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(54) Title: PARTICLE AGGLUTINATION ASSAY SYSTEM

(57) Abstract

A system for assaying a fluid sample for one or more types of analytes, which employs at least one class of finely divided polystyrene spheroidal particles, each class being limited to a predetermined specific narrow range of particle diameters, the particles of each such class being coated with a specific reactant unique for that class. After the coated particles are mixed with the sample to specifically react to form conjugates of the particles and any of the analyte present, the mixture is irradiated with bursts of ultrasound swept over a range of frequencies resonant to the expected conjugate sizes. The presence of the conjugates and therefore the analyte is detected directly by measuring any selective absorption of waves of frequencies to which the conjugates are resonant.

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PARTICLE ACCLUTINATION ASSAY SYSTEM

This invention relates to chemical and biochemical assay systems, and more particularly to improved particle agglutination assay systems.

Background of the Invention

Assays are well known in which aliquots of a liquid test sample containing one or more analytes are variously reacted with one or more reagents in highly specific reactions to form analyte complexes such as antigen/antibody or similar complexes which may then be observed in order to assay the sample for the presence of a predetermined moiety or analyte in the sample. Typically, an antibody is used to assay for the presence of an antigen against which the antibody was specifically induced and reactable, but such assays have been extended to other specific reactions to quantitate analytes including haptens and other molecules such as hormones, alkaloids, steroids, antigens, antibodies, nucleic acids, enzymes and fragments thereof. It is in this broad sense that the term "analyte" as used herein should be understood to refer to that which is being sought in an assay and is deemed to include antigens, haptens, antibodies and other molecules, the presence and/or quantity of which is being assayed. It will therefore be appreciated that the assay of the present invention has broader applications than immunoassays per se. The phrase "specific reaction" and variants thereof are to be understood to refer to a reaction, such as an antigen/antibody reaction and the like that are understood to exhibit very high specificity.

A substantial body of prior art exists relating to a specific form of immunoassay known as particle agglutination assay, sometimes referred to as PACIA systems. Much important work on PACIA systems originated by a group in Belgium and is published in, for example, Particle Counting Immunoassay (PACIA). I. A General Method for the Determination of Antibodies, Antigens and Haptens, C.L. Cambiaso et

al, *J. Immuno. Meth.*, 18 (1977) 33-34; Particle Counting Immunoassay (PACIA). II. Automated Determination of Circulating Immune Complexes by Inhibition of the Agglutinating Activity of Rheumatoid Sera, C.L. Cambiaso et al, *J. Immuno. Meth.*, 23 (1978) 29-50; Particle Counting Immunoassay (PACIA). III. Automated Determination of Circulating Immune Complexes by Inhibition of an Agglutinating Activity of Mouse Serum, C.L. Cambiaso et al, *J. Immuno. Meth.*, 28 (1979) 13-23; and Particle Counting Immunoassay. IV. The Use of $F(ab')_2$ Fragments and N^{ϵ}-Chloroacetyl Lysine N-Carboxy-Anhydride for Their Coupling to Polystyrene Latex Particles, N. Limet et al, *J. Immuno. Meth.*, 28 (1979) 25-32. These articles and later papers based on this work, including U.S. Patent No. 4,427,781, teach mixing the sample with an agglutinating agent and reactant-coated latex particles, and counting the unagglutinated or free particles with a light-scattering optical device of the type intended to count blood cells in an autoanalyzer format, e.g. the Technicon PACIA System (Technicon International Division, Geneva, Switzerland). The authors claim that this is a particularly suitable technique to obtain practical and sensitive measurements of various analytes. It should be noted that because this method measures agglutination indirectly by counting the unagglutinated particles, it requires a prior knowledge of the initial amount of particles used in the assay. Thus, unless the total number of particles employed is known *ab initio*, the system cannot serve for qualitative detection. Further, the system is limited to measurement of one analyte at a time, so lacks speed and flexibility.

Nephelometry and turbidimetry, two alternative optical methods used to measure particle agglomeration, are discussed in Marker-Free Immunological Analytical Methods, E. Henkel, *J. Clin. Chem. Clin. Biochem.*, 22 (1984) 919-926 which points out the many disadvantages of these two methods.

