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(54) Title: METHOD FOR DETERMINING MULTIPLE IMMUNOCOMPLEXES ON ONE SURFACE USING SPECTROSCOPY

(57) Abstract

The invention provides a method of detecting the presence of more than one analyte in a fluid sample using a single reactive surface in a spectroscopic assay.

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METHOD FOR DETERMINING MULTIPLE IMMUNOCOMPLEXES ON ONE SURFACE USING SPECTROSCOPY

BACKGROUND OF THE INVENTION

Field of the Invention.

The invention relates to a process of detecting one or more analytes in a sample through light-based methodology, especially that of scattered total internal reflectance spectroscopy or reflectance, using a single, multi-functional reactive surface.

Technology Review.

As immunoassays become increasingly important in the medical diagnostic field, various methods for determining the results of immunoassays are being developed and used. For example, methods using light of some sort, such as reflected light, fluorescent light or scattered light, determine if appropriate immunological binding pair members have complexed. One such method uses reflected light. A binding pair member, such as antigen, is coated or "spotted" on a carrier, a solid support, then exposed to a sample that may or may not contain the matching binding pair member, an antibody, and after allowing time for the reaction to occur, is placed in a reflectometer. The reflectometer scans the surface of the carrier with a light, and the intensity of the light reflected will change if the immunocomplex has formed. Currently, only one "spot" of material is used on a solid surface, and therefore only one test assay can be run on each solid support.

Another light-based method is scattered total internal reflectance (STIR) spectroscopy. This is an analytical method that relies on the differences in the optical properties of two mediums to investigate a

sample deposited at, or very near to, the interface of the two mediums. In general, STIR analysis takes advantage of the phenomenon of total internal reflectance wherein light, when directed at or near a critical angle through a high refractive index material toward the interface of that material with a second material having a lower refractive index, is almost completely reflected from that interface except for a microscopic evanescent light wave which propagates into the second material for only a short distance. As disclosed in U.S. Patent 3,939,350, when materials that are fluorescently labeled are brought to the interface of materials with different refractive indexes and within the field of the evanescent wave, the molecules can be made to fluoresce. In STIR spectroscopic analysis what is detected is not the fluorescence of a labeled molecule near the interface but rather the elastic scattering of an evanescent light wave by a light-scattering material such as colloidal gold, red blood cells or latex particles that are brought near the interface. For example, U.S. Patent Nos. 4,979,821 and 5,017,009, herein incorporated by reference, disclose the use of STIR spectroscopy to detect the presence of an analyte in a solution using an immunological reaction between the analyte and one or more antibodies and/or materials possessing an antigenic determinant, either of which specifically binds to the desired analyte. These patents also disclose cuvettes, an apparatus and a method useful in STIR spectroscopic analysis.

In the present disclosure, an "evanescent wave" refers to a non-propagating light wave such as a wave in the region of a surface on the side of the surface opposite the side of illumination, produced when the illuminating light undergoes total internal reflection. Also, "light-scattering material" means a molecule or a material which causes the incident light to be elastically scattered.

Central to STIR spectroscopic analysis using a light- scattering material, is that an incident light wave must be propagated through the material of higher index of refraction at or near a critical angle. The "critical angle" is the angle (less than 90°) measured from the line perpendicular at an interface between materials of different refractive indexes, beyond which total internal reflectance can occur, and is defined by the equation

$$\theta c = \sin^{-1} (\eta 1/\eta 2)$$

wherein $\eta 1$ is the lower refractive index and $\eta 2$ is the higher refractive index of the two mediums forming the interface. The critical angle can only exist in the higher refractive index medium. Total internal reflectance occurs exclusively when an interface between materials of different refractive indexes is illuminated from the higher refractive index medium beyond the critical angle, causing the incident light wave to be reflected unless it is perturbed by diffraction, scatter or absorption. As also disclosed in U.S. Patent Nos. 4,979,821 and 5,017,009, the angle at which incident light is most suitable for STIR spectroscopic analysis of a material deposited on the interface of two materials is, in practice, slightly greater than the theoretical angle calculated as above. However, as described in co-pending patent application Swope et al., USSN 07/574,184, the angle at which incident light is suitable can be less than and equal to θc, which angles do produce evanescent wave, and also provide a useful signal.

In U.S. Patent Nos. 4,979,821 and 5,017,009, a method is described for detecting a single analyte in a fluid solution by running only one test per cuvette. In many cases, however, it would be preferable to be able to perform more than one test using but a single cuvette. For example, blood banks regularly screen donated blood samples for the presence of infectious

agents, particularly viruses, as well as for blood groupings. By employing a multi-analyte test on a single reactive surface, errors associated with the need for multiple disbursements of blood into several sampling chambers would be minimized, if not completely eliminated. Thus, the results of separate tests for an individual sample would be unambiguously determined by use of a multi-analyte test performed on a single, multi-functional reactive surface. For example, routine blood typing requires testing for both ABO and Rh antigens, and the correct identification of a blood type with a given sample is ensured in a single reactive surface test, whereas in a multi-cuvette test there always remains the possibility that the samples tested in the different cuvettes are not of the same origin.

In any light based analysis it would also be preferred to be able to incorporate controls into the assay procedure. In the previously described STIR spectroscopic analysis disclosed in U.S. Patents 4,979,821 and 5,017,009, controls were performed in either the same or a separate cuvette. In the former case, the control is a competing pre-reaction, which may lower the sensitivity of the test by raising background levels of scattering. In the latter case of more than one cuvette, there may be an increased probability that the control was not a valid reflection of the test performed on a separate reactive surface. Such a probability of poor controls would also be greatly reduced in a single reactive surface, multi-functional test.

It would be additionally preferred to be able to extend the dynamic range of detection of a single analyte in spectroscopic analysis, while still performing the analysis of a sample on a single reactive surface. For example, by varying the concentration of a solid phase binding pair member, both large and small

concentrations of analytes may be detected in a single cuvette or solid surface.

Still yet another advantage of a single reactive surface, multi-functional test would be the economic benefits afforded by such an assay. For example, many different fluid samples could be assayed for the presence or absence of different analytes at the same time. This is in contrast to currently available methods wherein each test requires use of a separate reactive surface (e.g., cuvette). Thus, where processing or screening a large number of samples is routine, as again for example in blood screening, the decrease in the time it takes to assay multiple samples for multiple binding pair members will result in a decrease in the overall cost of performing the assays. Additionally, such a lower per-assay cost allows for more assays to be performed per unit time, thereby allowing large numbers of assay results to become available more quickly than was previously possible. A still further economic benefit of a single reactive surface, multi-functional test is that less cuvettes would be needed per test, thereby reducing the cost of materials required.

Due to the deficiencies in the currently available methods of spectroscopic or light based analysis wherein only a single test is performed on a reactive surface, none of the above-stated benefits are readily attainable. There exists a need in the art for a method of these types of analysis wherein multiple tests can be performed on a single, multi-functional reactive surface.

SUMMARY OF THE INVENTION

The present invention provides a method wherein multiple tests can be performed on a single reactive surface using spectrophotometric or light-based methodology. In one process of the invention, using

STIR methodology, a multiplicity of binding pair members that are either the same or different are first immobilized in more than one location, such that they are spatially separated, on a single reactive surface of a solid support, such as a cuvette made from an optically transparent material. These spatially separated, immobilized materials are then contacted with a sample which is to be tested for the presence of one or more analytes. At least one additional binding pair member, which is labeled with a light-scattering material, is brought into contact with the sample under conditions wherein either the first immobilized member, the analyte and the light-scattering, labeled member or the first immobilized binding pair member and the light-scattering, labeled member form a specifically bound, light-scattering immunocomplex on or near the reactive surface. A light scattering labeled member may bind to the analyte of interest in the sample or to the first immobilized member, but not both. This labeled member will be either a single gold conjugate (eg. anti-IgG gold) or, more probably, a mixture of differently labeled gold conjugates. The reactive surface is next illuminated by an evanescent light wave resulting from total internal reflectance of a light beam incident upon the optically transparent material comprising the reactive surface and is sufficiently narrow to permit individual discrimination between each of the spatially separated, light-scattering complexes in the vicinity of the reactive surface. Elastically scattered light, derived from the interaction of the evanescent wave with the spatially separated, light-scattering complexes, is then detected and correlated with the region of the reactive surface from which the evanescent light wave was scattered. The detection may also be made as a function of time of the reaction on the reactive surface.

In addition to a light scattering label, binding pair members may be labeled with fluorescent, chemiluminescent or other compounds made active through light interactions.

5 In another embodiment of the invention, where reflectance is the methodology used, no light-scattering labeling is needed, as only reflected light, not scattered light, is measured. The spatially separated, immobilized binding pair members are contacted with the 10 test sample and analyzed for the presence of bound analytes.

The invention provides for spectroscopic detection of multiple variants of a single analyte, simultaneous detection of different analytes, and/or quality control 15 features, where in each case only a single, multi-functional reactive surface is used.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a cross-sectional view of a cuvette 20 which may be used to practice the method of detecting binding pair members in accordance with the invention.

Figure 2 is a diagram depicting spatially separated binding pair members immobilized on a reactive surface of an optically transparent material such as the cuvette 25 in Figure 1, and a path of a continuously scanned light wave incident upon the optically transparent material for practicing the method according to the invention.

Figures 3 to 8 graphically depict the results of Examples 1 to 6, wherein select embodiments of the STIR 30 spectroscopic determination of multiple analytes in a fluid solution using a single reactive surface, as well as control analyses, are disclosed.

Figure 9 depicts the set-up of a reflectometer measuring the thickness of a flat carrier's coating.

