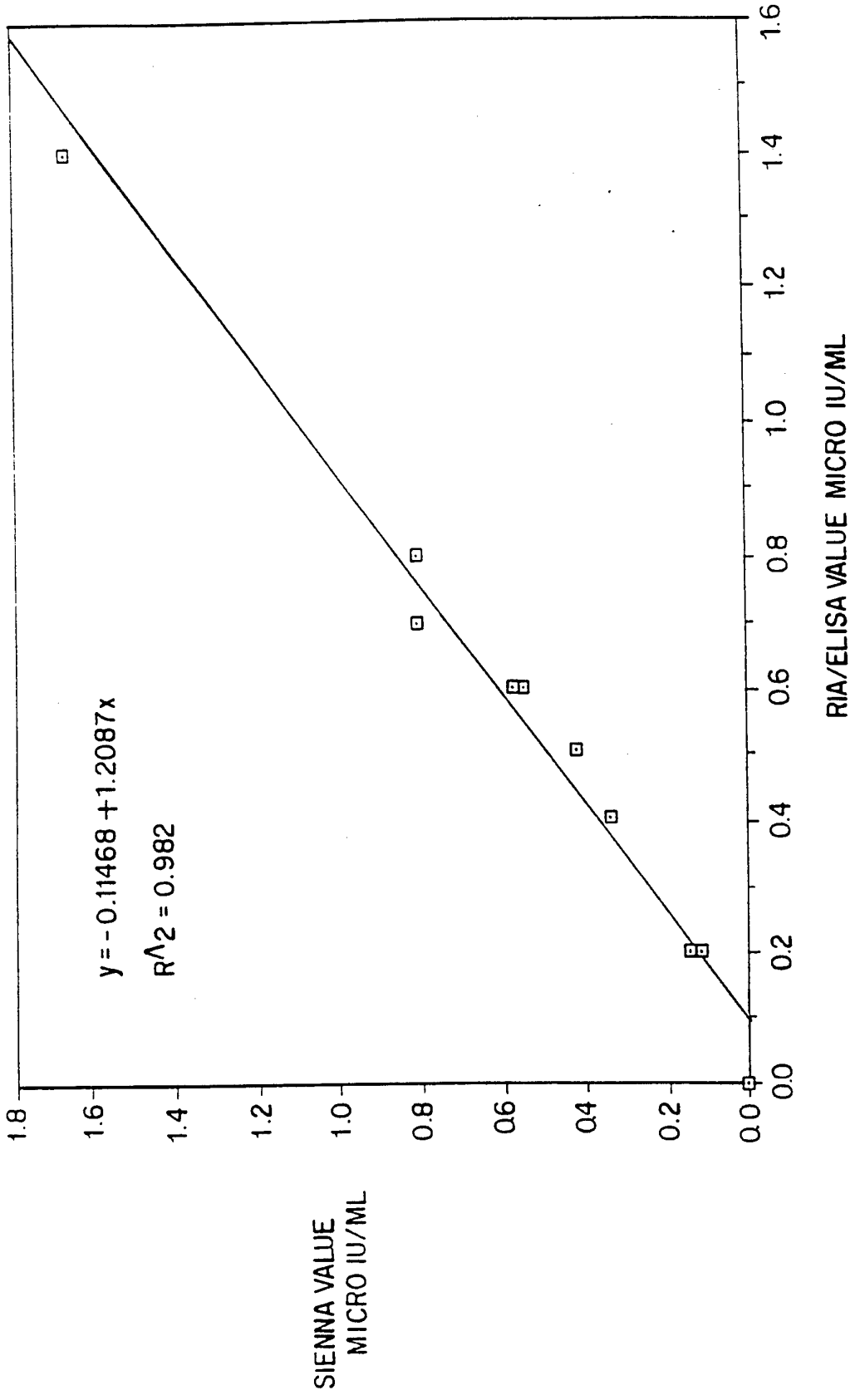

NPW
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
[TSH]MICRO IU/ML

FIG. 3A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R^2 = 0.982
SIENNA VALUE MICRO IU/ML
RIA/ELISA VALUE MICRO IU/ML
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0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