A principal object of the present invention is therefore to provide an improved assay system of the type described, in which the problems

of the prior art are overcome by providing a simple, low-cost system which provides direct information regarding the presence and/or quantity of agglutinated particles. Other objects of the present invention are to provide such a system in which the presence of one or more analytes are detectable qualitatively in the same sample, to provide such a system in which the presence of more than one analyte being assayed can be detected substantially simultaneously, and to provide such a system in which agglutinates or conjugates formed of two to four particles (hereinafter respectively "diglutinates", "triglutinates", etc.) are readily detected by absorption of pressure waves of resonant frequency. Other objects of the present invention will in part be obvious and will in part appear hereinafter.

Summary of the Invention

To effect the foregoing and other objects, the present invention generally is a system for assaying a fluid sample, which system may employ one or more classes of minute, substantially spheroidal particles, each such class being characterized as limited to only a specific narrow range of particle diameters. The particles of each such class are pre-coated with a unique specific reactant to a respective analyte. Where the analyte, for example is present in the sample solution, those particles of each such class will react with the analyte and where the latter has more than one attachment site, form conjugates typically initially as diglutinates or triglutinates. The presence of the latter is detected by the selective attenuation, as by scattering, absorption and the like, of compression waves of frequencies to which the conjugates are resonant. Where the reactant coated onto the particles is the same as the analyte sought to be assayed, a reagent, specifically reactable with that analyte is introduced to provide linkage between the immobile, bound reactant on the particles and the mobile or free analyte in the sample. It is preferred that the compression waves used are ultrasonic, and desirably, the ultrasonic energy generator serves also as a sensor to measure changes in the attenuation of the ultrasound.

The invention accordingly comprises the apparatus possessing the construction, combination of elements and arrangement of parts, and the method comprising the several steps and the relation of one or more of such steps with respect to each of the others, all as exemplified in the following detailed disclosure, and the scope of the application of which will be indicated in the claims.

Brief Description of the Drawings

For a fuller understanding of the nature and objects of the present invention, reference should be had to the following detailed description taken in connection with the accompanying drawing wherein:

Fig. 1 is a diagrammatic representation, partly in cross-section, of an assay system embodying the principles of the present invention;

Fig. 2 is an enlarged schematic representation of a coated particle used in the present invention; and

Fig. 3 is a graphical representation in which frequency is plotted against sound intensity to indicate resonant absorption peaks corresponding to agglutinate sizes.

Detailed Description of the Drawings

In the drawing there is shown an exemplary system for assaying fluid sample 20, and which preferably employs at least one of a plurality of classes of different narrow ranges of diameters of minute (e.g. over a total range of about 0.1μ to about 50μ, preferably from about 0.5μ to about 10μ, in average diameter) spherical or spheroidal particles 22 substantially chemically inert to sample solution 20 and to the reagents to be used in the assay method. Particles 22 are typically of white polystyrene latex beads(commercially available from Interfacial Dynamics Corp., Seattle, WA), and can readily be made of other polymers, glass and the like. Each class of particles is characterized as being limited to a definitive, predetermined small range of particle diameters, e.g each class lying within a range of typically 0.2μ, and each particle of a class is

coated with a sheath or coat 26 of at least one reactant,such as an antibody, unique to that class. The particles of each such class may also be coated with a plurality of types of reactant unique to that class, each of that plurality of types of reactant being couplable with a distinct analyte to form corresponding conjugates. The reactants can be not only antibodies, but low molecular weight haptens, antigens, enzymes, and other biological and chemical receptors that characteristically enter into highly specific reactions with other ligands.

In one particular example, two classes of white polystyrene latex beads 22, one class having average diameters of $2.0 \pm 0.1 \mu$, the other having average diameters of $5 \pm 0.1 \mu$, are each provided with a respective unique coat 26 as shown in Fig. 2. For example, one group is coated with an antigen, purified rDNA polypeptide CBr3 (a recombinant protein derived from HIV-1 *env* genes) and the other group with purified rDNA polypeptide K1 (a recombinant protein derived from HIV-2 *env* genes). The HIV-1 *env* proteins (gp41 and gp120) and HIV-2 *env* proteins (gp41 ad gp120) are provided by recombinant DNAs expressed in *E. coli* and the polypeptides purified to remove all immunologically-detectable bacterial proteins. Because proteinaceous materials are readily adsorbed onto the surface of hydrophobic polystyrene, simple immersion in a suspension or solution containing the proteins will result in the particles becoming coated. Of course, other techniques, such as covalent bonding, for coating such particles with antibodies, antigens and the like, have been extensively documented in the prior art and need not be further described here.