35 Figure 10 depicts the interaction of antibodies spotted and adsorbed onto wafers with various antigens.

Figure 11 is a graph showing the interactions of three different antigens with three antibodies adsorbed onto one piece of wafer.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method that solves the above-described problems currently associated with conventional spectroscopic and light-based analysis, and in particular, STIR analysis, by providing an assay method wherein multiple tests can be performed on a single reactive surface. For example, the presence of more that one analyte in a sample 20 can be detected on a single reactive surface 22 of a cuvette 11. The structure and the geometry of the reactive surface is not limited to the cuvette described above. However, from this point on, the term "cuvette" will be used to refer to any reactive surface regardless of geometry and structure. Controls may also be detected on the same reactive surface 22 as that used for detecting the presence of a binding pair member in sample 20. The STIR-based detection method of the present invention utilizes measurement of elastically scattered light derived from a scanning evanescent wave which is disturbed by the presence of a light-scattering material 15 brought near the interface of reactive surface 22 upon which an immunochemical reaction is taking, or has taken, place. When another method, such as reflectance, is used, the measurement of reflected light from multiple test loci will detect immunocomplexes formed.

Throughout this disclosure and in the claims appended hereto, the terms "binding pair member" or "member" are meant to designate materials which are capable of specifically interacting with one another so as to form a stable immunocomplex. For example, typical binding pair members include analytes, antibodies, antigens, haptens, intact cells containing antigenic determinants, proteins, polypeptides, nucleic acids,

chelators, both DNA-binding and immunoglobulin-binding proteins, biological receptors, lipids, lectins, carbohydrate residues, streptavidin and biotin-derivatized macromolecules.

5 A suitable apparatus and reactive surface for the STIR spectroscopic detection of the present invention is the same as that disclosed in U.S. Patent No. 5,017,009, while another suitable reactive surface is that provided by the cuvette disclosed in U.S. Patent No. 4,979,821.

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Cuvettes are comprised of suitable optically transparent materials such as glass, quartz, silicon, plastics such as polycarbonate, acrylic, or polystyrene, or oils comprising silicone or high molecular weight 15 hydrocarbons. Preferred in the method of the invention is the use of transparent polycarbonate plastic cuvettes, as these are economical and yet durable.

In the process of the invention, one or more binding pair members 12, such as, preferably antibodies 20 or molecules carrying antigenic determinants, are first immobilized, in a spatially separated way, onto a reactive surface of an optically transparent material, for example, the inner wall of a cuvette. Preferably, control binding pair members are also placed upon the 25 same reactive surface.

The binding pair members may be immobilized on the reactive surface of the optically transparent material in any convenient pattern or shape in the practice of the invention. However, there must be sufficient 30 separation of the immobilized binding pair members that the reaction of each of these members individually with the corresponding binding pair member in a fluid sample and/or a light-scattering labeled member can be measurably and discriminately detected without 35 substantial interference due to nearby immobilized binding pair members. It is preferred that the binding pair members be applied in the shape of either a circle

or a rectangular stripe. The first is most conveniently accomplished by applying a small droplet of binding pair member onto the reactive surface and allowing the droplet to dry. In many cases it is preferable to paint a rectangle or stripe of the binding pair member since such shapes provide sharp definition between those regions of the reactive surface having materials immobilized thereon and those regions of the reactive surface that spatially separate the aforementioned members. When applying a rectangular stripe of binding pair member to the reactive surface, the length of the stripe should exceed the width of the light beam. It should be readily apparent, however, that the method of the invention is not meant to be limited to members immobilized on the reactive surface in only these geometric shapes, but rather that any shape which allows for accurate determination of one or more than one member in a fluid sample is suitable for use in the method of the instant invention.

For application of the binding pair member onto the reactive surface in the practice of the present invention, any means that is convenient may be employed. Thus, manual use of micropipets or microcapillary tubes may be conveniently used for spotting members onto the reactive surface. It is also preferred to use mechanical means for the application of these members to the reactive surface, since such a method is convenient, reproducible and cost-saving. The advantages of mechanization make mechanical application of a binding pair member particularly desirable when the STIR assay is used in large-scale testing, such as routine screening applications. Examples of this use can be seen in infectious disease testing or typing of donated blood. Mechanical application methods include those such as used in ink jet spraying similar to those commonly used in electronic printers or touch pad

strips. For more specialized or individualized tests, manual application may be preferred.

When appropriate, the binding pair members may first be put into a solution to facilitate the process of depositing these samples onto the reactive surface. Suitable solutions for this purpose have only the general requirement that their use not substantially interfere with any subsequent specific binding reaction between binding pair members in the sample, on the reactive surface and a light-scattering, labeled member 15. Again, when light-scattering is not a part of the spectroscopic methodology, a labeled member may not be needed. Suitable solutions include aqueous and organic solutions as required by the particular binding pair member that is to be deposited. Such solutions may also be buffered with, for example, phosphate, TRIZMA (Sigma) or HEPES (Sigma) and preferably, carbonate-bicarbonate buffer, pH 9.5, or borate buffers at pH 9.3. It is preferred in the process of the invention that either carbonate or borate buffer be employed when necessary for the application of a protein binding pair member onto the reactive surface.

Binding pair members can also be attached to the reactive surface by covalent forces. The reactive surface may be derivatized directly with a variety of chemically reactive groups which then, under certain conditions, form stable covalent bonds with the applied binding pair member. Alternatively, the reactive surface may first be coated with chemically-derivatized polymers, such as dextran or PEG, which then bind applied binding pair members covalently. Certain types of detergents may also be coated to the reactive surface, then derivatized _in situ_, and reacted with binding pair members. In yet another method, the reactive surface may be coated with streptavidin through physical adsorption, then reacted with a biotin-labeled

binding pair member to create a well characterized, biologically responsive surface.

After the binding pair member has been deposited on spatially separated areas of the reactive surface, the member is then allowed to dry and thereby become immobilized on the reactive surface. Evaporation is the preferred drying method, and may be performed at room temperature (about 25°C). When desired, the evaporation may be performed at elevated temperatures, so long as that temperature does not sufficiently inhibit the ability of the immobilized members to specifically interact with their corresponding binding pair members or light-scattering labeled member. For example, where the immobilized binding pair member is a protein, non-denaturing temperatures should be employed.

In the process of the invention and embodiments wherein proteins are employed as the immobilized binding pair member, their concentration on the reactive surface after immobilization and drying must be experimentally defined for each particular member.

Following the immobilization of the binding pair members onto the reactive surface, the reactive surface is preferably treated so as to block non-specific interactions between the immobilized member and corresponding members in a fluid sample which is to be tested. In the case of an antigen-antibody interaction on the reactive surface, one blocking material is a protein. Others can be detergents and long-chain water soluble polymers. Most preferred in this instance is bovine serum albumin (BSA), due to its availability and low cost. The blocking material may be conveniently applied to the reactive surface as an aqueous or buffered aqueous solution.

The blocking solution may be applied to the reactive surface at any time after the first binding pair members are immobilized. It is preferred that cuvettes be blocked as soon as is practical after

immobilization. A sample to be tested for the presence of corresponding binding pair members is then brought into contact with the immobilized members on the reactive surface. The only general requirement of this process step is that the sample be in direct contact with the spatially separated immobilized binding pair members. Mild mixing of the fluid sample after bringing it in contact with the immobilized member is also preferred in the process of the present invention, but is not required. Such mixing may help to ensure close contact between the fluid sample and the immobilized binding pair member.

In the STIR-based embodiment of the present invention, a second binding pair member that specifically binds to either the immobilized member or its corresponding binding pair member in the solution, is labeled with a light-scattering material such as colloidal gold, red blood cells or latex particles. The light-scattering labeled member in the method of the invention is chosen such that it specifically interacts with either the immobilized member on the reactive surface or with one or more corresponding binding pair members possibly present in the solution, thereby forming spatially separated light-scattering complexes with these materials. In this embodiment, the light-scattering, labeled member may be included in the sample prior to contacting the fluid sample to the immobilized member or may be added to the sample after the latter is brought into contact with the immobilized member. Alternatively, the fluid sample and the light-scattering labeled member may be brought into contact with the immobilized member simultaneously.

After each of the above materials are in contact with the fluid sample, a light wave 3 is directed at the optically transparent material comprising the reactive surface, which illuminates the region of the reactive

surface to which an individual light-scattering immunocomplex is located.

Suitable light sources for generating the incident light beam 3 in the method of the invention provide collimated or uncollimated light, polarized or unpolarized light, or monochromatic or polychromatic light. Preferred light sources include lasers, light emitting diodes, flash lamps, arc lamps, incandescent lamps and fluorescent discharge lamps. The light source used to illuminate the reactive surface is a low wattage helium-neon laser. However, sources of light such as ultra-violet, visible, and near-IR may also be used in the proper setting.

The incident light wave that is internally reflected is preferably continuously scanned across the optically transparent material such that an evanescent wave derived therefrom will interact with only a small number of light-scattering complexes on or near the reactive surface at any given time. However, continuous scanning of the incident light wave is not required in the process of the present invention as the incident light wave may be sequentially directed only at those areas of the reactive surface which will give rise to an evanescent wave in the area of the reactive surface on which materials have been immobilized. In one embodiment of the invention, the incident light wave is scanned across the transparent material and any scattered light derived from the evanescent wave is continuously monitored and correlated with the position of the optically transparent material being illuminated and as a function of time.

The scan rate of incident light wave across the optically transparent material is not particularly critical in the practice of the invention, with the only general requirement that the rate not be so fast that correlation of the scattered light detected with the

position of the transparent material being illuminated is not impaired.

In the process of the invention, the transparent material may be repeatedly illuminated in the same manner described above, such that the intensity of scattering can be determined as a function of time. However, it should be recognized that not in all situations will more than one scan be required. For example, if the reactive surface were coated with a negative control, positive control and binding pair member, each within distinct spatially separated regions, then the reaction on the reactive surface between the immobilized binding pair members, the binding pair members in the sample, and/or the light-scattering binding pair members could be measured after a suitable incubation period with only a single scattered detection, i.e., scan. However, if multiple scans are desired or required, the number of scan cycles can vary as dictated by the assay. It should also be recognized that the number of scans required is independent of the scanning rate of the incident light wave.