FIG. 3B

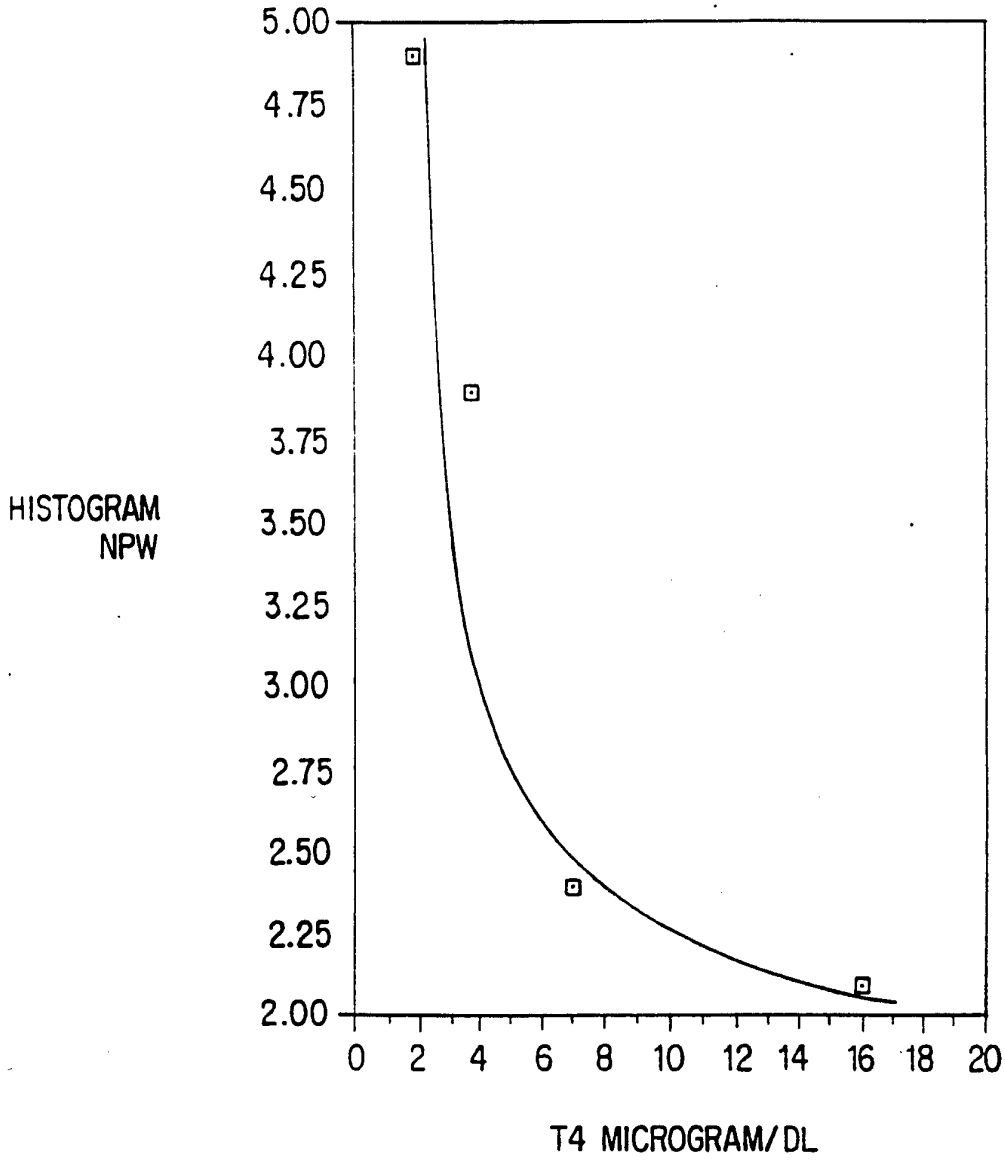
HISTOGRAM
NPW
5.00
4.75
4.50
4.25
4.00
3.75
3.50
3.25
3.00
2.75
2.50
2.25
2.00
0
2
4
6
8
10
12
14
16
18
20
T4 MICROGRAM/DL