Such exemplary coated particles are then used to assay a body fluid such as blood, for HIV-1 and HIV-2 by mixing the two groups of coated particles into sample 20 of body fluid. This exemplary assay is particularly useful in cases where antigens such as HIV tend to be present in tissues such as liver and the like, but usually only at extremely low concentrations in blood serum, if at all, whereas the antibody is much more likely to be available in the serum. Antigen coatings 26 on

particles 22 will specifically react with the precise viral antibodies, if present, in sample 21. Because typically an antibody has more than one reactive site, it will initially tend to couple to one or more particles coated with the specific antigen or hapten to that antibody, specifically reacting to form agglutinates or conjugates. This agglutination process is rate-sensitive in that the agglutinates formed initially are diglutinates, triglutinates and the like, although ultimately larger and larger conjugates will tend to grow or be created.

Thus, the class of smaller diameter particles described above would tend to form agglutinates or conjugates initially having major cross-sectional dimensions of about 4μ and 6μ, and the class of larger diameter particles then would tend to form conjugates initially having major cross-sectional dimensions of 10μ and 15μ.

The present invention can readily use as many as four different classes of coated particles, in which case one must select the range of sizes of the particles carefully to avoid the possible creation of diglutinates and triglutinates that might have the same or closely matched resonant frequencies as other conjugates or classes of particles.

Agglutination can be demonstrated by the following example which is presented merely as exemplary of a specific embodiment and is not intended to provide any limitation to the invention as claimed hereinafter. For an assay for human thyroid stimulating hormone (hereinafter TSH), polystyrene spheres of average 1.62 μm diameter are coated with anti-TSH monoclonal antibodies by incubating the spheres overnight in a solution of 100mg/ml of antibody in 10mM HEPES buffer, pH 7.5. The coated spheres are recovered by brief centrifugation, and washed three times in a 4-fold volume of 10mM HEPES buffer, pH 7.5, containing 0.1% (w/v) BSA and 0.01% NaN_3. The recovered, coated spheres are then suspended in 20mM glycine buffer, pH 9.3, containing 0.1% (w/v) BSA, 0.01% NaN_3, and 300mM NaI to form a reagent. The initial concentration of single spheres in this reagent are about 5×10^7 spheres/mL. Diglutinates, present in the

unreacted reagent as the result of the manufacturing and coating processes, are typically about 2% of the initial single sphere concentration. This reagent is then added to an equal volume of standard human serum (OEM Concepts, Inc., Toms River, N.J.) containing known concentrations of human TSH, and stirred continuously to effect a reaction between the coated spheres and the human TSH. Mixing can also be achieved without stirring. Sampling of reaction mixtures by the apparatus of the present invention during the first several seconds following initiation of the specific reaction between the particles and the analyte, detected the presence of diglutinates and triglutinates well in excess of those created by the manufacturing and coating processes.

Means, such as source 30, are provided for bulk insonifying the reacted mixture of body fluid 20 and particles 22 with a flux of compressional waves such as ultrasound, typically swept through a frequency range over which resonances for particles of the size involved can be expected. It is apparent that the range of frequencies employed depends on the propagation velocity of the compressional waves through the bulk of the sample medium, which itself depends on such factors as temperature, pressure and density. Thus, using the exemplary values of cross-sectional dimensions of 4μ and 6μ for the expected two and three-particle conjugates in the first class of particles and 10μ and 15μ for similar groups or conjugates of the second class of particles, for a liquid sample at normal pressure and 25°C with a density close to sea water so that the nominal velocity of a compressional wave in that medium would be about 1530 m/sec., the frequencies corresponding to wave lengths resonant with these conjugates would lie in the range of between about 10OMHz to 50OMHz. It will be recognized that the agglutinated particles are typically asymmetrical, so the resonant frequencies absorbed will not necessarily lie in a sharply defined frequency band. Because the system is quite temperature sensitive, it is preferred to provide known temperature controlling apparatus shown schematically at 32, to maintain the temperature of the sample solution during assay at a fixed,