It is preferred in the process of the invention that multiple scans be used when the assay is intended to be quantitative, since the time-dependency (i.e. rate) of the increase or decrease in the amount of light scattered may be more accurately indicative of the levels of the binding pair members present in the fluid sample than the total amount of scatter by the reaction at any given reaction point in time. Additionally, the use of multiple scans can provide a data set over which the increase in scattered light detected is of a known function with respect to time. Measuring the rate of change of the intensity of scattered light from a given region of the reactive surface versus time provides a reaction rate. By using reaction kinetics, the rate is

correlated to a quantitative measure of analyte concentration in the sample solution.

Another method is to integrate the scattered light intensity versus time. The area obtained by this integration is correlatable to the concentration of the analyte in solution.

In the method of the invention, scattered light derived from the interaction of the evanescent light wave with light- scattering materials on or near the reactive surface is then detected. Suitable photodetection means in the process of the present invention include photon detectors such as photomultiplier tubes, photodiodes such as PIN diodes or gallium- aluminum-arsenide diodes, or cadmium sulfide photoresistive cells, phototubes and pyrolytic detectors. Preferred is use of a photodiode, such as a Hamamatsu No. G1742 photodiode, in the STIR method of the invention. Preferably the detection means are positioned relative to the light source such that the scattered light detected is back-scattered light. It is further preferable that the detection means detect elastically back-scattered light.

In a preferred embodiment of the process of the invention, the binding pair members employed are antigens or antigenic determinants such as antibody-binding epitopes and antibodies or antibody fragments to these determinants. These types of binding pair members are preferred due to both the high specificity and selectivity of the antibody-antigen reaction as well as the great utility of such a test in the determination of the components of, in particular, blood or serum. It must be recognized, however, that the process of the invention is not meant to be limited to only such interactions, as the method disclosed herein is equally applicable and is highly useful to the detection of, for example, specific DNA or RNA analytes in a sample. In these cases, the required specificity of the reaction

can be found in a complimentary nucleic acid sequence to that which has been immobilized on the reactive surface. In still another useful embodiment, the process of the invention provides detection of the specific interaction between proteins and nucleic acids or lipids, immunoglobulins and immunoglobulin binding proteins such as protein A and protein G, biological receptor molecules and their corresponding ligands, lectins and specific carbohydrate moieties, and streptavidin and biotin-labeled macromolecules. As in the case of the antigen-antibody reaction exemplified below and described in the patents incorporated herein, these additional embodiments of the present invention may be either direct reactions or may be based upon competitive reactions.

The reactions that take place on the reactive surface may be of several types, the following of which are not meant to be an exhaustive list but rather merely exemplary of suitable techniques.

In one embodiment of the process of the invention, antibodies to different antigens are individually deposited onto the reactive surface. The analytes to be detected in the fluid sample in this embodiment are the antigens to which the immobilized antibodies specifically bind. In this non- competitive assay, the light-scattering, labeled member of this embodiment may then be a second antibody which has been labeled with a light-scattering material and binds the same or a different antigenic determinant than the immobilized member in a non-competitive way. When the specific analyte is present in the fluid sample, a reaction occurs on the reactive surface between the antibody coated thereon and the antigen in the fluid sample. A second reaction takes place simultaneously between the antigen in the fluid sample and the labeled antibody, resulting in the formation of an antibody-antigen-labeled antibody immunocomplex on or

near the reactive surface. As this light-scattering complex forms, the amount of an evanescent light wave scattered by the light-scattering complex provides an accurate indication of the presence of the complex formed on or near the reactive surface and, thereby, the presence of the analyte in the fluid solution. The amount of scattered light detected provides a quantitative analysis of the concentration of the analyte in the fluid solution.

In another embodiment of the present invention, both the immobilized member and the light-scattering labeled member are the same antigen or series of antigens or antigenic determinants. The analytes to be detected in the fluid sample in this embodiment are antibodies which bind specifically to both the immobilized member and the light-scattering labeled member, forming a specific link between the two on or very near the reactive surface. This embodiment is demonstrated in the examples.

In yet another embodiment of the present invention, the immobilized binding pair member is a material with an antigenic determinant, while the assay tests for the presence of a specific antibody to that determinant in the sample. In this embodiment, the labeled, light-scattering member is an antibody specific for the antigenic determinant binding pair member. The sample is contacted with the immobilized member such that an antigen-antibody reaction is allowed to occur. The light-scattering, labeled antibody is then introduced into the fluid sample and the amount of labeled antibody forming an immunocomplex with the antigen on the reactive surface is determined by the amount of elastically-scattered light derived from an evanescent light wave. Using immobilized samples containing a known amount of antigen, along with known concentrations of light-scattering labeled antibody, this embodiment of

the instant invention also provides for a quantitative analysis of the amount of analyte in the fluid sample.

In reference to Figure 1, an optically transparent cuvette 11 providing a reactive surface 22 with immobilized member 12 deposited thereon is depicted. Also depicted is a fluid sample 20 containing a light-scattering labeled member 15 in contact with the immobilized material 12. Only one of a plurality of spots of deposited material is seen from the view depicted. An incident light beam 3 is directed to the optically transparent material at an angle that produces an evanescent lightwave which illuminates complexes on or near the reactive surface. The scattered light derived from an evanescent light wave is detected in the process of the invention and correlated with the region of the reactive surface which generated the scattered light.

Referring to Figure 2, the placement of the immobilized binding pair members 12 on the reactive surface 22 and with respect to the area of illumination of the optically transparent material that comprises the reactive surface 22 is schematically depicted. The relation of the deposited material to a scan (arrow) of the incident light wave 3 (stippled area), is also schematically depicted. As the evanescent wave derived from the scanned incident light wave 3 moves across the reactive surface 22, the scattered light generated by any elastically scattered light derived from individual light-scattering complexes located at or near the reactive surface 22 are individually detected. The region of the reactive surface 22 that does not have an immobilized member 12 placed thereon serves to both differentiate regions of the reactive surface 22 whereon member 12 has been immobilized from those on which no material resides, and also provides background light scatter data over which any specific light-scattering reaction can be discriminated. In further reference to

Figure 2, the relative width of the incident internally reflected light wave 3 at the reactive surface as compared to the immobilized binding pair member 12 is also schematically depicted.

5 In another embodiment of the invention, experiments were carried out to test the simultaneous detection of three different antigens using reflectometric methodology. Reflectometric detection of binding pair members proved to be possible using corresponding
10 binding pair members adsorbed on a carrier polystyrene substrate. Cross-reactivity and the influence of the concentration of the immobilized binding pair members was also investigated.

When silicon wafers onto which a single antibody
15 was adsorbed were used, interaction was only found with the corresponding antigen e.g. hCG and anti-hCG, HBsAg and anti-HBsAg, and Rubella and anti-Rubella. For the nine non-corresponding antigen-antibody combinations the interaction was
20 < 0.2 mg/m^2. The interaction of HBsAg with α-HBsAg increased with increasing concentration of both components.

Experiments were also done in another way, in which, before the reflectometric measurement started, a
25 drop of three different antibodies were placed on three different spots on one wafer, and allowed to absorb. The interactions of the three corresponding antigens with the antibody on each of the three places were determined reflectometrically after the antibodies had
30 dried for one day. Results show that the antibodies used here are all highly specific towards the antigens. Sensitivity of the detection was different as the concentration changed in the three antibody-antigen combinations.

35 We may conclude that in this way reflectometry, another light-based methodology, enables simultaneous detection of many antigens.

EXAMPLES

In the following examples of the multi-analyte STIR spectroscopic detection system of the invention, the immobilized binding members are either the human immunodeficiency virus (HIV-1) 160 kD glycoprotein (gp160; Advanced Biosciences Laboratories, Inc., Kensington, MD) or the hepatitis B virus surface antigen (HBsAg; BioTech Resources Inc., San Antonio, TX). The light-scattering binding pair members are the above-mentioned proteins labeled with colloidal gold of approximately 70nm in diameter, prepared as described by Turkevich et al. (J. Phys. Chem. 57:670-673, 1953), and incorporated herein by reference. In the experiments depicted in Example 2, the positive control binding pair member was the α-HBsAg monoclonal antibody 233D (Biotechnology Research Institute, Rockville, MD).

EXAMPLE 1

Multi-analyte cuvettes were prepared by placing one 1.0 microliter droplet each of gp160 (2 μg/ml) and HBsAg (1μg/ml) about 1 millimeter apart on the reactive surface of the cuvette in a horizontal plane along the incident light wave path, as depicted in Figure 2. A cuvette of the structure described in U.S. Patent No. 4,979,821 was used. These deposited materials were then immobilized onto the cuvette's reactive surface by air drying overnight at room temperature. The reactive surface was blocked by filling the cuvette with a solution of 2% BSA in 25mM Hepes/Tris, pH 7.4 for 60 minutes at room temperature. The blocking solution was aspirated and the cuvettes washed once with the same buffer, then air dried and stored at room temperature in an atmosphere of low humidity.

The cuvettes were placed into a circular carousel and illuminated with a rotating laser beam, as depicted in Figure 1. The test human serum sample was added to the cuvette in sufficient volume to cover the region of

the reactive surface to which the two binding pair members had been immobilized. After a 5 minute incubation period at 37°C, a solution containing assay buffer and a 50:50 mixture of gp160-gold sol conjugate and HBsAg-gold sol conjugate was added. The contents of the cuvette were mixed, then the amount of light-scattering was recorded continuously for 10 minutes.

The data is presented as a series of plots representing an increase in light-scattering (y-axis) due to the presence of gold-labeled immunocomplexes accumulating on or above distinct regions of the cuvette's reactive surface (x-axis). These complexes are detected by a laser beam scanning across the cuvette. Due to this fact, the 0-50% region of the x-axis was coated with gp160 while the 50-100% region was coated with HBsAg, as described above. The plots depict light scattering readings every two minutes up to ten minutes, with the lowest line representing the first reading taken approximately ten seconds after the addition of the mixed gold sol conjugate.

Figures 3-6 demonstrate the results obtained with four different human serum samples, containing antibodies to gp160, antibodies to HBsAg, antibodies to both or to neither.

Figure 3 shows the response obtained when normal human serum was added to the cuvette. This serum contains no antibodies to either gp160 or HBsAg and thus does not generate a light scattering signal in any region of the cuvette.