FIG. 4A

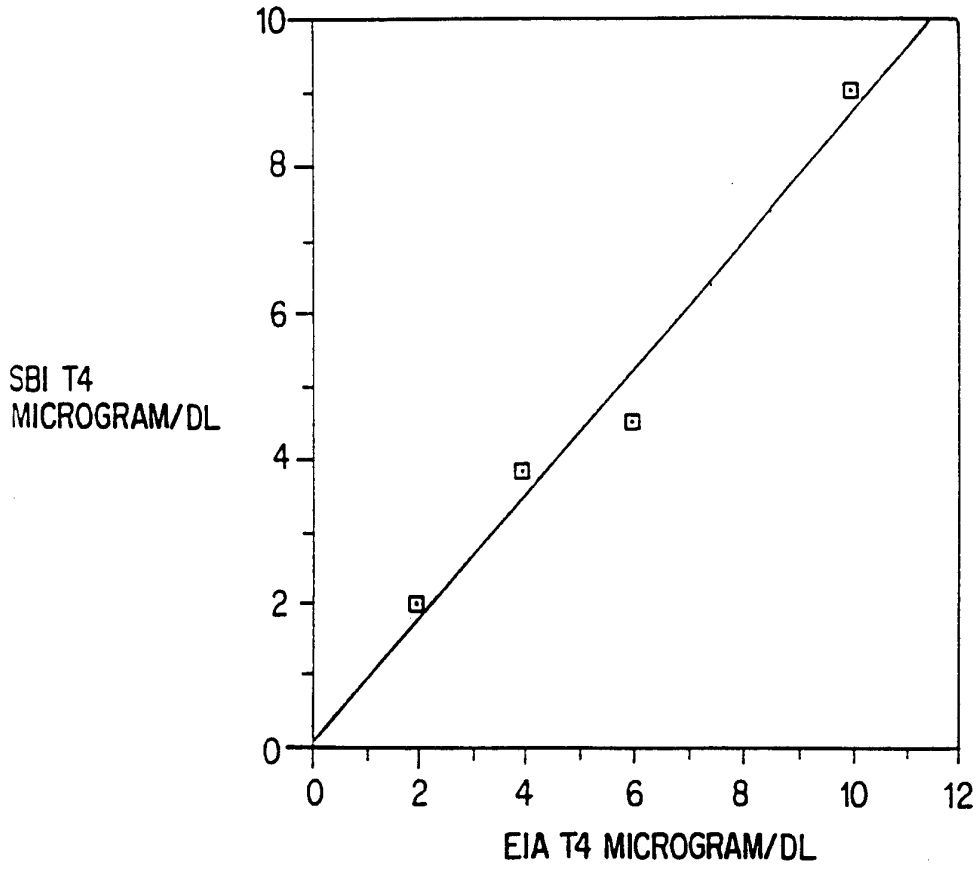

FIG. 4B

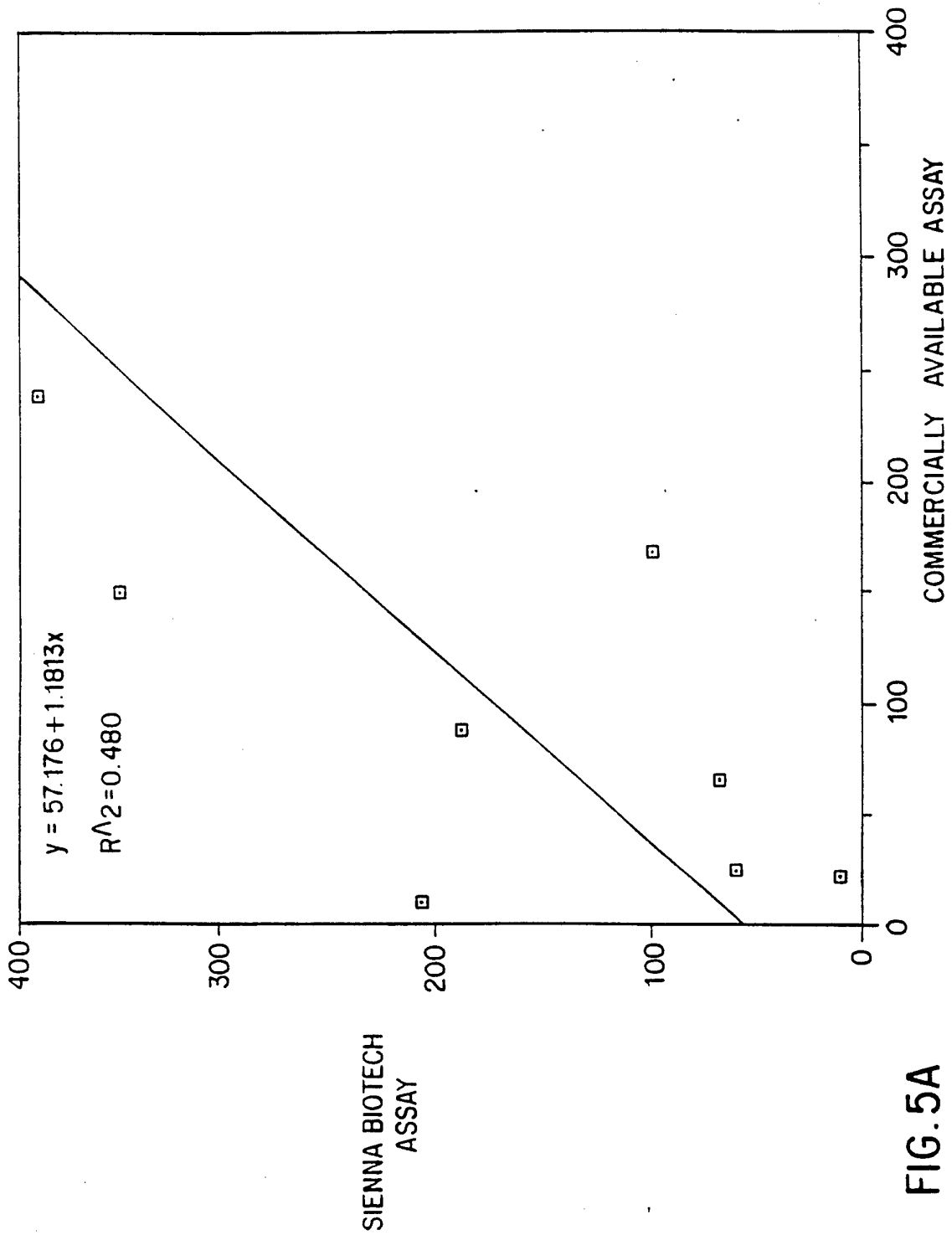
y = 57.176+1.1813x
R^2=0.480
SIENNA BIOTECH ASSAY
COMMERCIALLY AVAILABLE ASSAY
0
100
200
300
400