predetermined level. Preferably, ultrasound is applied from source 30 in bursts, each of which is swept through the desired frequency range, to permit one to broadcast and then listen, preferably with the same transducer, so that each agglutinated complex can resonantly absorb a characteristic frequency band or otherwise change the characteristics of the compression waves within the swept range of the input waveburst. Measurements are preferably taken only during the first several seconds following initiation of the specific reaction between the particles and the analyte in order to detect the presence of diglutinates and triglutinates before formation of much larger agglutinate masses that may interfere with the assay. It will be appreciated that with four different classes of particles, each class having a unique size of diglutinates and triglutinates, there can be twelve resonant frequencies which when spread across the swept frequency band provides separations of about 25 MHz between absorption peak centers. Attempts to obtain resonance data regarding agglutinates larger then four particles tend to become extremely complex, so the present assay is preferably limited to measurement of the smaller agglutinates formed during the earlier or initial phases of the agglutination reaction. The extent of attenuation, typically through resonant absorption, by agglutinates of particles 22 depends, inter alia, on the difference between the density of particles 22 and the density of the surrounding medium. For example where the densities of the particles and the medium are close, resonance of the particles will be heavily damped and sharp resonant absorption peaks will not be observed. Hence it is preferred to employ particles having at least 10% difference in density than the surrounding or supporting fluid medium. The resonant absorption peaks of agglutinates of particles 22 can be readily detected by the sonic transducer, or a separate microphone 34, the output of which is preferably connected to appropriate signal processing devices or display apparatus 36 such as a chart recorder or the like. For example, if the input ultrasound has a flat spectrum over the entire 100 to 500 MHz range, then there will be absorption peaks in the output, as shown in Fig.

3, displayed on chart recorder 36 and taken within 10 to 15 seconds following initiation of the agglutination reaction. Each such peak shown in Fig. 3 is indicative of a resonant structure that can be correlated with a particular two or three particle complex.

As noted above, the present invention readily lends itself to an assay of a plurality, preferably up to four, of different analytes simultaneously. For example, one can provide a plurality of classes of finely divided particles 22, each of such classes being, of course, limited to its own unique, predeterminedly narrow range of particle diameters. The particles of each such class are precoated with a corresponding, different reactant capable of entering into a reaction with high specificity. For example, one can coat the four classes respectively with HIV-1 and HIV-2 polypeptides as described earlier herein, and HVA (hepatitis virus A) and HVB (hepatitis virus B) protein coat materials. The four coated classes are then simultaneously mixed with a sample solution to be assayed so as to incite a plurality of specific reactions each involving one of the antigenic coating. When swept with the ultrasonic bursts, the resulting record of resonant absorptions will indicate which agglutinates formed and can be readily correlated with the specific antigens involved. It is preferred to presweep sample 20 before introducing coated particles 22, so as to provide a background spectrum showing any resonances associated with the sample and not caused by agglutination. Correlation of peaks appearing on the record with particular agglutinates can easily be accomplished by precalibrating the system with predetermined class of particles against known analytes in a calibration test solution to thereby identify empirically which peaks correspond to particular agglutinates.

It will be appreciated that the present invention contemplates the formation of agglutinates by direct specific reaction between the analyte being assayed and the reagent coated on the particles. For example, where antigenic material is coated onto the particles, the latter will specifically react with antibody present in the sample fluid to form

agglutinates. Conversely a similar result will occur where the antibody is coated onto the particles and allowed to specifically react directly with the antigen in the sample fluid. Alternatively, the present invention is applicable to known indirect types of assays such as sandwich assays, competition assays and the like, in which cases, the dimensions of the initial agglutinates may be different. For example, in a typical sandwich assay, the particles are coated with the same antibody for which the sample solution is to be assayed. The assay then requires the addition of a linking agent such as an antigen that will react specifically with both the free antibodies in solution and the antibodies bound to the particles, creating agglutinates that are different in dimensions than the agglutinates resulting from a direct antibody/antigen reaction.

Since certain changes may be made in the above process and apparatus without departing from the scope of the invention herein involved, it is intended that all matter contained in the above description or shown in the accompanying drawing shall be interpreted in an illustrative and not in a limiting sense.

WHAT IS CLAIMED IS:

1. Apparatus for assaying a fluid sample for an analyte, said apparatus comprising:

at least one class of minute, substantially spheroidal particles, said class being limited to a predeterminedly narrow range of particle diameters, the particles of said class being coated with a reactant capable of entering into a reaction with high specificity;

means for mixing said reactant coated particles with said sample so as to form initially conjugates of from two to four of said particles by said reaction with analyte present in said sample;

means for irradiating said conjugates with a range of sound waves of frequencies at which said conjugates will attenuate said sound waves; and

means for detecting selective attenuation of frequencies within said range of frequencies by said resonant conjugates.