In Figure 4, the sample applied to the cuvette was human serum containing antibodies to gp160, but none to HBsAg. The response demonstrated a specific, time-dependent increase in scattered light from the region of the cuvette corresponding to the position where gp160 was immobilized. No response was detected within the HBsAg coated region.

In Figure 5, the sample applied to the cuvette was human serum containing antibodies to HBsAg, but none to gp160. The results obtained were the inverse of those in Figure 4. A specific, time-dependent increase in scattered light was found in the region of the cuvette corresponding to the position where HBsAg was immobilized, but not where gp160 was immobilized.

In Figure 6, a human serum sample containing antibodies to both gp160 and HBsAg was applied to the cuvette. Both these analytes were detected discreetly, as judged by an increase in scattered light due to the accumulation of specific colloidal gold immunocomplexes within the gp160 and HBsAg coated regions of a single reactive surface. Each reaction is clearly compartmentalized, demonstrating that each analyte may be quantitated independently of the other.

EXAMPLE 2

The multi-functional cuvettes used in this example were prepared in a slightly different manner to those described in Example 1. Thin stripes of HBsAg (2.5 μg/ml) and 233D (10μg/ml) were immobilized onto the reactive surface of the cuvette on a plane perpendicular to and bisecting the scanning incident light beam. The stripes were applied using modified single well sample applicators from Pharmacia's PhastSystem[R] (Pharmacia, Uppsala, Sweden). These deposited binding pair members were allowed to air dry at room temperature onto the cuvette's reactive surface. The cuvettes were blocked, washed and stored as described in Example 1.

The assay conditions were identical to those described in Example 1, except that only a single gold conjugate (HBsAg coated colloidal gold) was added to the cuvette containing the test serum sample.

The aim of these experiments was to demonstrate the feasibility of incorporating a reagent positive control into each cuvette. In this case, the 0-50% region of

the x-axis of the cuvette was coated with HBsAg while the 50-100% region was coated with 233D monoclonal antibody. This antibody will react directly with the HBsAg-gold conjugate generating a strong light-scattering signal with every cuvette, regardless of the presence of specific analyte or not.

Figure 7 demonstrates that when a serum sample negative for a specific analyte (in this case anti-HBsAg antibody) was applied to the cuvette, there was no time-dependent increase in scattered light from the HBsAg coated region of the reactive surface. However, there was a strong reaction in the region of the reactive surface coated with 233D antibody, as the HBsAg-gold conjugate bound directly and specifically to this antibody. This reaction can be quantitated and used to assess the reagents integrity.

In Figure 8, the human serum sample applied to the cuvette contained antibodies to HBsAg. A specific, time-dependent increase in scattered light was observed in the region of the cuvette where HBsAg was coated. In addition, there was a strong reaction in the 233D coated region, very similar to that seen in Figure 7, again demonstrating the integrity of the colloidal gold reagent.

Of considerable interest are the steep sides of the graphs (plots) of the strong positive control responses in Fig. 7 and 8, indicating that multiple analytes can be measured with excellent baseline separation within a narrow region of the cuvette's reactive surface.

EXAMPLE 3

The investigation of the selectivity of three types of antibodies and antigens was done using a reflectance-based method. Reflectometry is based on the detection of a change in the intensity of linearly polarized light by reflection at the adsorption/interaction layer. This

method is described in detail in US Patent 4,908,508, herein incorporated by reference.

Figure 9 shows the set-up of the reflectometer. Polarized light of a He/Ne laser passes through a solution and is reflected at a substrate of silicon (refractive index = 3.76). The angle of incidence is the Brewster angle for a silicon/water interface, at which the reflectance of the parallel polarized light is zero. This is used to position the laser and the cuvette, which has windows perpendicular to the incident and the reflected light.

When for example a polymer layer is coated on a silicon wafer, the intensities of both polarizations are changed compared to the reflection at pure silicon.

COATING THE SOLID SURFACE

At the Brewster angle a polarized light beam from a He/Ne laser is reflected at the substrate (Si with n=3.76) and analyzed in terms of the intensities in two polarization directions: Rp and Rs, as shown in Figure 9. From the value of M, which equals (Rs-3Rp)/(Rs+3Rp), the thickness or, in most cases, the mass of adsorption of the layer on the wafer can be calculated with an accuracy of about 0.0003 mg/m^2.

In these experiments, the carriers, silicon wafers (Wacker Chemitronic, Germany) were spin coated with polystyrene having a layer thickness of about 70nm. The immunocomplexes formed were detected by the determination of the adsorbed mass present at places where the antibodies were initially absorbed. When three antibodies were present at different places on one wafer within

2 cm of total space, a scan was made over the 2 cm to measure the interactions of all three antibodies and the antigens reflectometrically.

MATERIAL USED IN THE EXPERIMENTS

The following reagents, antibodies and antigens, were used in the experiments.

TABLE 1

MATERIALS	CONCENTRATIONS
α-hCG	1.4mg/cm^3
α-HBsAg	7.0mg/cm^3
α-Rubella	20.0mg/cm^3
hCG	100,000 IU/l
HBsAg	3,375 IU/cm^3
Rubella	diluted 30X

EXPERIMENT 3A - Single Spot Per Wafer

Generally, silicon wafers coated with polystyrene as described above were spotted with antibodies against hCG, HBsAg, and rubella. In the first experiment, one set of wafers consisted of individual wafers where only one antibody was spotted in one place on each wafer and allowed to adsorb onto the wafer. A different wafer was used for each determination. The concentrations of the reagents used are given in Table 2.

As Table 2, in the first adsorption column shows, the adsorption of the three antibodies are almost equal at the concentration used in these experiments.

TABLE 2

Reflectometric Detection of the Reaction of Different Antigens

1st Compound	Conc. g/cm^3	2nd Comp.	Conc.	1st Adsorp.	2nd Adsorp.
α-hCG	5x10^{-5}	hCG	10 IU*/cm^3	+2.27	+0.72
α-hCG	5x10^{-5}	HBsAg	200 IU/cm^3	+2.16	+0.10
α-hCG	5x10^{-5}	Rubella	1/30	+2.31	-0.95
α-HBsAG	1x10^{-4}	hCG	10 IU/cm^3	+2.22	+0.16
α-HBsAG	1x10^{-4}	HBsAg	200 IU/cm^3	+2.52	+1.52
α-HBsAG	1x10^{-4}	Rubella	1/30	+2.32	+0.05
α-Rubella	1x10^{-4}	hCG	10 IU/cm^3	+2.48	-0.01
α-Rubella	1x10^{-4}	HBsAg	200 IU/cm^3	+2.50	-0.02
α-Rubella	1x10^{-4}	Rubella	1/30	+2.27	+0.44

*IU=International Units

20 The Γ(anti-hCG) = 2.25, Γ(anti-HBsAg) = 2.34, and Γ(anti-rubella) = 2.42. The value of anti-hCG may be slightly lower due to the fact that a two times lower concentration of reagent was used. From these results we conclude that the molar weight of the antibodies are 25 similar and/or that the affinity to the polystyrene surface is essentially the same.

The various antigens were brought into contact with each individual spot of antibody and a second reflectance measured. Significant antibody-antigen 30 interactions were found between the matched binding pairs. For all other pairs, only very slight interaction values were found. See Figure 10. However, a large negative interaction of anti-hCG and rubella antigen was found. It is believed that rubella may be

able to partially desorb the anti-hCG from the polystyrene.

EXPERIMENT 3B - Multiple Spots Per Wafer

5 Another set of wafers consisted of wafers coated as above, where each antibody was spotted onto a single wafer in such a manner that each spot was distinct from the other, and allowed to dry for one day. Antibodies to hCG were placed on the left third of the wafer, 10 antibodies to HBsAg were placed in the center and antibodies to rubella were placed in the remaining third. Then a different antigen was applied to each wafer, and the reflectance measured. The concentrations of the antibodies and antigens used are given in Table 15 2 above.

The results are given in Figure 11. This figure once again shows that at the concentrations used in this study, cross-reactivity is not a problem; only matching binding pairs show a significant increase in mass.

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It is understood that various other modifications will be apparent to and can be readily made by those skilled in the art without departing from the scope and spirit of this invention. Accordingly, it is not 25 intended that the scope of the claims appended hereto be limited to the description set forth above but rather that the claims be construed as encompassing all of the features of patentable novelty which reside in the

present invention, including all features which would be treated as equivalents thereof by those skilled in the art to which the invention pertains.

WHAT IS CLAIMED IS:

1. A method of detecting one or more binding pair members in a sample comprising the steps of:

(a) immobilizing at least two first binding pair members that are either the same or different, onto a reactive surface of an optically transparent medium that has an index of refraction greater than the index of refraction of the sample, such that each immobilized binding pair member is separated from the other, thereby providing at least two spatially separate immobilized first binding pair members;

(b) contacting the immobilized first members with the sample and at least one light-scattering second labelled binding pair member;

(c) illuminating any immobilized light-scattering complexes formed in step (b) with an evanescent wave derived from total internal reflectance of incident light propagated in the optically transparent medium; and

(d) separately detecting light scattered by each immobilized light-scattering complex.

2. The method according to claim 1, wherein in said step (a) the immobilized first binding pair members are different from each other.

3. The method according to claim 1, wherein the reactive surface of an optically transparent medium is

selected from the group consisting of glass, quartz, silicon, polycarbonate, acrylic, polystyrene, silicone and hydrocarbons.

5 4. The method according to claim 1, wherein said first binding pair members are antibodies or antigens.

5. The method according to claim 1, wherein said light-scattering second labelled binding pair member is 10 labeled with a label selected from the group consisting of coated colloidal gold, metal sols, selenium sols, blood cells and latex particles.

6. The method according to claim 5, wherein said 15 second labelled binding pair member is labelled with colloidal gold.

7. The method according to claim 1, wherein said sample is selected from the group consisting of blood, 20 serum, plasma, urine, saliva, cell lysate, sputum, cerebral spinal fluid, and control reagents.

8. The method according to claim 1, wherein said step (c) further includes deriving the light that is 25 internally reflected from a light beam which is scanned across the optically transparent medium.