FIG. 5A

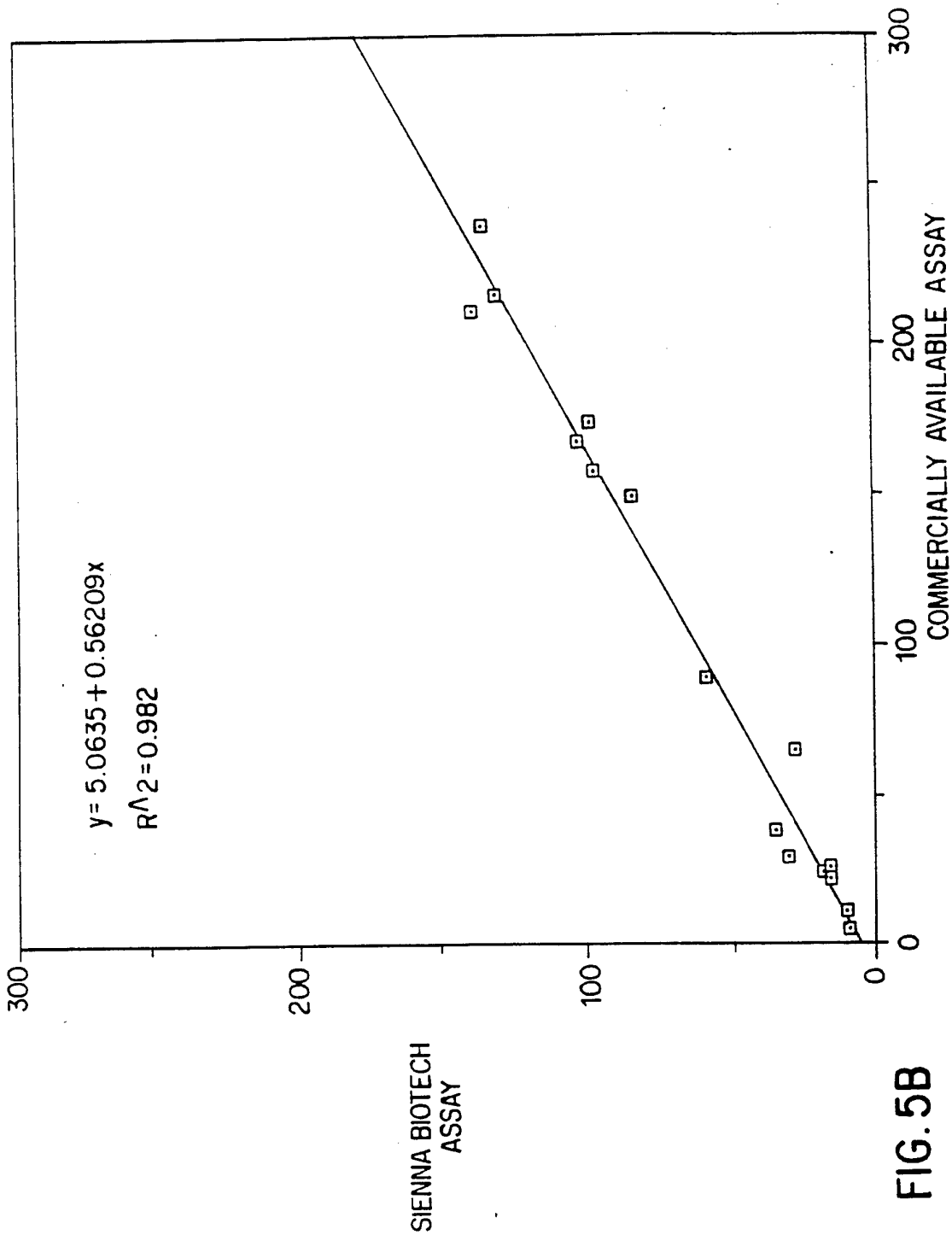
y= 5.0635+0.56209x
R^2=0.982
SIENNA BIOTECH ASSAY
COMMERCIALLY AVAILABLE ASSAY
0
100
200
300