2. Apparatus for assaying a fluid sample as set forth in claim 1 wherein said attenuation is through resonant absorption of said sound waves by said conjugates.

3. Apparatus for assaying a fluid sample as set forth in claim 1 wherein said reactant is specifically reactable with said respective analyte type.

4. Apparatus for assaying a fluid sample as set forth in claim 1 wherein said reactant is the same as said analyte or a specific analog thereof, and said system includes a reagent specifically reactable with said analyte for coupling said reactant with said analyte in said sample.

5. Apparatus for assaying a fluid sample as set forth in claim 3 wherein said reactant is an antibody to said analyte.

6. Apparatus for assaying a fluid sample as set forth in claim 3 wherein said analyte is an antibody to said reactant.

7. Apparatus for assaying a fluid sample as set forth in claim 1 wherein said analyte is selected from the group consisting of hormones, alkaloids, steroids, haptens, antigens, antibodies, nucleic acids, enzymes and fragments thereof.

8. Apparatus for assaying a fluid sample as set forth in claim 1 wherein said narrow range of particle diameters lies within the a broader range of between about 0.51A to about 10μ in diameter.

9. Apparatus for assaying a fluid sample as set forth in claim 1 wherein said means for irradiating comprises a source of ultrasonic waves.

10. Apparatus for assaying a fluid sample as set forth in claim 9 wherein said source is operable to provide a sweep of said ultrasonic waves across a frequency range of between about 100 to 500 MHz.

11. Apparatus for assaying a fluid sample as set forth in claim 9 wherein said source is operable as a microphone for detecting attenuation of said ultrasonic waves by said particle.

12. Apparatus for assaying a fluid sample as set forth in claim 1, including a plurality of said classes, the particles of each said class being coated with a reactant unique to that class and couplable with a different analyte to form corresponding conjugates.

13. Apparatus for assaying a fluid sample as set forth in claim 1, including a plurality of said classes, the particles of each said class being coated with a plurality of types of reactant unique to that class, each of said plurality of types of reactant being couplable with a distinct analyte to form corresponding conjugates.

14. Apparatus for assaying a fluid sample as set forth in claim 2, wherein said means for detecting selective absorption comprises microphonic means disposed to detect said sound waves after they have traversed the mixture of said particles and sample.

15. Apparatus for assaying a fluid sample as set forth in claim 14, wherein said means for detecting further includes spectrum analyzer means coupled to the output of said microphonic means.

16. Apparatus for assaying a fluid sample as set forth in claim 14, wherein said means for detecting fuirther includes means for displaying the spectrum of said absorption peaks corresponding to said selective absorption of frequencies within said range of frequencies by said resonant conjugates.

17. A method of assaying for at least one type of analyte in a liquid sample, including coating a plurality of at least one class of finely divided, substantially spheroidal particles with a specifically reactable reagent;

mixing said reagent coated particles with said sample so as to effect a specific reaction involving said reagent and said type of analyte to form conjugates of said particles;

introducing into the mixture containing said conjugates, sound waves of frequencies to which said conjugates are resonant; and

detecting the presence of said conjugates as a function of attenuation of said sound waves by said conjugates.

18. In a method as defined in claim 17 wherein said attenuation is by selective absorption of said sound waves by said conjugates at resonant frequencies.

19. In a method as defined in claim 17 including

providing a plurality of up to four of said classes of finely divided, substantially spheroidal particles, each of said classes being limited to a unique, predeterminedly narrow range of particle diameters;

coating the particles of each of said classes coated with a corresponding, different reactant capable of entering into a reaction with high specificity; and

forming a simultaneous mixture of said reagent coated particles with said sample so as to incite a plurality of specific reactions each involving one of said reagents and a corresponding type of analyte to form conjugates of said particles.

ANY REFERENCE TO DRAWINGS

SHALL BE CONSIDERED NON-EXISTENT

(See Article 14(2))

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

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Minimum documentation searched (classification system followed by classification symbols)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,615,984 (STOCKER) 07 October 1986, see entire document.	1-19

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