9. The method according to claim 8, wherein said light is selected from the group consisting of ultra-violet, visible and near-infra-red.

5 10. The method according to claim 8, wherein said light is internally reflected from a light source selected from the group consisting of lasers, light emitting diodes, flash lamps, arc lamps, incandescent lamps and fluorescent discharge lamps.

10

11. The method according to claim 1, wherein said scattered light is detected by detector means selected from the group consisting of photomultiplier tubes, photodiodes, cadmium sulfide photo resistive cells,

15 phototubes and pyrolytic detectors.

12. The method according to claim 1, wherein said step (d) further includes detecting light elastically back-scattered from the immobilized light-scattering

20 complexes illuminated in said step (c).

13. The method according to claim 1, wherein said step (d) further includes detecting light scattered elastically.

25

14. A method of detecting one or more binding pair members in a sample comprising the steps of:

(a) immobilizing at least two first binding pair members that are either the same or different, onto a carrier such that each immobilized binding pair member is separated from the other, thereby providing at least two spatially separate immobilized first binding pair members;

(b) contacting the immobilized first members with the sample;

(c) reflecting a polarized light beam at the carrier; and

(d) analyzing the intensity of two polarization directions and separately detecting the thickness or the mass of adsorption of each immobilized complex.

15. The method according to claim 14, wherein in said step (a) the immobilized first binding pair members are different from each other.

16. The method according to claim 14, wherein the carrier is a silicon wafer coated with polystyrene.

17. The method according to claim 14, wherein said first binding pair members are antibodies or antigens.

18. The method according to claim 14, wherein said sample is selected from the group consisting of

blood, serum, plasma, urine, saliva, cell lysate, sputum, cerebral spinal fluid, and control reagents.