FIG. 5B

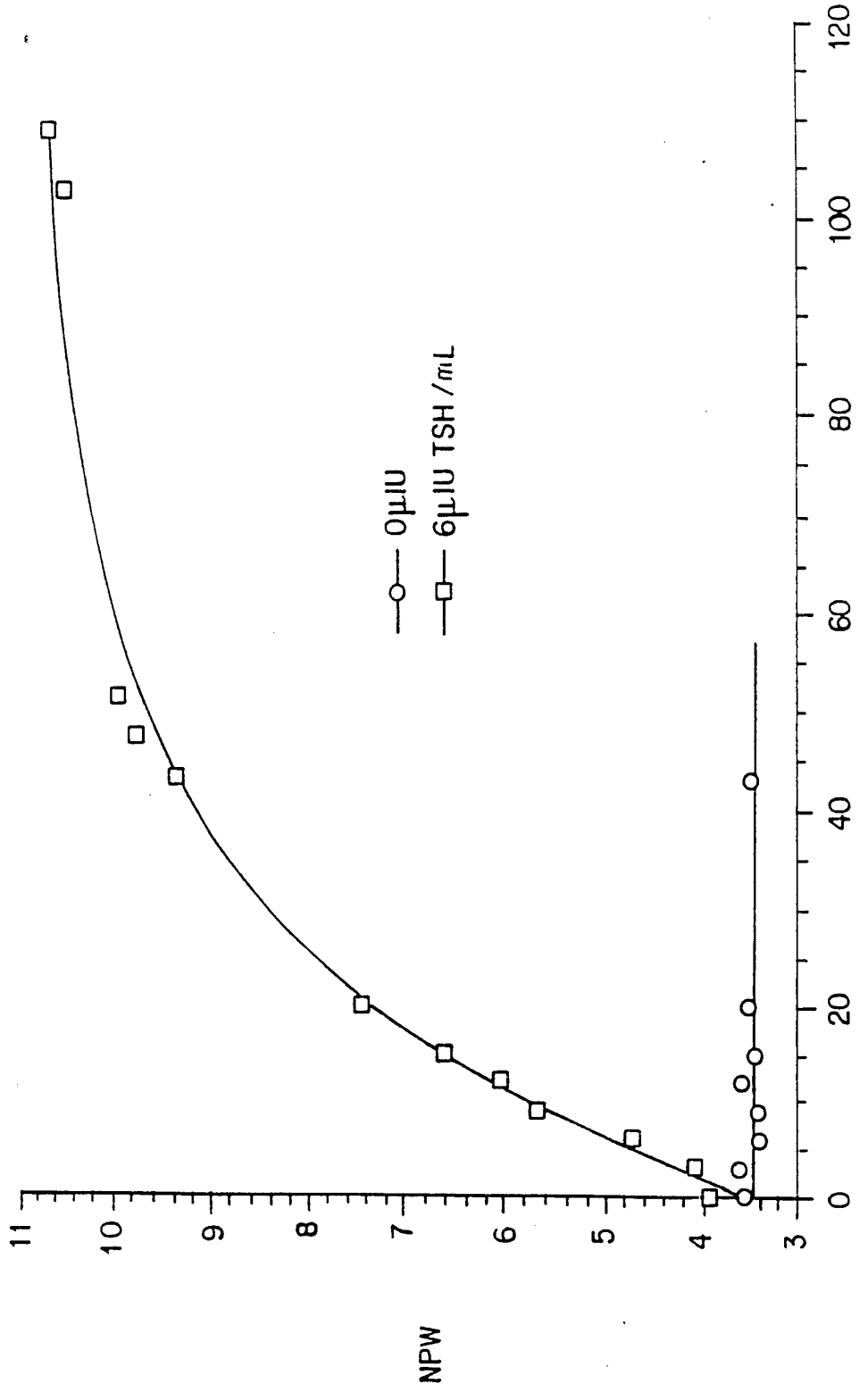

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/12428

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 15/14; 21/53, 21/64; 33/544, 33/546, 33/553
US CL :356/336, 337; 435/7.2, 287; 436/172, 525, 528, 533
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 356/336, 337; 435/7.2, 287; 436/172, 525, 528, 533

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS
search terms: particle analyzer, flow cytometer, colloidal gold, microsphere, particle, light scatter.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 5,079,172 (HARI ET AL) 07 JANUARY 1992, see column 1, lines 59-68; column 3, lines 43-65; column 7, lines 62-68; column 8, lines 32-39.	32 1-32
X	DUKE SCIENTIFIC CORPORATION, Bulletin 88C, 01 September 1990, "COVASPHERES® REAGENTS", see entire bulletin.	32
X	DUKE SCIENTIFIC CORPORATION, Bulletin 93D, 01 April 1992, "FLUORESCENT MICROSPHERES AND PARTICLES", see entire bulletin.	32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 February 1994	Date of mailing of the international search report 14 MAR 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer NANCY J. PARSONS Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/12428

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLINICAL CHEMISTRY, Volume 31, Number 12, issued 1985, G.C. Saunders et al, "Amplified Flow-Cytometric Separation-Free Fluorescence Immunoassays", pages 2020-2023, see especially pages 2020-2021 and Figure 1A-1C.	1-34
Y	US, A, 4,581,334 (KIRCHANSKI ET AL) 08 APRIL 1986, see column 2, lines39-66; column 8, lines 53-60.	1-34
Y	W.D. ODELL et al, "PRINCIPLES OF COMPETITIVE PROTEIN-BINDING ASSAYS", published 1983 by John Wiley & Sons, see pages 243-254, especially page 244, Figure 1.	22-24,27-29