5

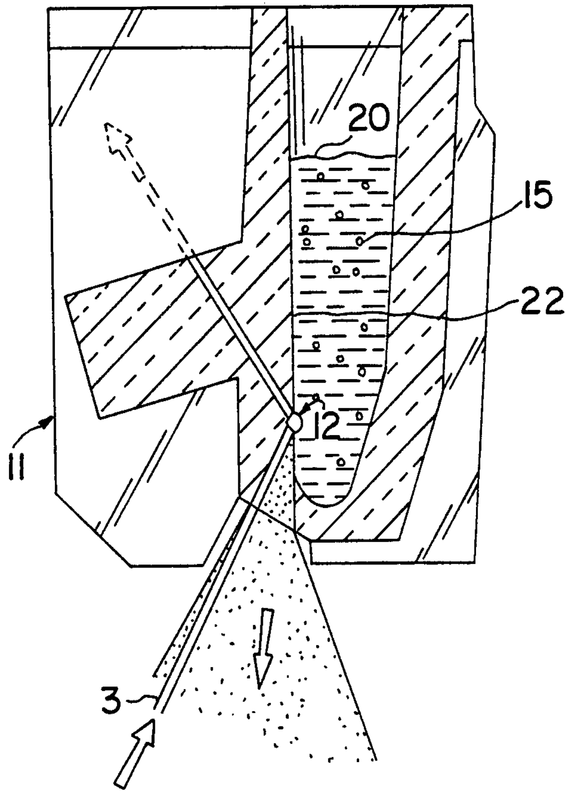

FIG.1

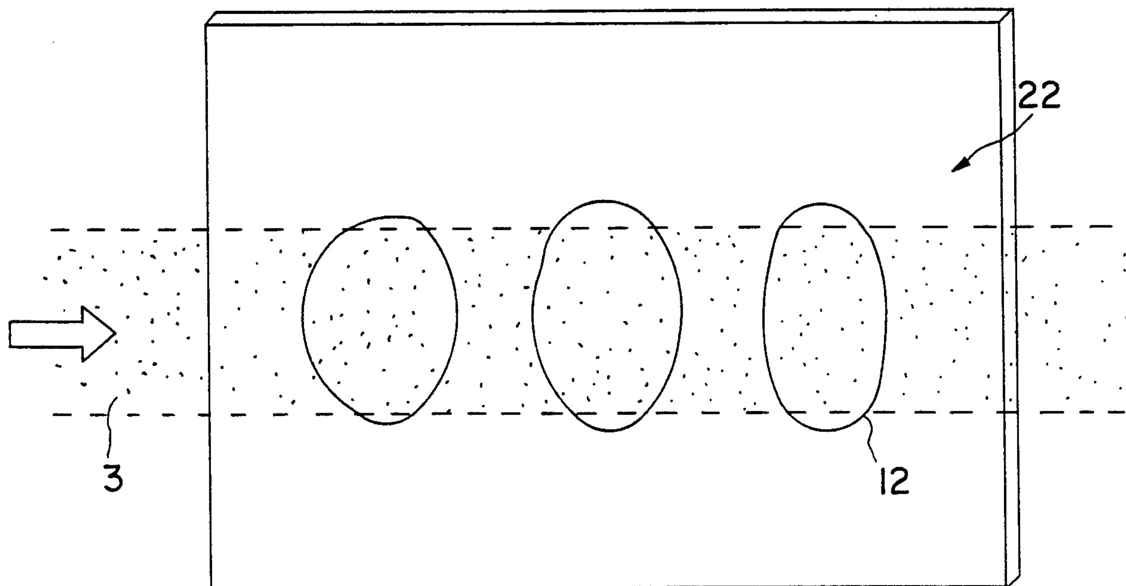

FIG.2

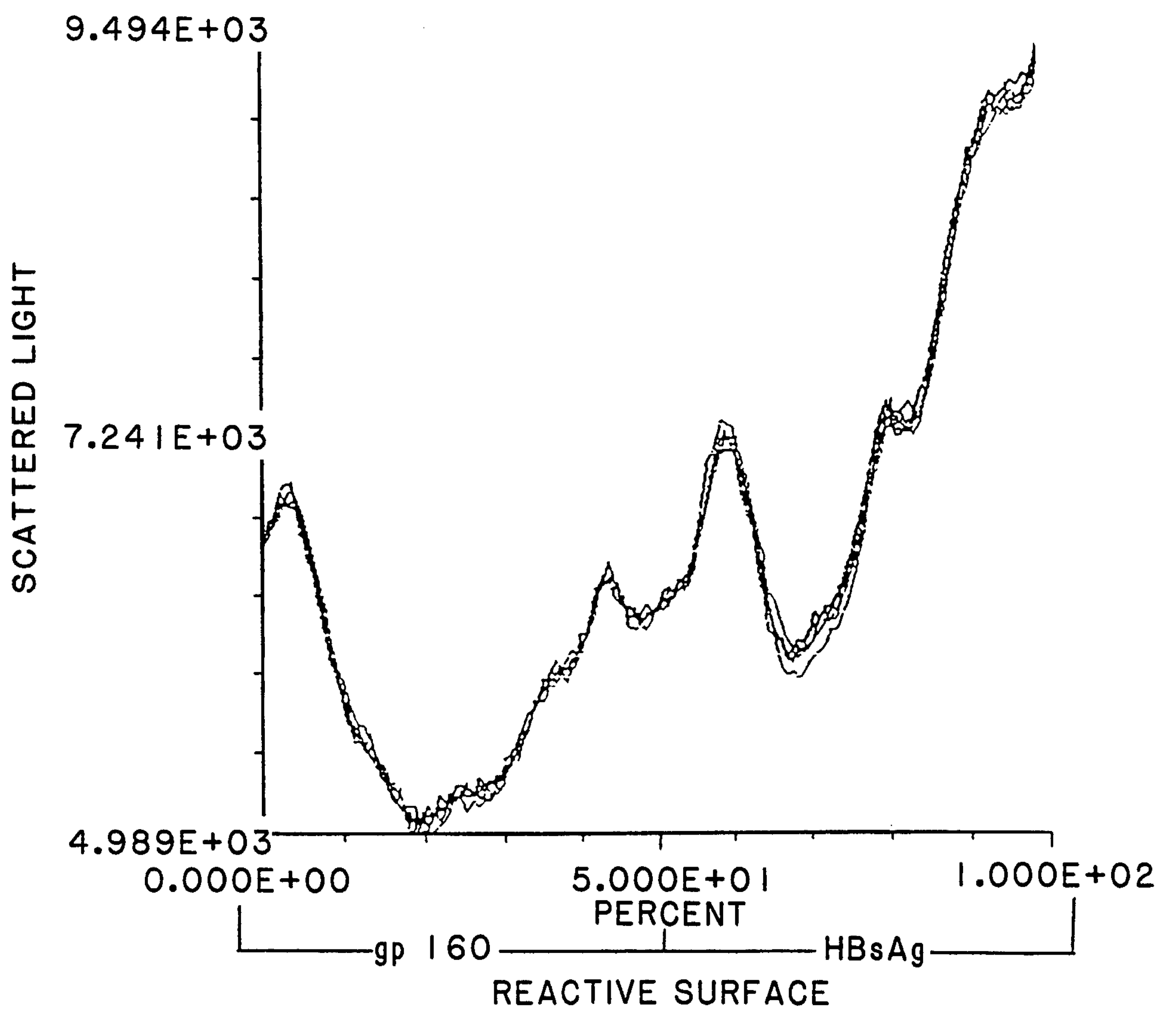

FIG.3

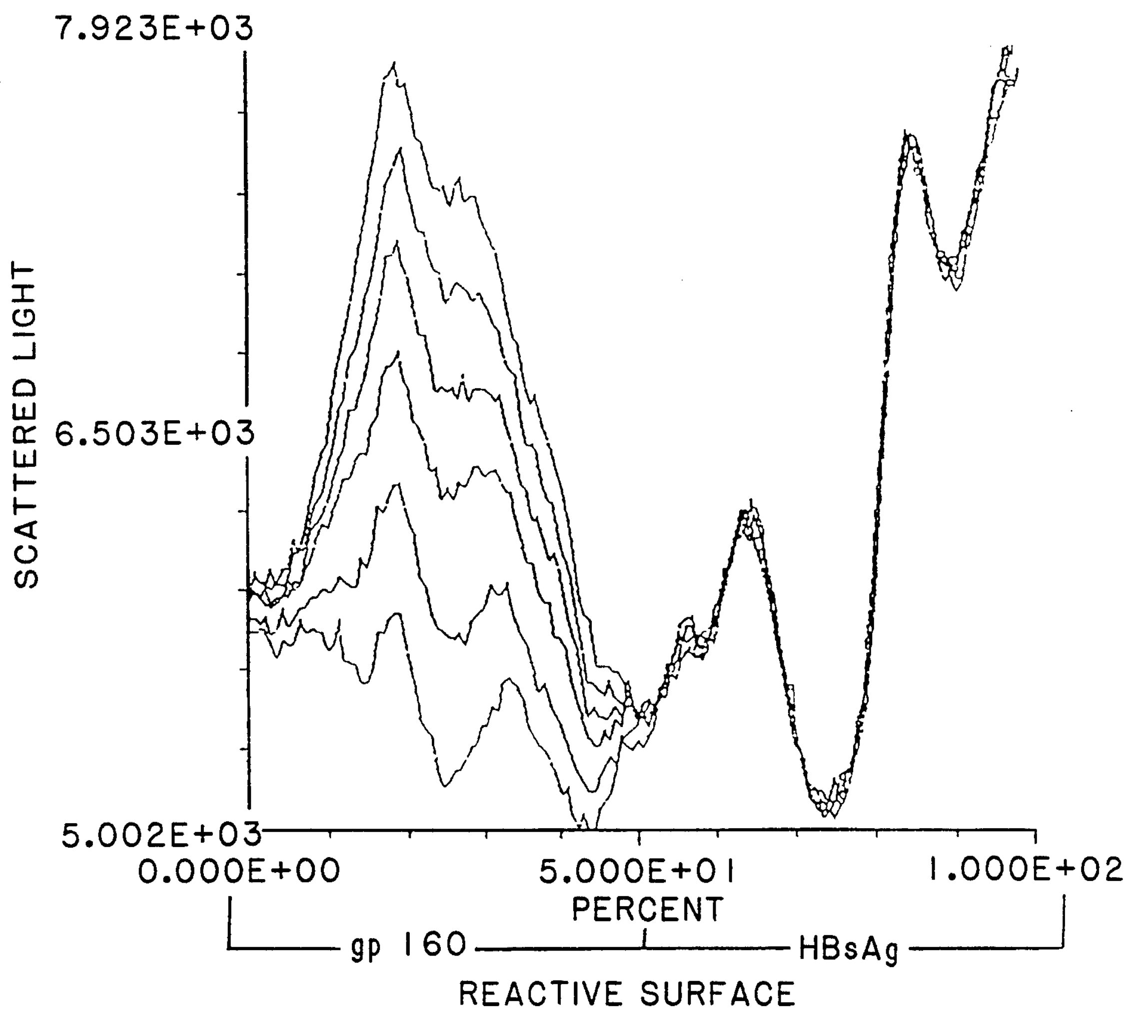

FIG.4

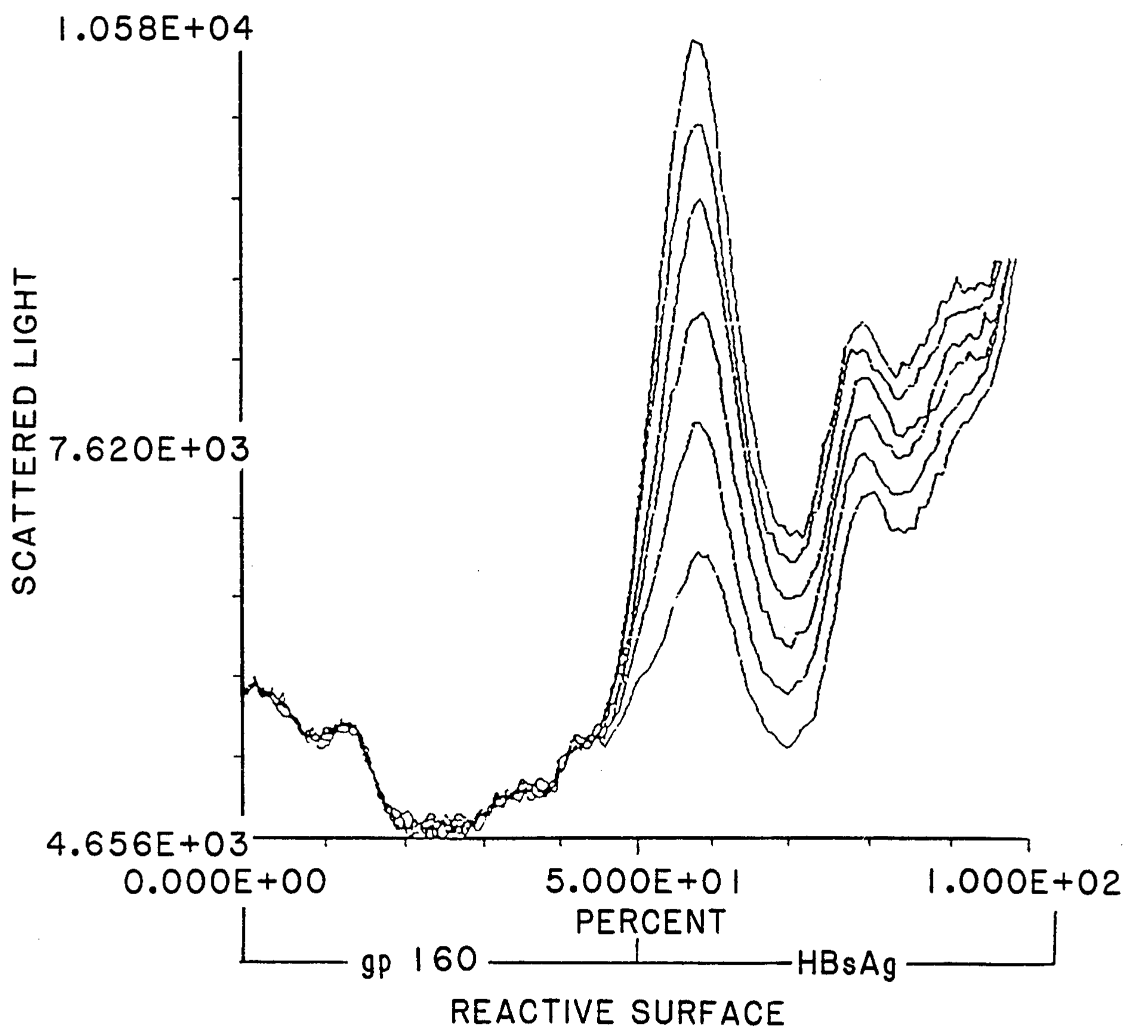

FIG.5

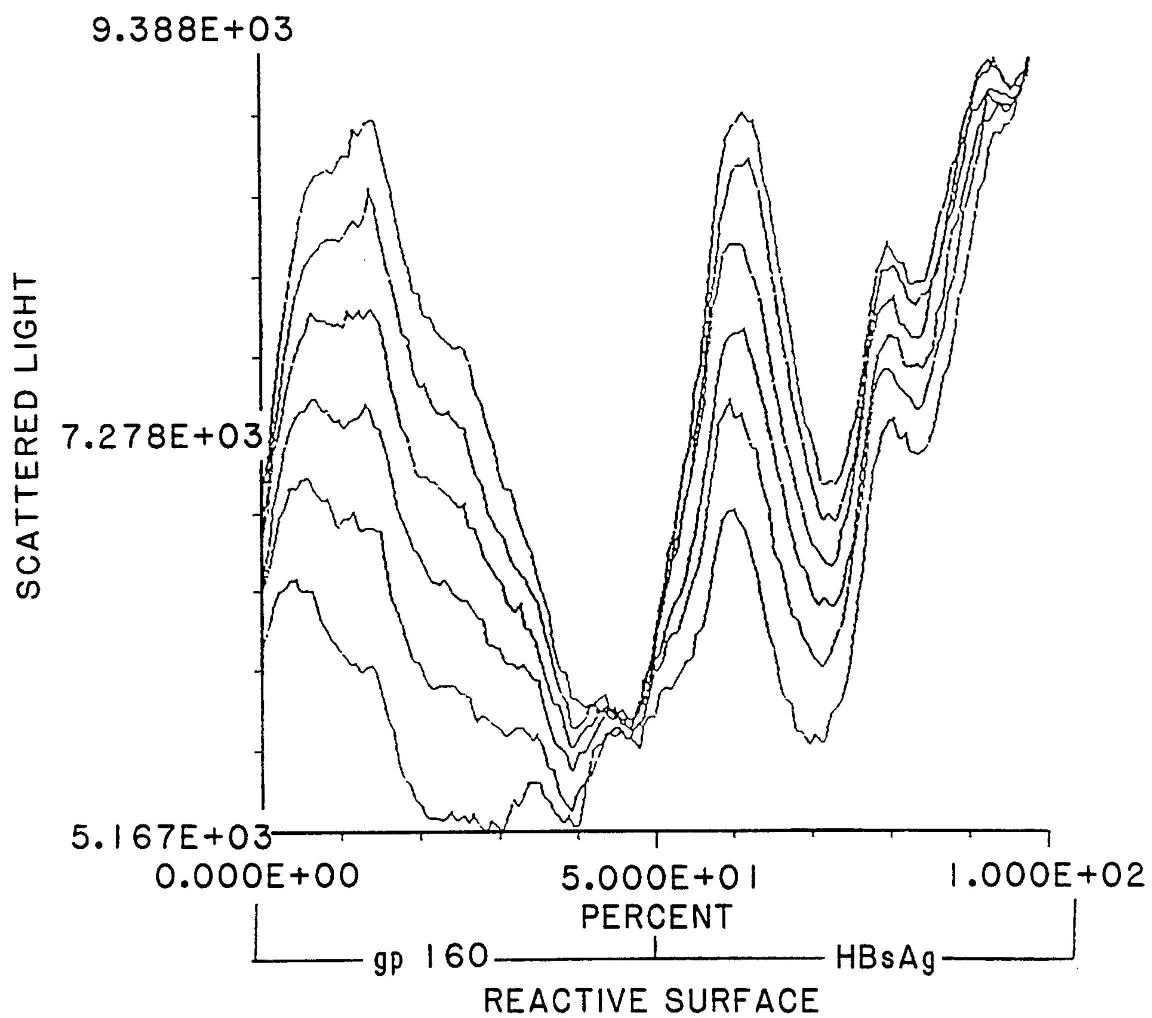

FIG.6

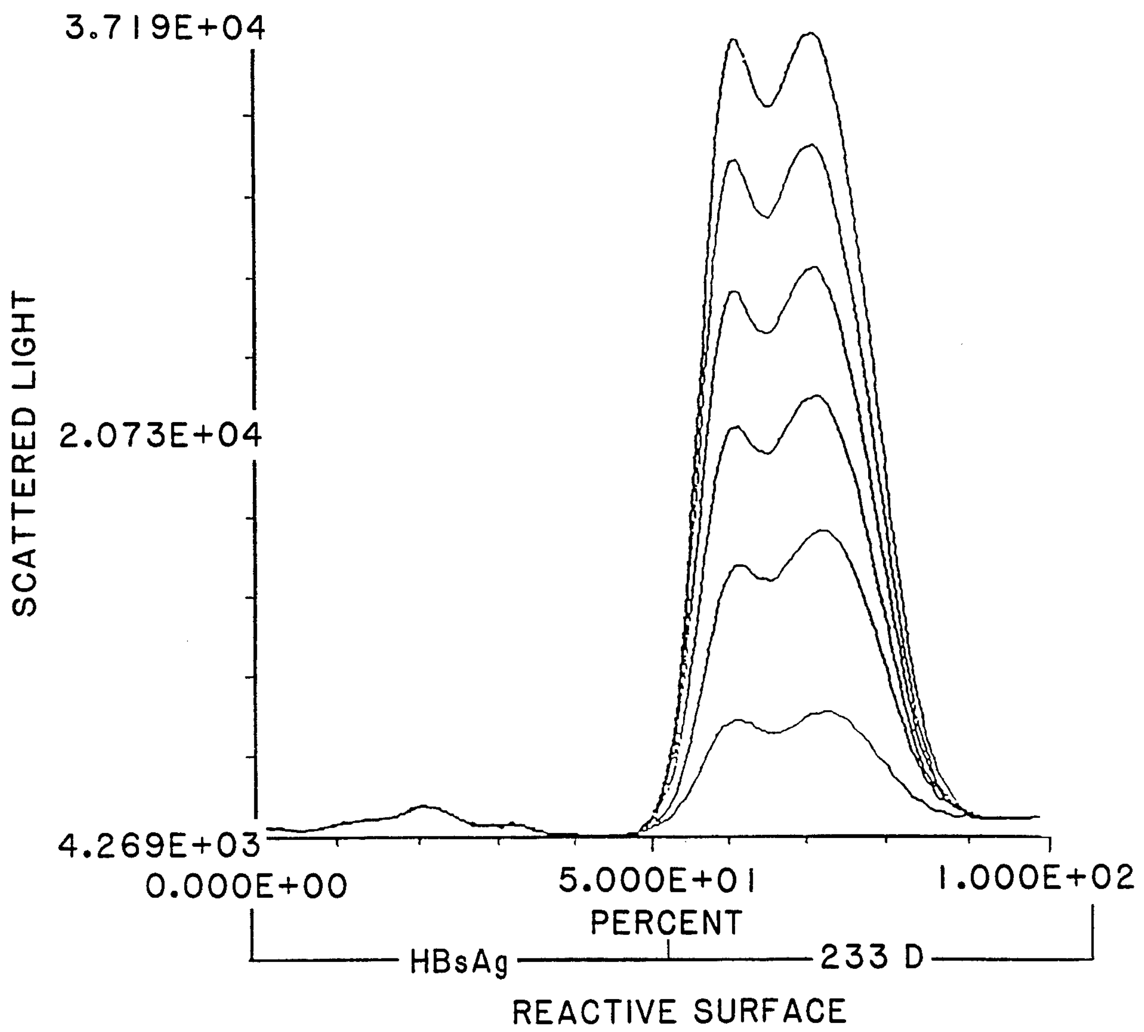

FIG.7

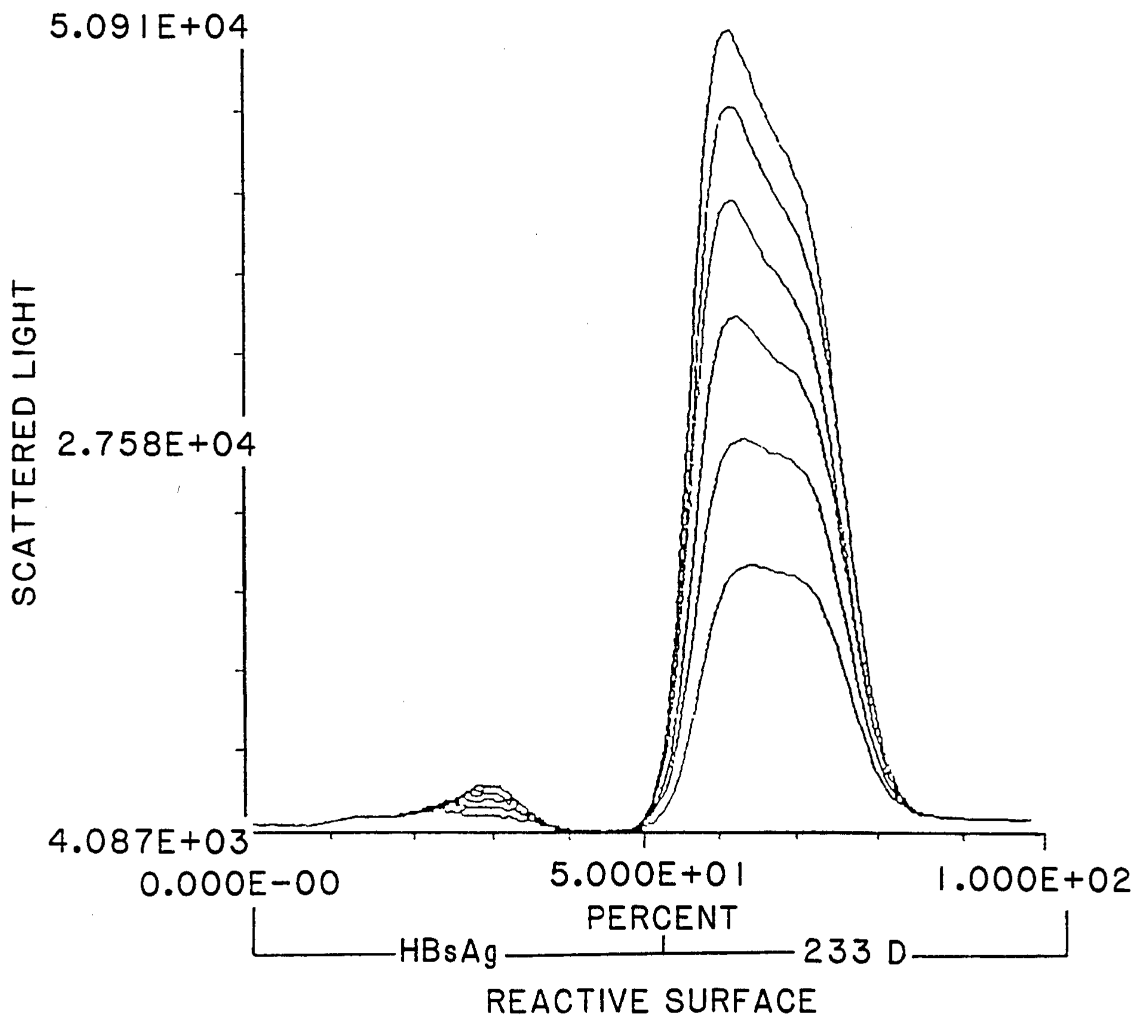

FIG.8

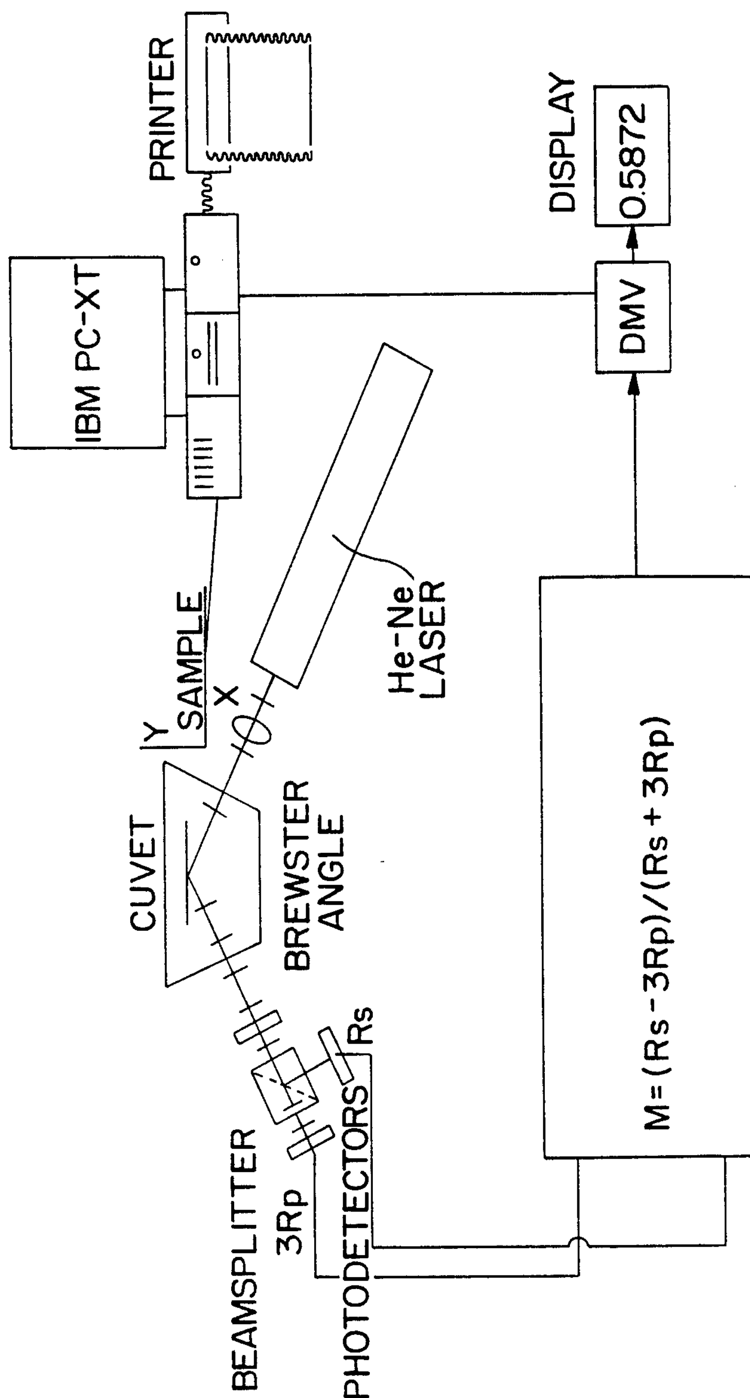

FIG.9

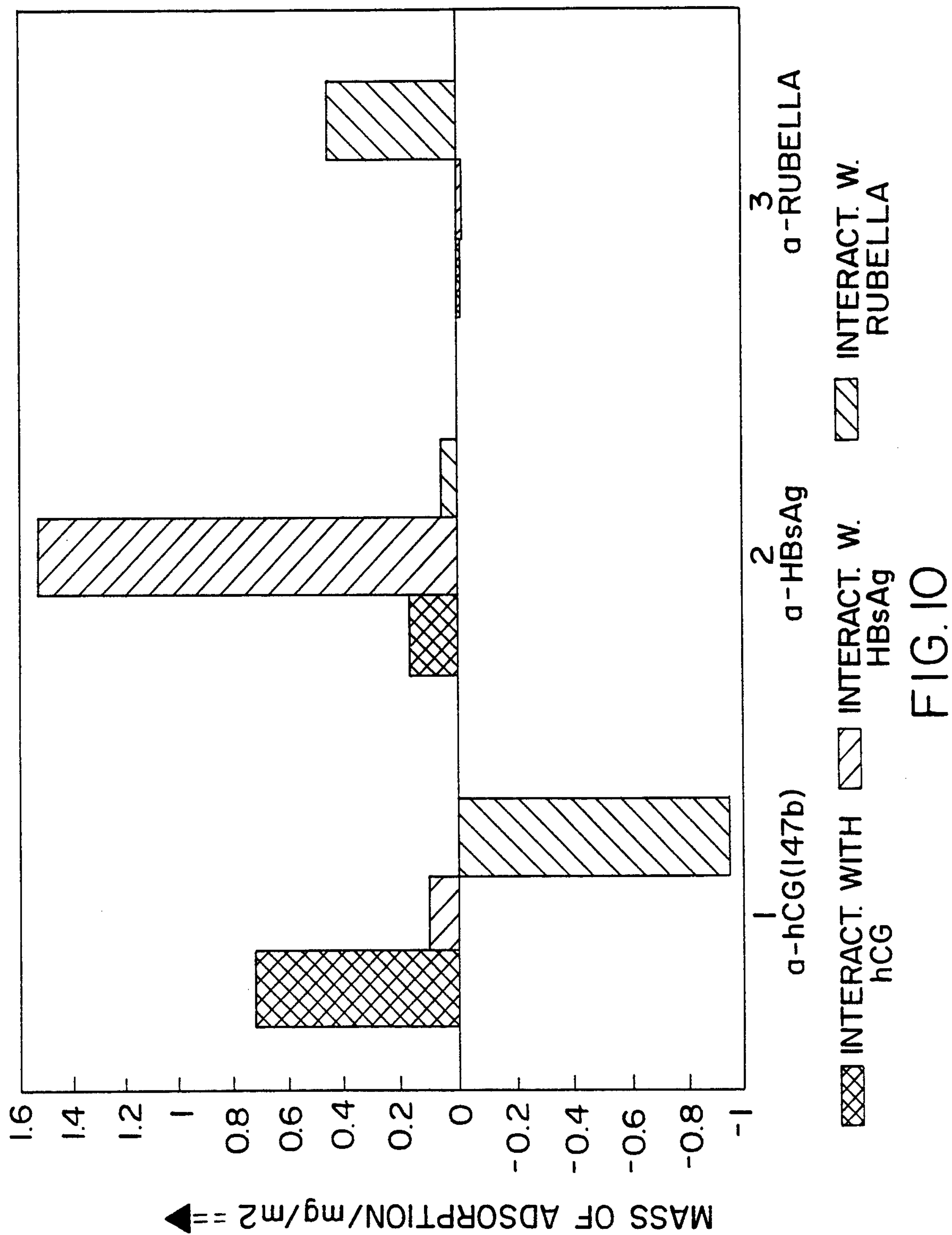

FIG. 10

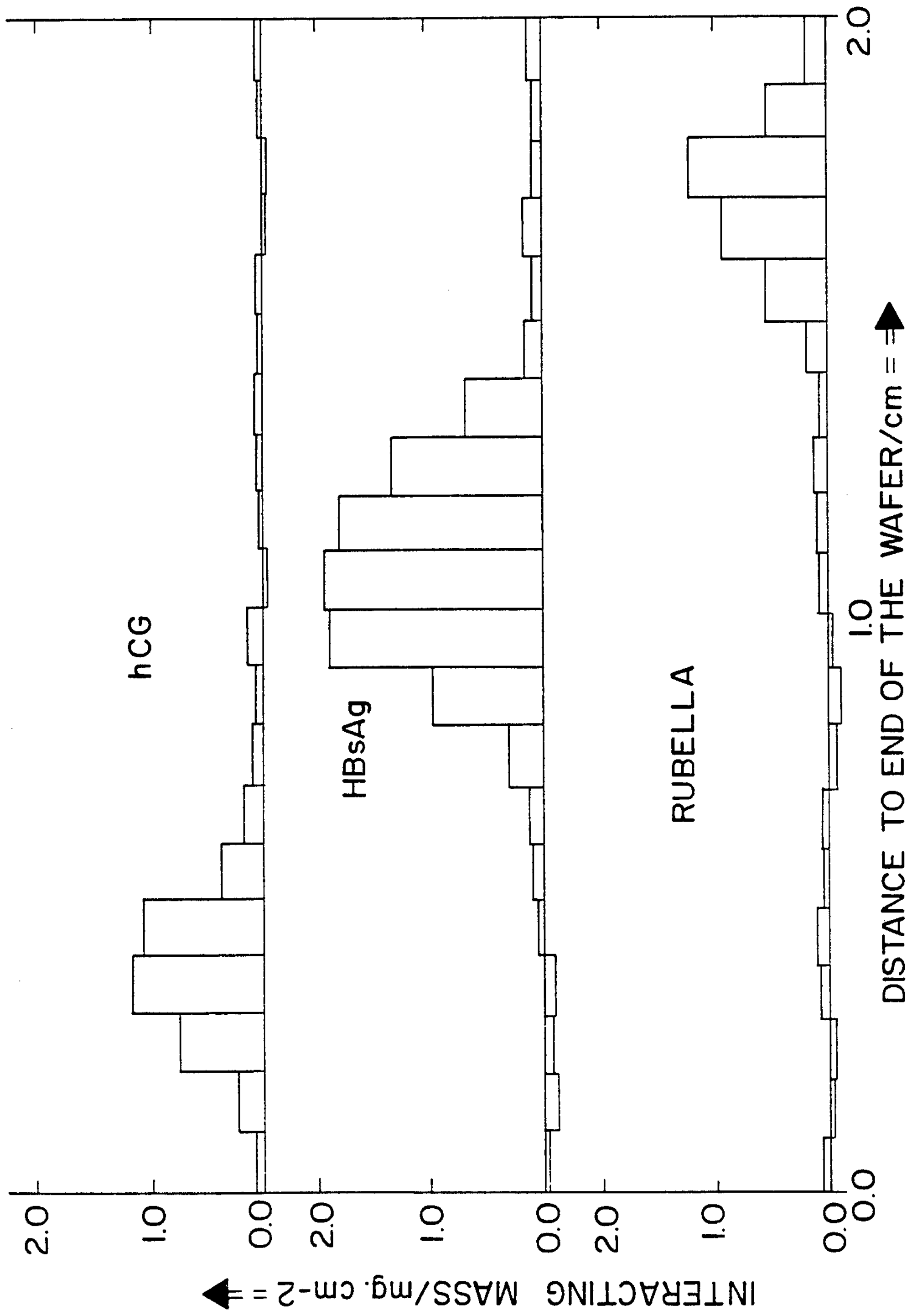

FIG.11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06020

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/553
US CL :436/525
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. : 436/519, 525, 527, 531, 164, 805; 435/5, 7.2, 973; 356/318, 336, 338, 342

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
search terms: total internal reflectance, antibod?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 84/02578 (Pettigrew et al.) 05 July 1984, see entire document, especially abstract; page 1, lines 15-19; page 4, lines 14-16; page 6, lines 32-37; page 11, lines 12-23.	1-18
Y	US, A, 5,017,009 (Schutt et al.) 21 May 1991, see entire document, especially abstract; column 1, lines 46-51; column 5, lines 37-44; column 6, lines 49-62; column 15, lines 59-63; column 16, lines 4-5; column 17, lines 58-63; Examples 1, 4, 6.	1-13

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

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be part of particular relevance
- "E" earlier document published on or after the international filing date
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- "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
- "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
- "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
- "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
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 1993	05 AUG 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SUSAN C. WOLSKI
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06020

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,908,508 (Dubbeldam) 13 March 1990, see entire document, especially abstract; column 1, lines 14-17; column 3, lines 43-50; column 4, lines 20-31.	14-18